Universidad de Oviedo

Departamento de Ingeniería Química y Tecnología del Medio Ambiente
Programa de Doctorado en Ingeniería Química, Ambiental y Bioalimentaria

Tratamientos fisicoquímicos y biológicos de residuos con compuestos fenólicos complejos

TESIS DOCTORAL

Ana Isabel Díaz González

Diciembre 2021

Universidad de Oviedo
Universidá d'Uviéu
University of Oviedo

RESUMEN DEL CONTENIDO DE TESIS DOCTORAL

1.- Título de la Tesis	
Español/Otro Idioma: Tratamientos fisicoquímicos y biológicos de residuos con compuestos fenólicos complejos	Inglés: Physico-chemical and biological treatment of wastes with complex phenolic compounds

2.- Autor	
Nombre: Ana Isabel Díaz González	DNI/Pasaporte/NIE:
Programa de Doctorado: Programa en Ingeniería Química, Ambiental y Bioalimentaria	
Órgano responsable: Centro Internacional de Postgrado	

RESUMEN (en español)

FOR-MAT-VOA-010 (Reg.2018)

La gestión y el tratamiento de efluentes industriales con alto contenido en materia orgánica es uno de los principales retos a los que se enfrenta la sociedad actual. Generalmente, su tratamiento se realiza mediante métodos biológicos aerobios o anaerobios que incluyen la eliminación de nutrientes. Sin embargo, con frecuencia nos encontramos con residuos que contienen contaminantes con estructuras fenólicas complejas, lo que les confiere un fuerte carácter refractario que dificulta su tratamiento por vía biológica y obliga a incorporar alternativas que permitan eliminar la materia orgánica recalcitrante.

En el presente trabajo de tesis se abordó el estudio de las poblaciones bacterianas involucradas en procesos biológicos que tratan residuos que contienen contaminantes fenólicos. Así, se llevó a cabo un estudio metagenómico de los microorganismos presentes en un proceso de biometanización de lodos de depuradora, analizando los cambios tras los procesos de digestión anaerobia, centrifugación y decantación. Los resultados obtenidos permitieron caracterizar microbiológicamente las fracciones resultantes en función del tratamiento de separación, y conocer la abundancia relativa de las comunidades microbianas.

Cuando la biometanización de lodos se realiza en un centro integral de residuos, es frecuente que el licor resultante sea tratado conjuntamente con los lixiviados del vertedero mediante un proceso biológico de nitrificación-desnitrificación. De esta manera, también se ha analizado metagenómicamente las poblaciones presentes en el efluente de entrada y en las distintas etapas del proceso, observándose una composición muy homogénea entre las distintas corrientes debido a la elevada tasa de recirculación existente entre los distintos tanques biológicos.

Por otra parte, se ha evaluado el uso de diferentes tratamientos fisicoquímicos y biológicos con el fin de complementar los procesos de tratamiento habituales o revalorizar los residuos considerados. Así, se ensayaron métodos de oxidación húmeda, hidrólisis, ultrasonidos y ozonización para el tratamiento del licor de biometanización. Se analizó la evolución de la demanda química (DQO) y biológica (DBO_5) de oxígeno, la biodegradabilidad, el color, el pH y estado medio de oxidación del carbono (AOSC) del efluente. En base a los resultados obtenidos se propusieron modelos cinéticos y se analizó la influencia de diferentes parámetros operacionales. Todas las técnicas ensayadas mejoraron considerablemente la biodegradabilidad del efluente, siendo la

ozonización y la oxidación húmeda los más eficaces en la reducción de DQO. También, se ensayó el uso de diferentes técnicas de hidrólisis para el tratamiento de residuos de verduras, siendo el tratamiento térmico-enzimático la opción más eficaz para obtener azúcares reductores, aumentando su potencial para ser utilizados como sustratos en procesos fermentativos.

Finalmente, como alternativa a los tratamientos biológicos con bacterias, se estudió el empleo del hongo *Phanerochaete chrysosporium* para tratar dos residuos agropecuarios (purines y alperujo), dos efluentes biológicamente pretratados (licor de biometanización y permeado de lixiviado) y un residuo de la industria papelera (licor negro). En este último caso se empleó también el hongo *Aspergillus uvarum*. La efectividad en los tratamientos fue evaluada mediante la determinación de la concentración de DQO, DBO_5, carbohidratos y compuestos fenólicos, así como, la evolución del color y la actividad enzimática. En todos los casos, se logró reducir el contenido de materia orgánica, resultando clave el control del pH y siendo necesario en algunos casos suplementar con carbono y/o nitrógeno para maximizar la eficacia. Los resultados obtenidos en estos experimentos ponen de manifiesto el potencial de los hongos para la eliminación de color, compuestos fenólicos y materia orgánica recalcitrante, si bien, las condiciones operativas deben optimizarse para cada caso particular.

RESUMEN (en Inglés)

The management and treatment of industrial effluents with high organic matter content is one of the main difficult tasks that society has to face. Generally, their treatment is carried out by aerobic or anaerobic biological processes, which include the removal of nutrients. However, theses effluents frequently contain pollutants with complex phenolic structures, which give them a strong refractory character that complicates the biological treatment and makes necessary to incorporate alternatives that allow the removal of recalcitrant organic matter.

In this PhD thesis it was studied the bacterial populations involved in biological processes treating wastes that contain phenolic pollutants. Thus, a metagenomic study of the microorganisms present in a sewage sludge biomethanization process was carried out, analysing the changes after the processes of anaerobic digestion, centrifugation, and decantation. Results obtained allowed the microbial characterization of the fractions obtained in relation with the separation treatment used, and to determine the relative abundance of the microbial communities.

When sludge biomethanization takes place in an integrated waste management facility, the resulting liquor is often treated together with the leachate collected from the landfill by biological nitrification-denitrification processes. Therefore, populations present in the incoming effluent and throughout the biological process have also been metagenomically analysed, obtaining a very similar composition in the different streams sampled due to the high rate of recirculation between the different biological tanks.

Besides, the use of different physico-chemical and biological treatments has been evaluated to complement the usual treatment processes or to revalorise the considered industrial wastes. Thus, wet air oxidation, hydrolysis, ultrasound, and ozonisation methods were tested for the treatment of the biomethanization liquor. The evolution of chemical (COD) and biological (BOD_5) oxygen demand, biodegradability, colour, pH,

and average oxidation state of carbon atoms (AOSC) of the effluent were analysed. Taking into account the results obtained, kinetic models were proposed, and the influence of different operational parameters was analysed. All the tested techniques significantly improved the biodegradability of the effluent, and ozonation and wet air oxidation were the most effective methods for COD reduction. In addition, the use of different hydrolysis techniques for the treatment of vegetable wastes was also tested. The thermal-enzymatic treatment was the most effective method to obtain reducing sugars, increasing the waste potential to be used as substrate in fermentative processes.

Finally, as an alternative to bacterial biological treatments, the use of the fungus *Phanerochaete chrysosporium* was studied to treat two agricultural and livestock wastes (slurry and olive processing waste), two biologically pre-treated effluents (biomethanization liquor and leachate permeate) and a waste from the paper industry (black liquor). In the last case, the use of the fungus *Aspergillus uvarum* was also tested. The effectiveness of the treatments was evaluated by analysing the concentration of COD, BOD_5, carbohydrates and phenolic compounds, as well as the evolution of colour and enzymatic activity. In all cases, the organic matter content was reduced. The pH control turned out to be key and, in some cases, the supplementation with external carbon and/or nitrogen sources was necessary to maximize the degradation efficiency. The results obtained in these experiments show the potential of fungi for the removal of colour, phenolic compounds, and recalcitrant organic matter, even if the operating conditions must be optimized for each particular case.

SR. PRESIDENTE DE LA COMISIÓN ACADÉMICA DEL PROGRAMA DE DOCTORADO EN INGENIERÍA QUÍMICA, AMBIENTAL Y BIOALIMENTARIA

Universidad de Oviedo
Universidá d'Uviéu
University of Oviedo

FORMULARIO RESUMEN DE TESIS POR COMPENDIO

1.- Datos personales solicitante	
Apellidos: Díaz González	Nombre: Ana Isabel

Curso de inicio de los estudios de doctorado	2015 / 2016

	SI	NO
Acompaña acreditación por el Director de la Tesis de la aportación significativa del doctorando	X	
Acompaña memoria que incluye		
Introducción justificativa de la unidad temática y objetivos	X	
Copia completa de los trabajos *	X	
Resultados/discusión y conclusiones	X	
Informe con el factor de impacto de la publicaciones	X	
Se acompaña aceptación de todos y cada uno de los coautores a presentar el trabajo como tesis por compendio (Art. 32.4.b)	X	
Se acompaña renuncia de todos y cada uno de los coautores no doctores a presentar el trabajo como parte de otra tesis de compendio (Art. 32.4.c)	X	

* Ha de constar el nombre y adscripción del autor y de todos los coautores asi como la referencia completa de la revista o editorial en la que los trabajos hayan sido publicados o aceptados en cuyo caso se aportará justificante de la aceptación por parte de la revista o editorial

FOR-MAT-VOA-033

Artículos, Capítulos, Trabajos

Trabajo, Artículo 1

Titulo (o título abreviado)	Treatment of supermarket vegetable wastes to be used as alternative substrates in bioprocesses
Fecha de publicación	18 de mayo de 2017
Fecha de aceptación	11 de mayo de 2017
Inclusión en Science Citation Index o bases relacionadas por la CNEAI (indíquese)	Si
Factor de impacto	4.723

Coautor2 x Doctor □ No doctor . Indique nombre y apellidos	Amanda Laca Pérez
Coautor3 x Doctor □ No doctor . Indique nombre y apellidos	Adriana Laca Pérez
Coautor4 x Doctor □ No doctor . Indique nombre y apellidos	Jose Mario Díaz Fernández

Universidad de Oviedo
Universidá d'Uviéu
University of Oviedo

Trabajo, Artículo 2

Titulo (o título abreviado)	Impact of anaerobic digestion and centrifugation/decanting processes in bacterial communities fractions
Fecha de publicación	10 de julio de 2018
Fecha de aceptación	29 de mayo de 2018
Inclusión en Science Citation Index o bases relacionadas por la CNEAI (indíquese)	Si
Factor de impacto	2.032

Coautor2 x Doctor □ No doctor . Indique nombre y apellidos	Paula Oulego Blanco
Coautor3 x Doctor □ No doctor . Indique nombre y apellidos	Sergio Collado Alonso
Coautor4 x Doctor □ No doctor . Indique nombre y apellidos	Adriana Laca Pérez
Coautor5 x Doctor □ No doctor . Indique nombre y apellidos	Jose Manuel González La Fuente
Coautor6 x Doctor □ No doctor . Indique nombre y apellidos	Jose Mario Díaz Fernández

Trabajo, Artículo 3

Titulo (o título abreviado)	Metagenomic Analysis of Bacterial Communities from a Nitrification–Denitrification Treatment of Landfill Leachates
Fecha de publicación	8 de noviembre de 2019
Fecha de aceptación	19 de septiembre de 2019
Inclusión en Science Citation Index o bases relacionadas por la CNEAI (indíquese)	Si
Factor de impacto	1.603

Coautor2 x Doctor □ No doctor . Indique nombre y apellidos	Paula Oulego Blanco
Coautor3 x Doctor □ No doctor . Indique nombre y apellidos	Adriana Laca Pérez
Coautor4 x Doctor □ No doctor . Indique nombre y apellidos	Jose Manuel González La Fuente
Coautor5 x Doctor □ No doctor . Indique nombre y apellidos	Jose Mario Díaz Fernández

Trabajo, Artículo 4

Titulo (o título abreviado)	Physico-chemical pre-treatments of anaerobic digestion liquor for aerobic treatment
Fecha de publicación	12 de agosto de 2020
Fecha de aceptación	1 de agosto de 2020
Inclusión en Science Citation Index o bases relacionadas por la CNEAI (indíquese)	Si
Factor de impacto	6.789

Coautor2 x Doctor □ No doctor . Indique nombre y apellidos	Paula Oulego Blanco

Universidad de Oviedo
Universidá d'Uviéu
University of Oviedo

Coautor3 x Doctor □ No doctor . Indique nombre y apellidos	Jose Manuel González La Fuente
Coautor4 x Doctor □ No doctor . Indique nombre y apellidos	Adriana Laca Pérez
Coautor5 x Doctor □ No doctor . Indique nombre y apellidos	Jose Mario Díaz Fernández

Trabajo, Artículo 5

Titulo (o título abreviado)	Fungal treatment of an effluent from sewage sludge digestion to remove recalcitrant organic matter
Fecha de publicación	7 de mayo de 2021
Fecha de aceptación	5 de mayo de 2021
Inclusión en Science Citation Index o bases relacionadas por la CNEAI (indíquese)	Si
Factor de impacto	3.978

Coautor2 x Doctor □ No doctor . Indique nombre y apellidos	Adriana Laca Pérez
Coautor3 x Doctor □ No doctor . Indique nombre y apellidos	Jose Mario Díaz Fernández

Trabajo, Artículo 6

Titulo (o título abreviado)	Biodegradation of olive mill effluent by white-rot fungi
Fecha de publicación	24 de octubre de 2021
Fecha de aceptación	20 de octubre de 2021
Inclusión en Science Citation Index o bases relacionadas por la CNEAI (indíquese)	Si
Factor de impacto	2.679

Coautor2 □ Doctor x No doctor . Indique nombre y apellidos	Marta Ibáñez Lázaro
Coautor3 x Doctor □ No doctor . Indique nombre y apellidos	Adriana Laca Pérez
Coautor4 x Doctor □ No doctor . Indique nombre y apellidos	Jose Mario Díaz Fernández

En caso de compendio de un número de artículos superior a seis, se incorporarán hojas suplementarias conforme a este modelo

"Fue el tiempo que pasaste con tu rosa, lo que la hizo tan importante".

El principito (Antoine de Saint-Exupéry, 1943).

AGRADECIMIENTOS

En primer lugar, quiero expresar mi más sincero agradecimiento al Dr. Mario Díaz, por la darme la oportunidad de desarrollar esta Tesis Doctoral bajo su dirección, por todos los consejos y el asesoramiento recibido, haciendo que esta etapa haya sido muy enriquecedora para mí.

A mi co-directora y tutora, la Dra. Adriana Laca, por su capacidad para transmitir todos los conocimientos que han hecho posible el desarrollo de esta tesis, por su habilidad para ordenar las ideas en la dirección correcta, dar sentido a los resultados y facilitarme siempre este camino. Por brindarme las herramientas necesarias para alcanzar los resultados que buscaba. Estas líneas se quedan cortas, gracias por toda la ayuda profesional, pero sobre todo por toda la ayuda personal, destacando la paciencia, la amabilidad y la disponibilidad absoluta.

Deseo transmitir también todo mi agradecimiento al Dr. Manuel Rendueles, por su ayuda incondicional y sus sabios consejos. Agradezco su cercanía, su optimismo y sus palabras de apoyo cuando los momentos no han sido fáciles. A la Dra. Amanda Laca, gran investigadora y amiga, que me ha acompañado, ayudado y enseñado mucho durante todo este proyecto, con el que ha reído (y sufrido) como si fuese suyo. Por compartir todos sus conocimientos y enseñarme a transmitirlos. En definitiva, gracias por estar ahí.

Quiero hacer extensible este agradecimiento al resto de compañeros del grupo de investigación de Tecnología de Bioprocesos y Reactores (TBR) de la Universidad de Oviedo, del que afortunadamente para mí, he formado parte durante este trabajo. Quiero expresar mi profundo agradecimiento a todos los miembros del grupo, por los momentos compartidos en los laboratorios y las salas, por siempre estar siempre dispuestos a echarme una mano cuando lo he necesitado. Especialmente a la Dra. Paula Oulego, por todo el tiempo brindado y los conocimientos transmitidos, por esa sincronicidad en el trabajo, por estar siempre dispuesta a ayudarme y facilitarme la investigación.

Quiero mostrar mi especial agradecimiento a la sala 229, mi segunda "casa", al "ahora sí" Dr. Manuel García, y a los futuros doctores, Lucia, Dani, Marta y Andrea, y

al Dr. Dani (que no Daniel) Sol. Gracias por todos los momentos vividos, por ser además de compañeros, amigos, por apoyarme y siempre intentar sacarme una sonrisa. Gracias por ayudarme a crecer profesional y personalmente. Gracias por estar ahí cuando más os he necesitado. Este trabajo no se hubiese podido llevar a cabo sin este equipo. Hago extensivo este agradecimiento a todas las personas del Departamento de Ingeniería Química y Tecnología del Medio Ambiente que, de un modo u otro, han hecho posible la realización y presentación de esta Tesis Doctoral.

Quisiera dar las gracias también a la Escuela Técnica Superior de Ingenierías Agrarias de Palencia perteneciente a la Universidad de Valladolid, especialmente a la Dra. Mercedes Sánchez, por su agradable recibimiento y asesoramiento, facilitando en todo momento la estancia realizada.

Asimismo, me gustaría agradecer al Centro de Ingeniería Biológica (CEB) de la Universidad de Minho (Braga, Portugal), especialmente al Dr. Nelson Lima, por creer en mi y darme la oportunidad de realizar con ellos mi estancia internacional. A todo el grupo de Micología Aplicada, especialmente a Carla y Célia, por su cercanía, amabilidad y paciencia. Por hacerme sentir desde el primer momento una más. Gracias por todo. Muito obrigado por toda a ajuda recebida. Por me fazer recordar esses momentos sempre com um sorriso no rosto.

A mis amigos, por escucharme y animarme constantemente a alcanzar mis sueños. Especialmente, a la futura doctora María Pombal, por estar siempre ahí y seguir arreglando la vida a través de un largo café. A Sergio, por su apoyo incondicional tanto en lo académico como en terreno personal, por escucharme, animarme y brindarme su confianza cada día, sobre todo en los momentos no tan fáciles.

Quiero hacer una mención especial a mi familia, que siempre ha estado ahí para animándome, apoyándome y haciendo este proyecto parte de ellos. Resumir aquí toda mi gratitud hacia vosotros es imposible, sería necesaria otra tesis. Gracias por llenar mi vida de alegría y de sentido. Por darme las alas para alcanzar mis sueños por altos que estos parezcan. Esta tesis es tanto mía como vuestra. Especialmente de mi padre, mi amigo, mi compañero y mi ángel, quien "pasito a pasito" me ha enseñado que uno puede llegar tan lejos como se proponga. Esta Tesis es resultado de vuestro esfuerzo, del Proyecto "Díaz González" comenzado en 1990.

ÍNDICE

RESUMEN…….………………..……………………………………………………… **IV**

ABSTRACT…....……………..……………………………………………………… **VI**

Lista de Figuras…....……………………………………………………………..**VIII**

Lista de Tablas……..……………………………………………………………..**XIV**

Capítulo 1. INTRODUCCIÓN ***1***

1.1. Consideraciones previas 3

1.2. Objetivos de la Tesis Doctoral 5

1.3. Estructura de la Tesis Doctoral 6

Capítulo 2. CONSIDERACIONES TEÓRICAS ***9***

2.1. Residuos procedentes de la digestión anaerobia de lodos 11

2.1.1. Proceso biológico de digestión anaerobia.. 14

2.1.2. Tratamientos habituales de los efluentes de digestión anaerobia 16

2.2. Residuos procedentes del tratamiento biológico de lixiviados 21

2.2.2. Etapas del proceso de nitrificación y desnitrificación 23

2.2.3. Tratamientos terciarios de lixiviados 25

2.3. Residuos agropecuarios 26

2.3.1 Residuos agroalimentarios 26

2.3.2. Efluentes ganaderos 28

3.3.3. Tratamientos habituales de los residuos agropecuarios 29

2.4. Residuos procedentes de la industria papelera 31

2.4.1. Proceso de obtención del licor negro 33

2.4.2. Tratamientos del licor negro 34

2.5. Tratamiento biológico mediante hongos 35

2.5.1. Aspectos generales de los hongos 36

2.5.3. Enzimas extracelulares 38

2.6. Referencias.. 40

Capítulo 3. MATERIAL Y MÉTODOS *59*

3.1. Materias primas 61

3.1.1. Licor de biometanización 61

3.1.2. Permeado de ultrafiltración 62

3.1.3. Descartes alimentarios 63

3.1.4. Licor negro 65

4.1.6. Alperujo 65

3.1.7. Aguas residuales porcinas 67

3.2. Metodología experimental 68

3.2.1. Caracterización microbiológica 68

3.2.2. Tratamientos fisicoquímicos 70

3.2.3. Tratamientos biológicos 77

3.3. Métodos analíticos 81

3.3.1. Análisis taxonómico 81

3.3.2. Análisis de parámetros fisicoquímicos 82

3.3.3. Cálculo de parámetros adicionales 88

3.4. Referencias 89

Capítulo 4. RESULTADOS Y DISCUSIÓN *93*

4.1. Tratamiento post-anaerobiosis 96

4.1.1. Impact of anaerobic digestion and centrifugation/decanting processes in bacterial communities fractions 97

4.1.2. Physico-chemical pre-treatments of anaerobic digestion liquor for aerobic treatment 120

4.1.3. Fungal treatment of an effluent from sewage sludge digestion to remove recalcitrant organic matter 148

4.2. Tratamiento de lixiviados 175

4.2.1. Metagenomic analysis of bacterial communities from nitrification-denitrification treatment of landfill leachates by Ion PGM System 176

4.2.2. Evaluation of using white-rot fungi as post-treatment of biologically and physical-chemically pre-treated landfill leachate 196

4.3. Tratamiento de residuos agropecuarios 219

4.3.1. Treatment of supermarket vegetable wastes to be used as alternative substrates in bioprocesses 220

4.3.2. Biodegradation of olive mill effluent by white-rot fungi 246

4.3.3. Evaluation of *Phanerochaete chrysosporium* for swine wastewater treatment 268

4.4. Tratamiento de efluentes de la industria papelera 287

4.4.1. Treatment of kraft black liquor using basidiomycete and ascomycete fungi 288

Capítulo 5. CONCLUSIONES 314

ANEXOS 321

ANEXO I: Supplementary material for “Impact of anaerobic digestion and centrifugation/decanting processes in bacterial communities fractions”... 323

ANEXO I: Supplementary material for “Physico-chemical pre-treatments of anaerobic digestion liquor for aerobic treatment” 329

ANEXO III. Supplementary material for “Metagenomic analysis of bacterial communities from a nitrification-denitrification treatment of landfill leachates”. 332

DIFUSIÓN DE LA TESIS DOCTORAL 339

Publicacións 341

Participación en congresos y seminarios: 342

RESUMEN

La gestión y el tratamiento de efluentes industriales con alto contenido en materia orgánica es uno de los principales retos a los que se enfrenta la sociedad actual. Generalmente, su tratamiento se realiza mediante métodos biológicos aerobios o anaerobios que incluyen la eliminación de nutrientes. Sin embargo, con frecuencia nos encontramos con residuos que contienen contaminantes con estructuras fenólicas complejas, lo que les confiere un fuerte carácter refractario que dificulta su tratamiento por vía biológica y obliga a incorporar alternativas que permitan eliminar la materia orgánica recalcitrante.

En el presente trabajo de tesis se abordó el estudio de las poblaciones bacterianas involucradas en procesos biológicos que tratan residuos que contienen contaminantes fenólicos. Así, se llevó a cabo un estudio metagenómico de los microorganismos presentes en un proceso de biometanización de lodos de depuradora, analizando los cambios tras los procesos de digestión anaerobia, centrifugación y decantación. Los resultados obtenidos permitieron caracterizar microbiológicamente las fracciones resultantes en función del tratamiento de separación, y conocer la abundancia relativa de las comunidades microbianas.

Cuando la biometanización de lodos se realiza en un centro integral de residuos, es frecuente que el licor resultante sea tratado conjuntamente con los lixiviados del vertedero mediante un proceso biológico de nitrificación-desnitrificación. De esta manera, también se ha analizado metagenómicamente las poblaciones presentes en el efluente de entrada y en las distintas etapas del proceso, observándose una composición muy homogénea entre las distintas corrientes debido a la elevada tasa de recirculación existente entre los distintos tanques biológicos.

Por otra parte, se ha evaluado el uso de diferentes tratamientos fisicoquímicos y biológicos con el fin de complementar los procesos de tratamiento habituales o revalorizar los residuos considerados. Así, se ensayaron métodos de oxidación húmeda, hidrólisis, ultrasonidos y ozonización para el tratamiento del licor de biometanización. Se analizó la evolución de la demanda química (DQO) y biológica (DBO_5) de oxígeno, la biodegradabilidad, el color, el pH y estado medio de oxidación del carbono (AOSC) del efluente. En base a los resultados obtenidos se propusieron modelos cinéticos y se

analizó la influencia de diferentes parámetros operacionales. Todas las técnicas ensayadas mejoraron considerablemente la biodegradabilidad del efluente, siendo la ozonización y la oxidación húmeda los más eficaces en la reducción de DQO. También, se ensayó el uso de diferentes técnicas de hidrólisis para el tratamiento de residuos de verduras, siendo el tratamiento térmico-enzimático la opción más eficaz para obtener azúcares reductores, aumentando su potencial para ser utilizados como sustratos en procesos fermentativos.

Finalmente, como alternativa a los tratamientos biológicos con bacterias, se estudió el empleo del hongo *Phanerochaete chrysosporium* para tratar dos residuos agropecuarios (purines y alperujo), dos efluentes biológicamente pretratados (licor de biometanización y permeado de lixiviado) y un residuo de la industria papelera (licor negro). En este último caso se empleó también el hongo *Aspergillus uvarum*. La efectividad en los tratamientos fue evaluada mediante la determinación de la concentración de DQO, DBO_5, carbohidratos y compuestos fenólicos, así como, la evolución del color y la actividad enzimática. En todos los casos, se logró reducir el contenido de materia orgánica, resultando clave el control del pH y siendo necesario en algunos casos suplementar con carbono y/o nitrógeno para maximizar la eficacia. Los resultados obtenidos en estos experimentos ponen de manifiesto el potencial de los hongos para la eliminación de color, compuestos fenólicos y materia orgánica recalcitrante, si bien, las condiciones operativas deben optimizarse para cada caso particular.

ABSTRACT

The management and treatment of industrial effluents with high organic matter content is one of the main difficult tasks that society has to face. Generally, their treatment is carried out by aerobic or anaerobic biological processes, which include the removal of nutrients. However, theses effluents frequently contain pollutants with complex phenolic structures, which give them a strong refractory character that complicates the biological treatment and makes necessary to incorporate alternatives that allow the removal of recalcitrant organic matter.

In this PhD thesis it was studied the bacterial populations involved in biological processes treating wastes that contain phenolic pollutants. Thus, a metagenomic study of the microorganisms present in a sewage sludge biomethanization process was carried out, analysing the changes after the processes of anaerobic digestion, centrifugation, and decantation. Results obtained allowed the microbial characterization of the fractions obtained in relation with the separation treatment used, and to determine the relative abundance of the microbial communities.

When sludge biomethanization takes place in an integrated waste management facility, the resulting liquor is often treated together with the leachate collected from the landfill by biological nitrification-denitrification processes. Therefore, populations present in the incoming effluent and throughout the biological process have also been metagenomically analysed, obtaining a very similar composition in the different streams sampled due to the high rate of recirculation between the different biological tanks.

Besides, the use of different physico-chemical and biological treatments has been evaluated to complement the usual treatment processes or to revalorise the considered industrial wastes. Thus, wet air oxidation, hydrolysis, ultrasound, and ozonisation methods were tested for the treatment of the biomethanization liquor. The evolution of chemical (COD) and biological (BOD_5) oxygen demand, biodegradability, colour, pH, and average oxidation state of carbon atoms (AOSC) of the effluent were analysed. Taking into account the results obtained, kinetic models were proposed, and the influence of different operational parameters was analysed. All the tested techniques significantly improved the biodegradability of the effluent, and ozonation and wet air oxidation were the most effective methods for COD reduction. In addition, the use of

different hydrolysis techniques for the treatment of vegetable wastes was also tested. The thermal-enzymatic treatment was the most effective method to obtain reducing sugars, increasing the waste potential to be used as substrate in fermentative processes.

Finally, as an alternative to bacterial biological treatments, the use of the fungus *Phanerochaete chrysosporium* was studied to treat two agricultural and livestock wastes (slurry and olive processing waste), two biologically pre-treated effluents (biomethanization liquor and leachate permeate) and a waste from the paper industry (black liquor). In the last case, the use of the fungus *Aspergillus uvarum* was also tested. The effectiveness of the treatments was evaluated by analysing the concentration of COD, BOD_5, carbohydrates and phenolic compounds, as well as the evolution of colour and enzymatic activity. In all cases, the organic matter content was reduced. The pH control turned out to be key and, in some cases, the supplementation with external carbon and/or nitrogen sources was necessary to maximize the degradation efficiency. The results obtained in these experiments show the potential of fungi for the removal of colour, phenolic compounds, and recalcitrant organic matter, even if the operating conditions must be optimized for each particular case.

Lista de Figuras

CAPITULO 2

Figura 2.1. *Destino de los lodos generados por comunidades y ciudades autónomas en España durante 2018.* *12*

Figura 2.2. *Etapas del proceso de digestión anaerobia y microorganismos implicados.*.. *15*

Figura 2.3. *Posibles tratamientos de los subproductos de la digestión anaerobia.*.. *18*

Figura 2.4. *Residuos generados en España durante el año 2016 [47].* *21*

Figura 2.5. *Proceso de nitrificación mediante bacterias amonio oxidantes (AOB) y bacterias nitrito oxidantes (NOB)*.. *24*

Figura 2.6. *Proceso de desnitrificación*.. *24*

Figura 2.7. *Pérdidas y desperdicio de alimentos en diferentes países (kg/año) [* *27*

Figura 2.8. *Tratamientos de los residuos agroalimentarios y productos obtenidos.*.. *30*

Figura 2.9. *Producción total de celulosa en los países miembros CEPI en 2020.* *32*

Figura 2.10. *Esquema del proceso Kraft*.. *33*

Figura 2.11. *Pudrición blanca producida por Phanerochaete chrysosporium*. *37*

Figura 2.12. *Ciclo catalítico de MnP* *38*

Figura 2.13. *Reacción catalítica LiP*... *39*

CAPITULO 3

Figura 3.1. *Proceso de obtención del licor de biometanización.* *61*

Figura 3.2. *Residuos agroalimentarios empleados* *63*

Figura 3.3. *Residuo de alperujo*.. *66*

Figura 3.4. *Equipo de ultrasonidos.* *71*

Figura 3.5. *Equipo de ozonización* *72*

Figura 3.6. *Equipo de hidrólisis y oxidación húmeda*..................................... *73*

Figura 3.7. *Phanerochaete chrysosporium y Asperillus uvarum*...................... *77*

Figura 3.8. *Proceso de obtención de los pellets fúngicos de Phanerochaete chrysosporium* *79*

CAPITULO 4.

Figura 4. 1. *Resumen gráfico del trabajo 1* 96

Figura 4. 2. *Flow diagram of anaerobic digestion plant.* 101

Figura 4. 3. *Relative abundance for the phyla detected in the initial sludge (A) and digestate (B) samples (S1 and S2, respectively). In boxes are shown the relative abundance obtained for the classes within Firmicutes, Bacteroidetes and Proteobacteria phyla.* 107

Figura 4. 4. *Relative abundance for the phyla detected in the solid phase (A) and clarified (B) samples (S3 and S4, respectively).* 110

Figura 4. 5. *Relative abundance for de phyla detected in the decanted product (S5)..* 113

Figura 4. 6. *Resumen gráfico del trabajo 2* 120

Figura 4. 7. *a) Changes of tCOD (dashed lines), sCOD (solid lines) and nsCOD (dotted lines) during ultrasound treatment at 50% of amplitude (dark colour and triangle markers) and 90% of amplitude (light colour and circle markers).* ***b)*** *Changes of BOD_5 (lines and markers) and biodegradability (columns).* ***c)*** *Change of AOSC and* ***d)*** *Changes of pH (lines and markers) and colour number (columns). The standard deviations (SD) of the experimental data were in all cases lower than 3% of the mean value* 131

Figura 4. 8. *a) Changes of tCOD (dashed lines), sCOD (solid lines) and nsCOD (dotted lines) during ozonation treatment. The experiment A corresponds to the sample with solids (dark colour and triangle markers) and the experiment B corresponds to the sample without solids (light colour and circle markers). b) Changes of BOD_5 (lines and markers) and biodegradability (columns). c) Changes of AOSC. d) Changes of pH (markers) and CN (columns). The standard deviation (SD) of the experimental data were in all cases less than 4% of mean value.* 133

Figura 4. 9. *a) Changes of tCOD (dashed lines), sCOD (solid lines) and nsCOD (dotted lines) during hydrolysis treatment at 160 ºC (light colour and triangle markers), at 180 ºC (intermediate colour and square markers) and at 200 ºC (dark colour and circle markers).* ***b)*** *Changes of BOD_5 (markers) and biodegradability (columns).* ***c)*** *Changes of AOSC* ***d)*** *Changes of pH (markers) and CN (columns). The standard deviation (SD) of the experimental data were in all cases lower than 4% of the mean value.* 135

Figura 4. 10. *a) Changes of tCOD (dashed lines), sCOD (solid lines) and nsCOD (dotted lines) during wet air oxidation treatment at 160 ºC amplitude (light colour and triangle markers), at 180 ºC (intermediate colour and square markers) and at 200 ºC (dark colour and circle markers).* ***b)*** *Changes of* BOD_5 *(markers) and biodegradability (columns).* ***c)*** *Changes of AOSC.* ***d)*** *Changes of pH (markers) and CN (columns). The standard deviation (SD) of the experimental data were in all cases lower than 10% of mean value. 137*

Figura 4. 11. *Results of solubilization degree* ***(a)*** *and tCOD degradation* ***(b)*** *for the different treatments. 139*

Figura 4. 12. *Resumen gráfico del trabajo 3 .... 148*

Figura 4. 13. *Changes in COD concentration during batch test for the different samples. The dashed lines shown the non-inoculated tests* ***C1 (●)*** *and* ***C2 (●)****, used as controls. The solid lines shown the inoculated tests* ***C3 (●)****,* ***S1*** *(○),* ***S2*** *(▲),* ***S3*** *(◆) and* ***S4*** *(■). A concentration of 3 g/L, 5 g/L and 8 g/L of glucose was added to S2, S3 and S4, respectively. The tests C2 and C3 were supplied with 5 g/L of glucose. In addition, the experiment C3 was carried out with sterile sample while C1, C2, S1, S2, S3 and S4 were carried out with non-sterile sample. The standard deviation (SD) of the experimental data were in all cases less than 9% of mean value. 157*

Figura 4. 14. *Data of* BOD_5 *values during batch test at initial time, 5 days, and 10 days for the non-inoculated tests* ***C1*** *(■) and* ***C2*** *(□), and inoculated tests* ***C3*** *(■),* ***S1*** *(■),* ***S2*** *(■),* ***S3*** *(■) and* ***S4*** *(■). A concentration of 3 g/L, 5 g/L and 8 g/L of glucose was added to S2, S3 and S4, respectively. The tests C2 and C3 were supplied with 5 g/L of glucose. In addition, the experiment C3 was carried out with sterile sample while C1, C2, S1, S2, S3 and S4 were carried out with non-sterile sample. The standard deviation (SD) of the experimental data were in all cases less than 6% of mean value. 161*

Figura 4. 15. *Changes in colour index (CN) during batch test for the different samples. The dashed lines shown the non-inoculated tests* ***C1 (●)*** *and* ***C2 (●)****, used as controls. The solid lines shown the inoculated tests* ***C3 (●)****,* ***S1 (●)****,* ***S2*** *(▲),* ***S3*** *(◆) and* ***S4*** *(■). A concentration of 3 g/L, 5 g/L and 8 g/L of glucose was added to S2, S3 and S4, respectively. The tests C2 and C3 were supplied with 5 g/L of glucose. In addition, the experiment C3 was carried out with sterile sample while C1, C2, S1, S2, S3 and S4 were carried out with non-sterile sample. The standard deviation (SD) of the experimental data were in all cases less than 4% of mean value. 162*

Figura 4. 16. *Changes in* ***a)*** *COD,* ***b)*** *BOD_5 and* ***c)*** *colour index (CN) during the continuous treatments with different HRT values: 104 h (●), 208 h (■) and 240 h (▲). The red dashed line shown the value of each parameter for the ADL treated.* 164

Figura 4. 17. *Percentages of COD (●) and colour removal (■) during continuous treatment (steady state) for each HRT. The biodegradability (▲) is shown in the right axis.* 165

Figura 4. 18. *Resumen gráfico del trabajo 4* 176

Figura 4. 19. *Process flow diagram of biological treatment plant.* 180

Figura 4. 20. *Relative abundance for the phyla detected in the raw leachate (S1).* 185

Figura 4. 21. *Relative abundance for the classes detected in the A) Proteobacteria and B) Bacterioidetes phyla.* 186

Figura 4. 22. *Relative abundance for denitrification reactor output (S2).* 188

Figura 4. 23. *Relative abundance for the phyla detected in the A,B) nitrification reactors output and C) ultrafiltration sludge samples (S3, S4, and S5, respectively)..* 191

Figura 4. 24. *Resumen gráfico del trabajo 5* 196

Figura 4. 25. ***A)*** *sCOD (solid lines) and CN (dashed lines) evolution during the fungal treatment. Filled markers symbolize the inoculated tests F1 (●), F2 (▲) and empty markers symbolize the non-inoculated tests C1 (○) and C2 (△), used as control.* ***B)*** *pH evolution during the fungal treatment for C1 (○) and F1 tests (●).* 204

Figura 4. 26. *Evolution of LiP* ***(A)*** *and MnP* ***(B)*** *enzymatic activities during the treatment of permeate by P. chrysosporium. Solid lines correspond to tests carried out with undiluted permeate, whereas dashed lines show the data for the tests performed with diluted permeate. Besides, empty markers show the non-supplemented tests F1 (◇), F2 (△) and F7 (○), whereas solid lines and filled markers show the supplemented tests F3 (▲), F4 (▲), F5 (▲), F6 (■) and F8 (●). The percentage of standard deviation (SD) of the experimental data were in all cases less than 6% of mean value.* 206

Figura 4. 27. *Evolution of sCOD concentration* ***(A)*** *and colour* ***(B)*** *during the treatment of permeate effluent by P. chrysosporium at controlled pH. In both cases, solid lines correspond to tests carried out with undiluted permeate, whereas dashed lines show the data for the experiments performed with diluted permeate. Besides, empty markers show the non-supplemented tests, F2 (△) and F7 (○), whereas solid lines and filled markers show the supplemented tests F3 (▲), F4 (▲), F5 (▲), F6 (■)*

and F8 (●). In F3, F4 and F5 tests, 1 g/L, 3 g/L and 5 g/L of glucose was added as co-substrate, respectively. In F6 and F8 tests, 1 g/L of glucose and 0,5 g/L of ammonium tartrate were supplied. The percentage of standard deviation (SD) of the experimental data were in all cases less than 8% of mean value. *209*

Figura 4. 28. *Resumen gráfico del trabajo 6* *219*

Figure 4. 29. *Soluble reducing sugars (A), soluble total sugars (B) and potential reducing sugars (C) extracted from different batches of vegetable wastes*.................. *230*

Figura 4. 30. *Reducing and total sugars extracted from vegetable wastes after the hydrolyses: (A) thermal 110ºC, (B) thermal 135ºC, (C) acid, (D) enzymatic and (E) thermal-enzymatic.* *233*

Figura 4. 31. *Evolution of solubilised reducing sugars (M:□), solubilised non-reducing sugar intermediates (D:◇) and potential reducing sugars in solid phase (S:△) with time. Symbols correspond to experimental data (or calculated from experimental data) and lines correspond to model results. All concentrations are expressed as equivalent glucose. Vertical grey line divides the thermic and the enzymatic steps. In all cases, SD < 2.0 g/100 g.* *237*

Figura 4. 32. *Resumen gráfico del trabajo 7* *245*

Figura 4. 33. *Changes in sCOD concentration during biological treatment. The dashed lines shown the non-inoculated tests C1 (■) and C2 (■), used as controls. The solid lines shown the inoculated tests E1 (●) and E2 (●). The standard deviation (SD) of the experimental data were in all cases less than 6.5% of mean value.*....................... *253*

Figura 4. 34. *Changes in $sBOD_5$ concentration and biodegradability index for the different experiments at initial, intermediate, and final time of the treatment. Bars corresponds with $sBOD_5$ concentration for non-inoculated tests C1 (■) and C2 (■), and inoculated tests E1 (■) and E2 (■). The dashed lines shown the biodegradability index (BI) for non-inoculated tests C1 (■) and C2 (■), used as controls, whereas the solid lines represent the inoculated tests E1 (●) and E2 (●)*.. *255*

Figura 4. 35. *Changes in reducing sugars concentration during biological treatment. The dashed lines shown the non-inoculated tests C1 (■) and C2 (■), used as controls. The solid lines shown the inoculated tests E1 (●) and E2 (●). The standard deviation (SD) of the experimental data were in all cases less than 11% of mean value.* *256*

Figura 4. 36. *Changes in total phenolic content for non-inoculated tests C1 (■) and C2 (■), and inoculated tests E1 (■) and E2 (■). The standard deviation (SD) of the experimental data were in all cases less than 4% of mean value................................ 258*

Figura 4. 37. *Changes in colour index during biological treatment. The dashed lines shown the non-inoculated tests C1 (■) and C2 (■), used as controls. The solid lines shown the inoculated tests E1 (●) and E2 (●). The standard deviation (SD) of the experimental data were in all cases less than 11.5% of mean value........................... 259*

Figura 4. 38. *Resumen gráfico del trabajo 8 .... 266*

Figura 4. 39. *Evolution of different parameters during the swine wastewater treatment by P. chrysosporium.* ***A)*** *pH* ***B)*** *soluble COD* ***C)*** *Colour number (CN)* ***D)*** *LiP enzymatic activity* ***E)*** *MnP enzymatic activity* ***F)*** *Lac enzymatic activity. The standard deviation (SD) of the experimental data were in all cases less than 10% of mean value. In A, B, and C, empty markers and dashed lines show the control test, C1 (●), C2 (▲) and C3 (■) and filled markers and solid lines show the inoculated tests, S1 (●), S2 (▲) and S3 (■). In D, E and F, solid lines show data of S1 (●), S2 (▲) and S3 (■), while columns show data of control samples C1 (green colour), C2 (blue colour) and C3 (grey colour)... 273*

Figura 4. 40. *Evolution of different parameters during the digestated slurry treatment by P. chrysosporium.* ***A)*** *soluble COD* ***B)*** *pH* ***C)*** *Colour number (CN)* ***D)*** *LiP enzymatic activity* ***E)*** *MnP enzymatic activity. The standard deviation (SD) of the experimental data were in all cases less than 13% of mean value. Empty markers and dashed lines show the control test C4 (■) and C5 (●), and filled markers and solid lines show the inoculated tests, D4 (■) and D5 (●). 278*

Figura 4. 41*. Resumen gráfico del trabajo 9 .... 287*

Figura 4. 42. *Evolution of different parameters during the treatment of KBL with P. chrysosporium and pH control.* ***A)*** *Soluble COD* ***B)*** *Reducing sugars* ***C)*** *Enzymatic activities* ***D)*** *BOD_5 and biodegradability index (BI)* ***E)*** *Phenolic compounds.* ***F)*** *Colour number (CN). The standard deviation (SD) of the experimental data were in all cases less than 11% of mean value. In A, B, E and F, empty markers show the inoculated tests, P6 (▲) and P4 (●) and filled markers show the non-inoculated tests, C-P6 (▲) and C-P4 (●). In C, filled markers and empty markers show data of P6 and P4, respectively. MnP (●, ○), LiP (■, □) and Lac (▲, △). In D, initial BOD_5 is shown in dark columns, final BOD_5 in light columns and final BI in circle markers. 299*

Figura 4. 43. *Evolution of different parameters during the treatment of KBL with A. uvarum and pH control.* ***A)*** *Soluble COD* ***B)*** *Reducing sugars* ***C)*** *Enzymatic activities* ***D)*** *BOD_5 and biodegradability index (BI)* ***E)*** *Phenolic compounds.* ***F)*** *Colour number (CN). The standard deviation (SD) of the experimental data were in all cases less than 12% of mean value. In A, B, E and F, empty markers show the inoculated tests, U6 (△) and U4 (○) and filled markers show the non-inoculated tests, C-U6 (▲) and C-U4 (●). In C, filled markers and empty markers show data of U6 and U4, respectively. MnP (●, ○), LiP (■, □) and Lac (▲, △). In D, initial BOD_5 is shown in dark columns, final BOD_5 in light columns and final BI in circle markers. 301*

Figura 4. 44. *Evolution of different parameters during the treatment of KBL with and without solids, and without pH control with A. uvarum.* ***A)*** *Soluble COD (solid lines) and pH (dashed lines)* ***B)*** *Reducing sugars* ***C)*** *Enzymatic activities* ***D)*** *BOD_5 and biodegradability index (BI)* ***E)*** *Phenolic compounds.* ***F)*** *Colour number (CN). The standard deviation (SD) of the experimental data were in all cases less than 12% of mean value. In A, B, E and F, empty markers show the inoculated tests, UL (△) and US (○) and filled markers show the non-inoculated tests, CL (▲) and CS (●). In C, filled markers and empty markers show data of U6 and U4, respectively. MnP (●, ○), LiP (■, □) and Lac (▲, △). In D, initial BOD_5 is shown in dark columns, final BOD_5 in light columns and final BI in circle markers. 304*

Lista de Tablas

CAPITULO 3

Tabla 3.1. *Caracterización del licor de biometanización.* 62

Tabla 3.2. *Caracterización del permeado de ultrafiltración* 63

Tabla 3.3. *Composición nutricional de los vegetales empleados.* 64

Tabla 3.4. *Caracterización del licor negro* 65

Tabla 3.5. *Caracterización del residuo de alperujo*... 66

Tabla 3.6. *Caracterización de los purines de cerdo* 67

Tabla 3.7. *Caracterización de efluentes procedentes de la digestión anaerobia de purines de cerdo* 68

Tabla 3.8. *Condiciones de la PCR para la amplificación del ADN*.................. 70

Tabla 3.9. *Tratamientos de biorremediación con hongos*................................ 80

CAPITULO 4

Table 4.1. *Characteristics of the samples analysed. The average values correspond to four samples taken along 2016. The ± value correspond to the standard deviation.* 103

Table 4.2. *Characteristics of the anaerobic digestion liquor (ADL)* 125

Table 4.3. *Kinetic constants calculated from experimental data* 141

Table 4.4. *Characteristics of anaerobic digestion liquor (after dilution with buffer)* 154

Table 4.5. *Kinetic constants calculated from experimental data obtained after batch tests* 160

Table 4.6. *Characteristics of the samples analysed. The values correspond to the averages (± standard deviations) of four samples taken along 2016.*......................... 183

Table 4.7. *Characteristics of the initial permeate*... 200

Table 4.8. *Experimental design for the fungal treatments (F1-F8) and control tests (C1-C8)*.. 202

Table 4.9. *Effect of fungal treatment on sCOD, colour, $sBOD_5$, BI and TS at intermediate (8 days) and final times (15 days) of treatment. Negative percentages of*

degradation indicate that the sample suffered an increase in the value of the parameter studied. 207

Table 4.10. *Nutritional composition of the vegetables employed as substrate (given per 100 g of fresh product).* 223

Table 4.11. *Solids weights throughout the thermal-enzymatic hydrolysis expressed on dry weight basis (%).* 227

Table 4.12. *Comparison of reducing sugars yield extraction of the different hydrolysis assayed. Average values are shown.* 234

Table 4.13. *Kinetic constant calculated from experimental data* 239

Table 4.14. *Characteristics of the diluted AL.* 249

Table 4.15. *TSS for control (C1 and C2) and inoculated (E1 and E2) tests at initial and final times of the fungal treatment.* 254

Table 4.16. *Characteristics of the initial samples subjected to fungal treatment.* 270

Table 4.17. *Concentrations of nitrites (NO_2^-), nitrates (NO_3^-), ammonium (NH_3), total inorganic nitrogen (TIN), dissolved organic nitrogen (DON) and total nitrogen (TN) for the inoculated (S1-S3) and control (C1-C3) samples after 10 days of swine wastewater treatment.* 276

Table 4.18. *Characteristics of KBL diluted 1:24 after pH adjusting.* 292

Table 4.19. *Experimental design for the biological treatments. In all cases, the medium used was diluted KBL and the amount of inoculated fungus was 3 g/L (dry matter).* 294

Capítulo 1

INTRODUCCIÓN

1.1. CONSIDERACIONES PREVIAS

El aumento de la población mundial, el creciente desarrollo industrial y los modelos actuales de producción y consumo, provocan que la generación residuos, sobre todo de aquellos con una elevada carga orgánica, sea cada vez mayor. La gestión de estos residuos es uno de los retos más complejos a los que la sociedad actual debe enfrentarse. Una gestión inadecuada podría implicar serios daños ambientales como la contaminación del suelo y de las aguas subterráneas, así como potenciar el efecto invernadero, afectando al cambio climático y a la salud humana. Por lo tanto, desarrollar procesos de prevención y tratamiento adecuados es fundamental para garantizar un modelo socioeconómico basado en el desarrollo sostenible.

La tendencia actual para la gestión de estos residuos apuesta por la economía circular, aplicando técnicas que minimicen el impacto ambiental de los residuos, al mismo tiempo que permitan su revalorización al obtener subproductos de valor añadido. En este sentido, los lodos de depuradora procedentes tanto de las plantas de tratamiento de aguas residuales urbanas como de lixiviados de vertedero son tratados generalmente mediante procesos de digestión anaerobia, obteniendo biogás que puede usarse para la obtención de energía eléctrica y térmica. Recientemente, esta técnica también se aplica para los residuos con alto contenido orgánico procedentes de la actividad agraria y del desperdicio alimentario, tanto del entorno doméstico como de la producción primaria o la industria agroalimentaria, ya que contienen compuestos potencialmente útiles que pueden revalorizarse y emplearse posteriormente en la agricultura, en la industria farmacéutica, cosmética o química.

Sin embargo, a pesar de que la digestión anaerobia es una vía de tratamiento prometedora para estos residuos, su efectividad se ve reducida cuando el residuo o efluente industrial a tratar tiene una elevada carga en compuesto fenólicos complejos, lo que dificulta su tratamiento por los métodos biológicos convencionales. Estas macromoléculas se caracterizan por ser altamente recalcitrantes, tener un elevado color y una compleja estructura química. Suponen la fracción mayoritaria de la materia orgánica presente en aguas residuales como lixiviados de vertedero o efluentes de la industria papelera. También representan un porcentaje importante en los residuos lignocelulósicos, siendo necesario su pretratamiento de los residuos para su correcta gestión.

Por consiguiente, es sumamente importante la evaluación de nuevas técnicas de tratamiento que permitan el procesado de este tipo de residuos. En los últimos años, las investigaciones se han centrado en el estudio de diferentes tratamientos fisicoquímicos o biológicos que permitan el procesado de estos efluentes, reduciendo su impacto en el medio ambiente o mejorando sus características para incorporarse en un tratamiento posterior.

Las técnicas de oxidación avanzada como la oxidación húmeda o la ozonización han mostrado resultados satisfactorios en la eliminación de materia orgánica altamente recalcitrante. Sin embargo, el empleo de estos métodos está vinculado a altos costes operacionales debido a su las elevadas presiones y temperaturas necesarias para llevar a cabo el proceso de tratamiento. Por lo tanto, es evidente la necesidad de buscar alternativas que permitan reducir los compuestos recalcitrantes sin suponer elevados costes de realización. En este sentido, los métodos de degradación biológica mediante hongos se muestran como una tecnología atractiva de tratamiento. A diferencia de las bacterias, estos microorganismos son capaces de romper la materia orgánica recalcitrante en compuestos más simples gracias a la síntesis de enzimas extracelulares.

Así pues, resulta un tema de fundamental importancia la caracterización microbiológica de las corrientes generadas durante el tratamiento de estos residuos, y estudio de la influencia de los procesos operacionales en la distribución de los microorganismos. Además, como se ha comentado anteriormente, la investigación de técnicas alternativas de tratamiento que reduzcan la materia recalcitrante o permitan incrementar la revalorización de los residuos es esencial.

En este trabajo se realiza un estudio de estos aspectos en diferentes residuos agroalimentarios y efluentes industriales, con la finalidad de desarrollar tratamientos adecuados que permitan minimizar el impacto de los residuos, ampliar las aplicaciones biotecnológicas de los hongos en el tratamiento de residuos con alto contenido orgánico conocer la influencia de los parámetros operacionales en la eficacia de los tratamientos con el fin de seleccionar las condiciones operativas más optimas.

1.2. OBJETIVOS DE LA TESIS DOCTORAL

Esta Tesis se enmarca en el contexto general del tratamiento de residuos con alto contenido en estructuras fenólicas complejas. El objetivo principal de este trabajo es

evaluar diferentes técnicas fisicoquímicas o biológicas para el tratamiento de estos residuos, con el fin de optimizar los procesos de revalorización o tratamiento habituales. Para ello se estudiaron diversos aspectos como: la microbiología presente en el proceso de tratamiento, el efecto del tratamiento en la demanda química (DQO) y biológica (DBO) de oxígeno, la variación del color o la concentración de carbohidratos y compuestos fenólicos.

Así, los objetivos específicos de la Tesis Doctoral se desarrollan según los siguientes puntos:

- Caracterizar microbiológicamente las distintas corrientes generadas durante el proceso de digestión anaerobia de lodos de depurada y evaluar el efecto de los parámetros operacionales en la distribución de los microorganismos.
- Evaluar diversas alternativas de tratamiento fisicoquímicas para el licor de biometanización resultante del proceso de digestión anaerobia de lodos.
- Estudiar del empleo del hongo *P. chrysosporium* para el tratamiento biológico del licor de biometanización
- Determinar la población bacteriana de un proceso de nitrificación-desnitrificación de lixiviados de vertedero y determinar el efecto de los parámetros operacionales sobre la distribución de las comunidades bacterianas y la efectividad del proceso.
- Evaluar el uso del hongo *P. chrysosporium* para el tratamiento del permeado procedente de una planta de tratamiento biológico de lixiviados con tecnología BIOMEMBRAT.
- Investigar diferentes métodos de hidrólisis como pretratamiento de residuos agroalimentarios para potenciar la obtención de productos de valor añadido.
- Estudiar el uso del hongo *P. chrysosporium* para el tratamiento de residuos agroalimentarios y ganaderos.
- Evaluar el uso de los hongos para el tratamiento de residuos altamente recalcitrantes procedentes de la industria papelera.

1.3. ESTRUCTURA DE LA TESIS DOCTORAL

Para la consecución de los objetivos descritos anteriormente se han desarrollado varios trabajos de investigación, autónomos entre sí, pero todos relacionados estructuralmente, cuyo conjunto ha conformado esta memoria.

Algunos de estos trabajos han sido aceptados para su publicación en revistas científicas incluidas en *Science Citation Index Expanded*. Por tanto, esta Tesis Doctoral se presenta como un compendio de publicaciones enmarcadas en la línea de investigación correspondiente al tratamiento, tanto fisicoquímico como biológico, de residuos con un elevado contenido en materia orgánica, principalmente de estructuras fenólicas complejas altamente recalcitrantes. Cada una de las publicaciones se ha divido a su vez en diferentes apartados, siguiendo la estructura tradicional de introducción, material y métodos, resultados y discusión, y conclusiones. La referencia completa de la revista o editorial en la que los trabajos han sido publicados, se encuentra detallada al inicio de cada investigación.

La estructura de esta memoria está dividida en 7 capítulos.

El **Capítulo 1** corresponde con la introducción de la Tesis Doctoral. En este primer apartado, se exponen los principales motivos que han impulsado la realización de este trabajo, y se definen los objetivos concretos de la investigación. Además, se incluye una explicación de cómo se ha estructurado la tesis doctoral y estructurada la memoria.

El **Capítulo 2**, consiste en un desarrollo teórico sobre la obtención de los residuos empleados en este trabajo, sus características y volumen, así como una visión general de los procesos de tratamientos posibles para cada uno de ellos. El objetivo fundamental de este capítulo es plantear y fundamentar teóricamente la investigación, centrando el campo de estudio en unos residuos concretos.

El **Capítulo 3**, describe los materiales y los métodos experimentales empleados de forma general en el desarrollo de la investigación, así como los métodos analíticos utilizados para la determinación de los parámetros evaluados. Esta información también se muestra en la sección "*Materials and Methods*" de cada una de las investigaciones,

donde puede consultarse la metodología específica que se ha empleado en cada uno de los estudios.

El **Capítulo 4**, muestra los resultados obtenidos en la investigación. Este capítulo está dividido a su vez en 4 subapartados, atendiendo a la naturaleza del residuo evaluado. Así, podremos encontrar el apartado **4.1** que hace referencia a los tratamientos post-anaerobiosis, donde los ensayos fueron realizados con el licor de biometanización como material de partida. El apartado **4.2**, recoge las investigaciones relacionadas con el permeado procedente del tratamiento biológico de lixiviados. Los estudios relativos al tratamiento de los residuos agroalimentarios y ganaderos se encuentran detallados en el apartado **4.3**. Finalmente, el apartado **4.4** recoge las investigaciones relativas al tratamiento del licor negro procedente de la industria papelera. Al inicio de cada subapartado se encuentra una breve descripción de la cada uno de los trabajos incluidos.

El **Capítulo 5**, expone las principales conclusiones obtenidas en la Tesis doctoral.

Se incluye también un apartado de Anexos que muestra la información suplementaria a los resultados expuestos en el capítulo 4. Finalmente, esta Tesis contiene una última sección donde se recoge los diferentes medios de difusión de los resultados obtenidos, incluyendo comunicaciones en congresos (póster u oral) y publicaciones en revistas.

Las referencias bibliográficas que se ha consultado durante el desarrollo de esta Tesis se muestran al final de cada uno de los capítulos, salvo en el caso del Capítulo 4, donde la bibliografía empleada se detalla para cada investigación en concreto, al final de cada subapartado.

Capítulo 2.

CONSIDERACIONES TEÓRICAS

2.1. RESIDUOS PROCEDENTES DE LA DIGESTIÓN ANAEROBIA DE LODOS

El aumento de la población y el rápido desarrollo industrial tiene como consecuencia directa un incremento en el volumen de las aguas residuales urbanas procedentes tanto del ámbito doméstico como de las actividades agrícolas e industriales desarrolladas en áreas urbanas. Estas aguas residuales junto con las aguas procedentes de la escorrentía pluvial pueden contener elementos contaminantes que dañen seriamente el medio ambiente. Tal y como se establece en la legislación vigente, concretamente en la Directiva 91/271/EEC (modificada por la Directiva 98/15/CE), estas aguas residuales necesitan tratarse mediante procesos fisicoquímicos y biológicos, con el fin de minimizar la concentración de los compuestos contaminantes hasta límites aceptables que permitan su vertido.

Las estaciones de depuración de aguas residuales urbanas (EDAR) son las responsables del tratamiento de este tipo de efluentes. Durante el proceso, además del efluente depurado, se obtienen lodos como consecuencia de los procesos de sedimentación a lo largo de los tratamientos primarios y secundarios de las aguas residuales. Estos lodos de depuradora, con más de un 95% de humedad, poseen una alta carga orgánica y microbiológica, así como los metales pesados, entre ellos cadmio (Cd), cromo (Cr), cobre (Cu), mercurio (Hg), níquel (Ni), plomo (Pb) y zinc (Zn) [1,2]. Generalmente, los lodos resultantes se someten a diferentes tratamientos de compostaje, estabilización aerobia o digestión anaerobia para disminuir su carga contaminante y asegurar su aplicación ambiental de forma segura.

Según el Instituto Nacional de Estadística de España (INE), a nivel nacional y desde 2018, en torno al 86% de los lodos tratados se aplican en el sector agrícola, incluyendo la jardinería y la silvicultura, mientras que sólo el 8% y el 6% se destinan a vertedero o aprovechamiento energético, respectivamente. Resulta interesante destacar que, tal y como se refleja en la Figura 2.1, estos porcentajes se invierten en el caso particular del Principado de Asturias, donde el 41% de los lodos generados en depuradoras se deriva a procesos de aprovechamiento energético, un 55% tiene como destino final el vertedero y solamente un 4% se emplea en el sector agrícola.

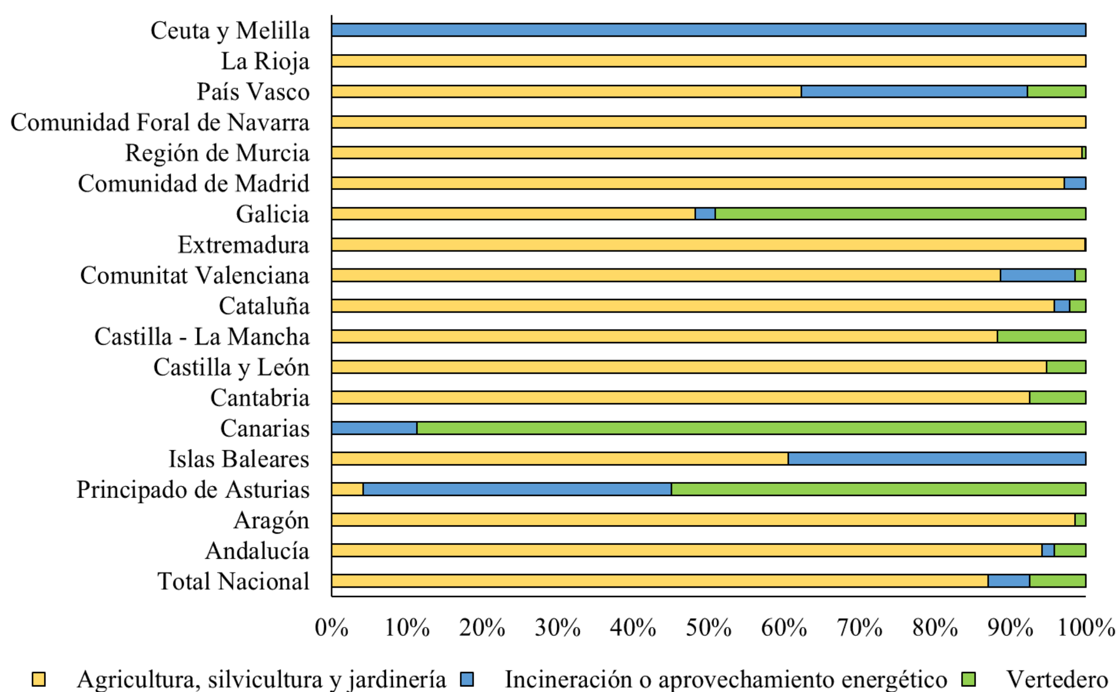


Figura 2. 1. *Destino de los lodos generados por comunidades y ciudades autónomas en España durante 2018. (Fuente: INE)*

Esta diferencia tan marcada en cuanto al aprovechamiento de los lodos de depuradora está directamente relacionada con el hecho de que, a finales del año 2013, la Compañía para la Gestión de los Residuos Sólidos en Asturias (COGERSA) puso en marcha, una planta de digestión anaerobia por vía húmeda para el tratamiento de lodos procedentes de la depuración de las aguas residuales urbanas, que ha permitido la revalorización de estos residuos. Actualmente, esta planta de tratamiento tiende a incorporar también la fracción orgánica de los residuos municipales (FORM).

El proceso de digestión anaeróbica (DA), también denominado biometanización, es un proceso bioquímico por el cual un conjunto heterogéneo de microorganismos degrada la materia orgánica en ausencia de oxígeno, generando como productos finales: biogás y digestato [3,4].

El biogás es un gas renovable, formado principalmente por metano (CH_4) y dióxido de carbono (CO_2), mezclado en menor proporción con otros gases como hidrógeno (H_2), sulfuro de hidrógeno (H_2S) o vapor de agua. Este subproducto puede

emplearse en distintos sectores como combustible o en procesos de generación de energía eléctrica, desplazando a las materias primas de origen fósil [5].

Las actuales estrategias en política ambiental impulsan la generación de este tipo de biocombustible. En concreto, la Directiva (UE) 2018/2001, relativa al fomento del uso de energía procedente de fuentes renovables, eleva el objetivo obligatorio de energía procedente de fuentes renovables hasta un 32% en el consumo final bruto de energía y hasta un 14% en el consumo final de energía para todos los tipos de transporte, para el año 2030. El desarrollo de estas políticas junto con el aumento constante de la demanda energética y el agotamiento de las reservas de combustibles fósiles hace que el biogás se convierta en un producto de alto valor añadido.

Por ello, la aplicación de la digestión anaerobia para el tratamiento de lodos de depuradora ha suscitado un gran interés, ya que permite el tratamiento y la valorización simultanea de este tipo de residuos. En los últimos años, esta técnica también se ha considerado una tecnología atractiva para el procesamiento de otro tipo de residuos producidos en los entornos urbanos, industriales o agrícolas, que al igual que los lodos de depuradora, poseen una elevada concentración de materia orgánica [6,7].

Estos residuos pueden tratarse o bien de forma independiente, o mezclados, lo que se conoce como procesos de co-digestión anaerobia [8]. Estos tratamientos tienen varias ventajas, ya que al tratar conjuntamente diferentes residuos se pueden obtener mayores eficacias en cuanto a la obtención de biogás, al combinar la composición química de las materias primas, reducir los gastos derivados de su gestión, y compensar la temporalidad de algunos residuos [9].

Como se ha mencionado anteriormente, además de biogás, el proceso de biometanización también genera un digestato. Este residuo semilíquido, parcialmente estabilizado, que tiene una elevada concentración de materia orgánica y minerales. Destaca por su concentración en nitrógeno, potasio y fósforo, por lo que puede emplearse como fertilizante orgánico para mejorar la calidad del suelo agrícola. Su aplicación agrícola está permitida siempre y cuando cumpla con los parámetros establecidos en la legislación vigente, principalmente los regulados por la Directiva Europea 91/676/CEE [10].

Sin embargo, hay que tener en cuenta que este efluente final es complejo, y puede contener una concentración variable de compuestos tóxicos o microorganismos patógenos que no permitan su aplicación directa [11]. Además, algunas características del digestato, como el olor, la viscosidad, el alto contenido en ácidos grasos volátiles o su elevada carga orgánica recalcitrante pueden ser perjudiciales para el medio ambiente [12]. Por tanto, en la mayoría de los casos es preciso aplicar métodos de almacenamiento y pretratamiento adecuados que eviten serios daños medioambientales por una gestión inadecuada del digestato. Esta necesidad de tratamiento ha sido uno de los aspectos que se ha considerado en esta Tesis Doctoral y que impulsado las investigaciones llevadas a cabo en el Capítulo 5.1.

2.1.1. Proceso biológico de digestión anaerobia

La digestión anaerobia es un proceso complejo que implica la actuación de diversos grupos de microorganismos para la degradación de los diferentes sustratos.

La composición de la población bacteriana en cada etapa del proceso de biometanización va a estar condicionada en gran medida por los parámetros operacionales del proceso (pH, temperatura, agitación, etc.) y por la composición química del residuo de partida [13]. La presencia de compuestos contaminantes puede inhibir o ralentizar el desarrollo de los microorganismos. Además, una elevada concentración de estructuras fenólicas complejas como lignina o ácidos húmicos puede dificultar el proceso, ya que la rotura de esos compuestos durante la fase inicial de hidrólisis es mucho más compleja. Varios autores han reportado mejoras en el proceso de biometanización y por consiguiente, un incremento en la cantidad de metano obtenido, sometiendo el residuo inicial a diferentes pretratamientos mecánicos, térmicos o con ultrasonidos [14–17].

Por otro lado, el contenido en sólidos del residuo va a determinar que el tratamiento de biometanización se desarrolle por vía húmeda o seca. En este contexto, se denominan procesos de anaerobia por vía húmeda, aquellos en lo que el residuo inicial contiene un porcentaje de materia seca por debajo del 15%, mientras que, por vía seca el contenido está entre el 15% y el 40% [18].

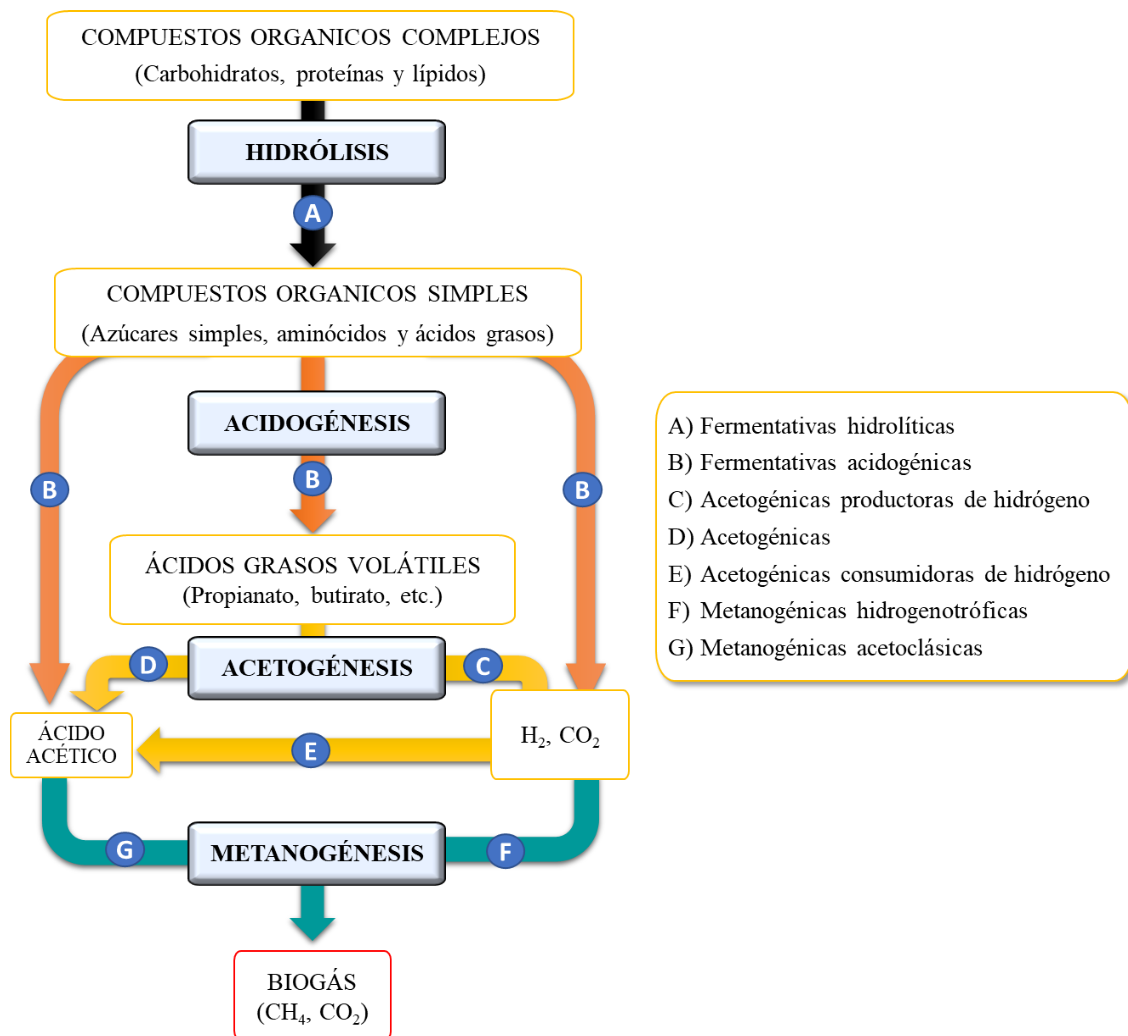


Figura 2. 2. *Etapas del proceso de digestión anaerobia y microorganismos implicados. Adaptada de Moraes et al. [15]*

A pesar de la complejidad del proceso de biometanización, atendiendo al sustrato degradado, se puede hablar de cuatro etapas principales [19] (ver Figura 2.2):

A) Hidrólisis:

Esta primera etapa, consiste en la transformación de los compuestos orgánicos complejos (proteínas, lípidos, polisacáridos, etc.) en compuestos de bajo peso molecular que los microorganismos pueden asimilar y utilizar como fuente de energía y nutrición. Esta biodegradación se lleva a cabo por las bacterias hidrolíticas, que segregan diferentes enzimas extracelulares como celulasas, lipasas, amilasas y proteasas, entre

otras [20]. La velocidad de descomposición durante la etapa de hidrólisis depende en gran medida de la naturaleza del sustrato. La mayoría de los microorganismos responsables de este proceso pertenecen a los géneros *Clostridium*, *Bacillus*, y *Staphylococcus* [21].

***B) Acidogénesis*:**

Durante esta fase los compuestos orgánicos simples solubilizados en la etapa hidrolítica anterior son absorbidos por las bacterias acidogénicas, produciendo principalmente ácidos grasos volátiles (AGV), alcoholes, ácido acético (CH_3-COOH), hidrógeno (H_2) y dióxido de carbono (CO_2) [22]. Se ha relacionado este proceso de biodegradación con la presencia de microorganismos pertenecientes a los géneros *Lactobacillus*, *Escherichia*, *Pseudomonas*, *Streptococcus*, y *Desulfobacter* [21].

C) Acetogénesis:

Esta etapa ocurre de forma simultánea a la acidogénesis. En este caso, las bacterias acetogénicas son las encargadas de convertir los ácidos grasos volátiles en ácido acético, el dióxido de carbono y el hidrógeno [23]. Entre las bacterias implicadas en este proceso de han descrito microorganismos pertenecientes a los géneros *Acetobacterium*, *Acetoanaerobium*, *Acetogenium*, *Butyribacterium*, *Clostridium*, *Eubacterium* y *Pelobacter* [24].

D) Metanogénesis:

Es la última fase del proceso biológico de digestión anaerobia y consiste en la producción de metano y dióxido de carbono a partir de los productos intermedios procedentes de la acidogénesis y la acetogénesis. En este proceso han identificado microorganismos pertenecientes a los géneros *Methanosaeta*, *Methanosarcina* *Methanobacterium*, y *Methanospirillum* [25,26].

2.1.2. Tratamientos habituales de los efluentes de digestión anaerobia

Como se ha mencionado anteriormente, tras el proceso de digestión anaerobia se obtienen como productos finales, biogás y digestato. En general, ambos subproductos necesitan un postratamiento antes de ser utilizados. En la Figura 2.3, se muestran

algunos de los tratamientos habituales a los que se somete tanto el biogás como el digestato para su acondicionamiento.

Tanto la calidad del biogás como su valor económico final son factores variables en función de la pureza del biocombustible. Su composición final depende en gran medida de la materia prima empleada en el proceso de digestión anaerobia y de las condiciones operacionales del tratamiento. Además de CH_4 y CO_2, el biogás puede contener trazas de amoniaco (NH_3), sulfuro de hidrógeno (H_2S), haluros, siloxanos y mercaptanos.

La presencia de estos compuestos puede generar problemas de ensuciamiento, corrosión y erosión cuando el biogás se utiliza en sistemas térmicos o catalíticos [27]. Además, el H_2S presente en el biogás se oxida a dióxido de azufre (SO_2) al quemarse, uno de los principales contaminantes atmosféricos. Por ello, la purificación y mejora del biogás ha sido un tema de investigación destacado en los últimos años [28].

Para la mejora del biogás se han investigado diferentes técnicas, todas ellas enfocadas principalmente a reducir la humedad y la concentración de H_2S y CO_2 en el biocombustible. Generalmente, los tratamientos aplicados se basan en el uso de técnicas de absorción mediante diferentes disolventes, que por contacto en contracorriente con el gas, logran la eliminación de H_2S y CO_2 [29–31]. También se ha reportado el uso técnicas de adsorción usando carbón activo o zeolitas [32,33] o métodos de separación por membranas [34–36].

Además de estos métodos fisicoquímicos, también se han descrito métodos biológicos, o combinaciones entre ambos, que logran una mejora satisfactoria. Estas tecnologías implican el uso de diferentes biofiltros, y la acción, en su mayoría, de las bacterias sulfuro-oxidantes [37–40]. Las investigaciones más recientes se han centrado de la aplicación de procesos de criogenización, logrando la separación de los gases en función de su temperatura de condensación y sublimación [41,42].

En cuanto al digestato, su tratamiento y gestión es más complicado. Como se indicó anteriormente, aunque puede aplicarse directamente como fertilizante, en la mayoria de los casos es nesario un postratamiento que acondione el digestato y evite

daños medioambientales como la contaminación y eutrofización de las aguas cercanas [43]. Generalmente este proceso sólo se lleva a cabo cuando el digestato obtenido proviene de un proceso de biometanización por vía seca ya que se obtiene un producto final con un contenido de materia orgánica entre el 30% y 40%, permitiendo su aplicación directa.

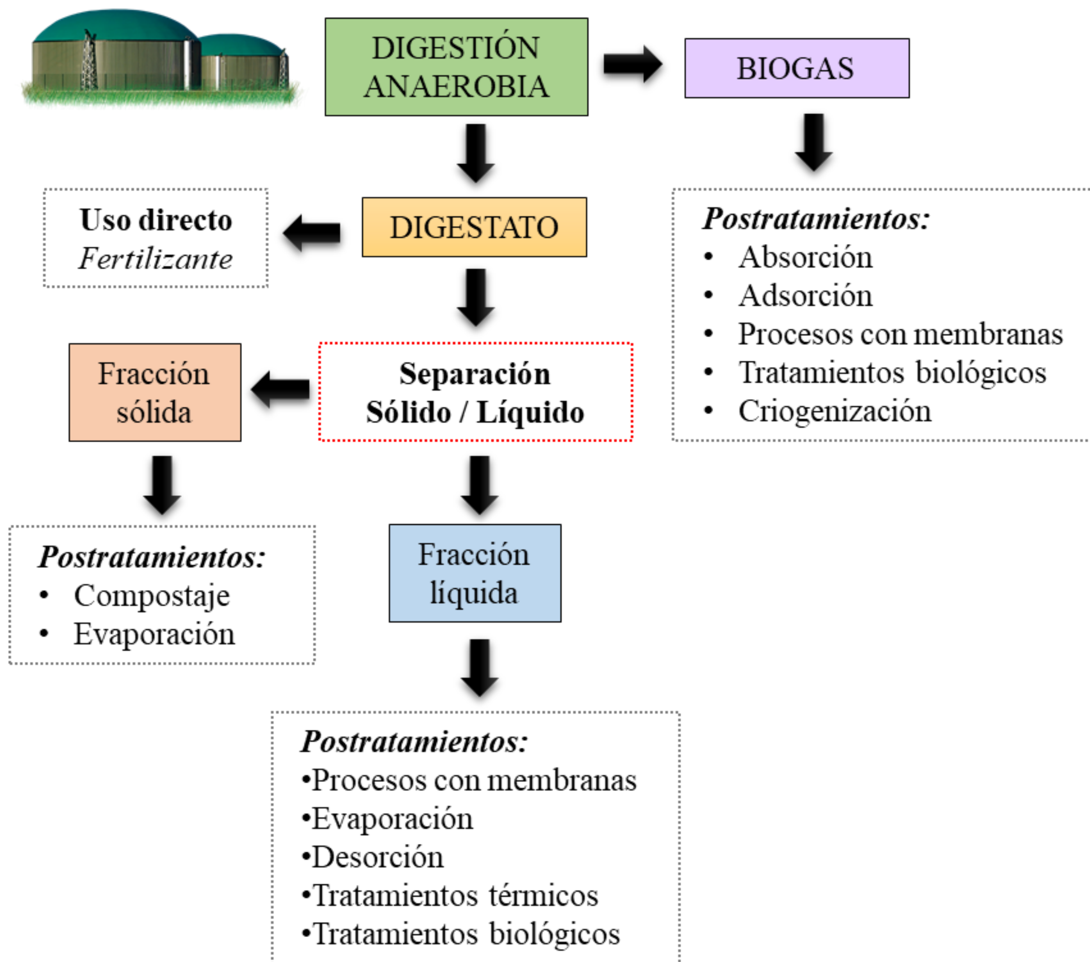


Figura 2. 3. *Posibles tratamientos de los subproductos de la digestión anaerobia.*

Cuando la digestión anaerobia se lleva a cabo por vía húmeda, la opción habitual es separar el digestato en fase líquida y sólida mediante procesos de filtración o centrifugación. Peng and Pivato [43], reportaron que tras un proceso de centrifugación la fracción sólida del digestato acumuló entre el 55% - 65% del fósforo, mientras que entre el 70% y el 80% del total del nitrógeno en forma de amonio se encontró en el

digestato líquido. La concentración de compuestos contaminantes y metales pesados en ambas fracciones dependerá en gran medida del residuo de partida.

La elección de emplear unas técnicas u otras de postratamiento se basa fundamentalmente en el destino final del efluente tratado. De forma general, se pretender abordar dos objetivos: el acondicionamiento del digestato para ser empleado como un biofertilizante o la eliminacion de los compuestos tóxicos y de la materia orgánica recalcitrante para permitir un vertido seguro. En la literatura podemos encontrar multiples trabajos de investigacíon relacionados con los métodos de tratamiento empleados tanto para la fracción sólida como líquida del digestato [44].

En relación a la fase sólida, destacan los procesos de secado que permiten concentrar su contenido en nutrientes y además facilitan su gestión al reducir su volumen. Dado que este proceso requiere una gran cantidad de energía, muchas investigaciones se han centrado en el aprovechamiento de la energía excedente del proceso de biometanización. Un vez seco, el digestato puede ser pelletizado y utilizado como fertilizante orgánico o biocombustible en procesos de combustión.

Por otro lado, se han evaluado técnicas de pirólisis que permiten la obtención de biochar, que a su vez puede emplearse como superficie de fijación para los microorganismos durante el proceso de biometanización [11]. Tambien se ha estudiado el empleo de otras técnicas como el uso de microondas, hidrólisis y ultrasonidos, que principalmente rompen la materia orgánica compleja, favoreciendo su solubilización en procesos posteriores [44]. En cuanto a métodos biológicos, destaca su tratamiento en plantas de compostaje debido a la gran cantidad de nutrientes y componentes orgánicos presentes en el digestato sólido. Sin embargo, el compost resultante puede contener metales pesados lo que supone un riesgo ambiental [45]. Otros procesos incluyen las hidrólisis enzimáticas empleando generalmente hongos capaces de sintetizar enzimas que rompan los compuestos complejos (lignina, celulosas, polisacáridos) en productos más biodegradables.

La gestión y el tratamiento de la fracción líquida del digestato es mucho más complicado, por lo que este efluente ha sido objeto de estudio en esta Tesis Doctoral. Generalmente, una porción de la fracción líquida del digestato se recircula al proceso de digestión anaerobia. Sin embargo, una gran parte necesita ser almacenada y tratada.

Los tratamientos más habituales incluyen la tecnología de membranas, la evaporación, los procesos térmicos, generalmente de hidrólisis, y los tratamientos biológicos. En cuanto a las técnicas de filtración por membranas, la mayoria de los estudios se han centrado en uso de métodos de ósmosis inversa y ultrafiltracion, que permiten la concentración de los nutrientes. Sin embargo, estos métodos permiten trabajar con pequeños volúmenes de efluente y requieren una gran energia, lo que encarece el proceso. Otro de los problemas es que el efluente obtenido sigue teniendo altas concentraciones de amonio [46].

Los métodos de evaporación han suscitado un creciente interés, sobre todo para su aplicación en aquellas plantas de biometanización donde hay exceso de energia o calor. Dentro de ellos, el empleo de evaporadores al vacio se ha considerado como una de las técnicas más prometedoras al permitir el tratamiento de grandes volúmenes de digestato sin necesidad de temperaturas muy elevadas (85ºC - 90ºC) [47].

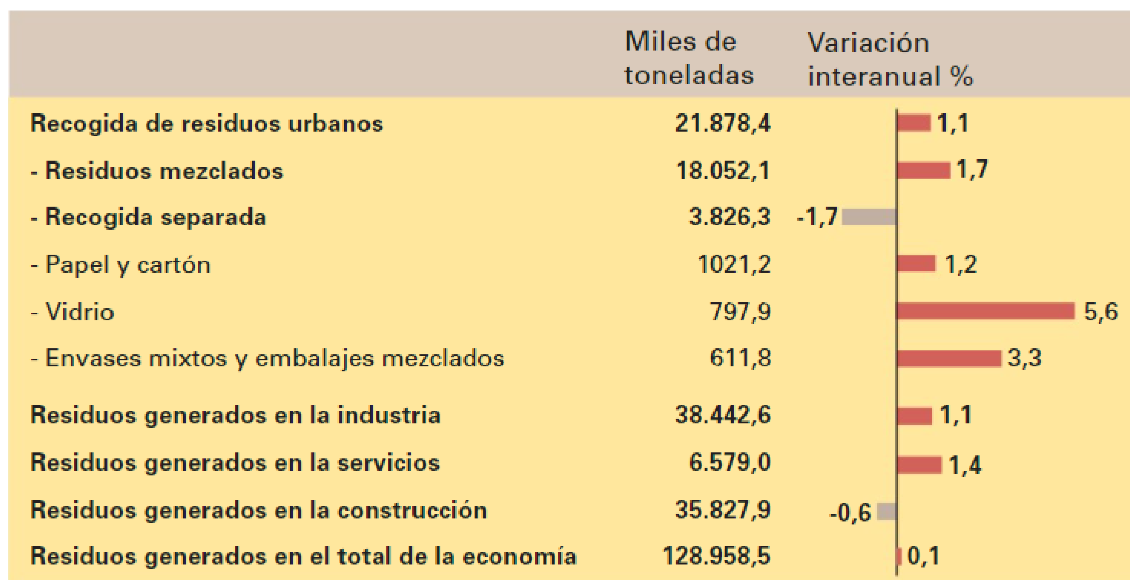
Otra opción para la recuperación de los nutrientes son las técnicas de desorción y precipitación que permiten obtener fertilizantes líquidos y sólidos (estruvita). Las técnicas de desorción se aplican cuando los digestatos contienen altas concentraciones de amoniaco. Como producto final se obtiene sulfato de amonio, utilizado como fertilizante principalmente en suelos alcalinos o neutros. La precipitación de estruvita es una tecnología que se utiliza principalmente para la recuperación de fósforo. Este compuesto es un fertilizante de liberación lenta con un contenido relativamente bajo de contaminantes que puede reemplazar a otros fertilizantes producidos a partir de roca fosfórica [48]. Otras tecnologías empleadas se fundamentan en procesos de oxidación que convierten el amonio y el nitrógeno orgánico en nitrógeno gaseoso o nitrato. Entre estas técnicas destacan la ozonización y la oxidación química, que usan oxidantes fuertes como el ozono y el peróxido de hidrógeno para aumentar la biodegradabilidad de la materia orgánica. Estos métodos permiten reducir el volumen de lodos y controlan las emisiones de olores.

El empleo métodos biologicos está muy limitado debido a las elevadas concentraciónes de nitrógeno en el efluente, por lo que la literatura al respecto es escasa [43,45]. Un tratamiento posible es su incorporación a un proceso de nitrificación-denitrificación, por ejemplo, al mezclarse con lixiviados de vertedero. Sin embargo, puede dar lugar a un efluente con niveles de DQO y nitrógeno, superiores a los

parámetros de diseño de la planta de tratamiento, incrementando los costes o disminuyendo la efectividad del proceso. En este sentido, la oxidación anaeróbica de amonio mediante procesos Anammox, es otra alternativa [49]. Otra opción puede ser el tratamiento biológico mediante hongos debido a su capacidad de sintetizar enzimas extracelulares que pueden degradar la materia orgánica recalcitrante del efluente [50–52]. Mientras que el uso de estos microorganismos como pretratamiento en el proceso de biometanización, o como tratamiento de la fracción sólida ha sido evaluado, su aplicación para el digestato líquido apenas ha sido estudiada [53].

2.2. RESIDUOS PROCEDENTES DEL TRATAMIENTO BIOLÓGICO DE LIXIVIADOS

El modelo actual de producción y consumo provoca que la generación de residuos a nivel mundial sea muy elevada. Según el INE, en España, a lo largo del año 2018 se generaron aproximadamente 22,7 millones de toneladas de residuos urbanos, lo que corresponde a unos 486 kilos de residuos urbanos por habitante. Esto supone un incremento del 3,5% en comparación con los residuos generados en el año 2016 (ver Figura 2.4) [54].



	Miles de toneladas	Variación interanual %
Recogida de residuos urbanos	21.878,4	1,1
- Residuos mezclados	18.052,1	1,7
- Recogida separada	3.826,3	-1,7
- Papel y cartón	1021,2	1,2
- Vidrio	797,9	5,6
- Envases mixtos y embalajes mezclados	611,8	3,3
Residuos generados en la industria	38.442,6	1,1
Residuos generados en la servicios	6.579,0	1,4
Residuos generados en la construcción	35.827,9	-0,6
Residuos generados en el total de la economía	128.958,5	0,1

Figura 2. 4. *Residuos generados en España durante el año 2016 [47].*

El dato más preocupante es que la mayoría de estos residuos (54%) se depositaron en vertedero, mientras que sólo un 34% se derivó a procesos de reciclaje. El 12% restante fue tratado en plantas incineradoras para su eliminación. El depósito de residuos urbanos en vertederos es una práctica sencilla y económica, pero supone una amenaza importante para el medio ambiente debido a los lixiviados que forman en este tipo de estructuras.

Los lixiviados son efluentes líquidos que se originan a partir de las filtraciones del agua a través de los depósitos de desechos. La composición específica de los lixiviados va a depender del tipo de residuos, de las condiciones climáticas y de la edad e hidrogeología del vertedero [51]. Los lixiviados obtenidos a partir de un vertedero de corta edad se caracterizan comúnmente por una alta demanda bioquímica de oxígeno (DBO) y demanda química de oxígeno (DQO), por lo que su tratamiento es más sencillo. Sin embargo, en los lixiviados maduros, la fracción orgánica dominante está compuesta principalmente por los compuestos altamente recalcitrantes [55]. Estos efluentes se caracterizan generalmente por altas concentraciones de materia orgánica, amonio y sales metálicas y cloradas potencialmente tóxicas [56].

Por tanto, es necesario llevar a cabo una gestión adecuada de estos efluentes y un tratamiento óptimo que permita eliminar los compuestos contaminantes y refractarios. Si los lixiviados de los vertederos no tratados se infiltran o se descargan en el suelo o las aguas subterráneas se causarán dañaos inconmensurables al entorno ecológico y a la salud humana. Varios artículos científicos se han centrado en recopilar detalladamente las técnicas más aplicadas para el tratamiento de lixiviados, siendo algunas de las revisiones más actuales las llevadas a cabo por Yu et al. [57], Bandala et al. [58] y Teng et al. [59]. En general, las tecnologías para el tratamiento de los lixiviados se pueden agrupar en tres categorías: tratamientos físicos, de oxidación avanzada y biológicos. Entre los tratamientos físicos, la coagulación y la adsorción son las técnicas más utilizadas, reportando ambas buenos resultados en cuanto a la eliminación de DQO, turbidez y sólidos en suspensión [60,61].

Los tratamientos de oxidación avanzada se han considerado tecnologías prometedoras al destruir eficazmente las estructuras complejas presentes en los lixiviados. Las técnicas más empleadas se basan en procesos de oxidación tipo Fenton, oxidaciones con persulfato y oxidaciones electroquímicas [62,63]. El problema de estas

técnicas es el alto coste y la necesidad de utilizar un compuesto oxidante, que podría afectar a la calidad del agua final [57].

Entre los tratamientos biológicos, destacan los tratamientos de nitrificación y desnitrificación. Estos métodos se han mostrado efectivos para la eliminación del nitrógeno y la materia orgánica de los lixiviados, sobre todo de lixiviados jóvenes, mostrándose menos efectivos cuando el efluente tratado contiene alta carga de compuestos refractarios, debido a que las bacterias no son capaces de degradar estos compuestos complejos [59]. Aun así, los tratamientos biológicos mediante bacterias nitrificantes y desnitrificantes son ampliamente utilizados. Si bien, en algunos casos, es necesario aplicar un tratamiento terciario a los lixiviados obtenidos ya que los niveles de DQO en el efluente son superiores al límite permitido para su vertido [56].

2.2.2. Etapas del proceso de nitrificación y desnitrificación

Los procesos de nitrificación y desnitrificación biológica son las técnicas más estudiado para la eliminación de nitrógeno de diferentes aguas residuales, entre ellas, los lixiviados de vertedero. Las plantas de tratamiento biológico de lixiviados combinan ambos procesos para alcanzar una degradación óptima de los efluentes [56].

A) Nitrificación

Durante esta fase se lleva a cabo la oxidación aeróbica del amoníaco a nitrito y luego a nitrato. El proceso lo realizan bacterias quimiolitoautótrofas que obtienen su energía a partir de la oxidación de los compuestos nitrogenados. Este proceso ocurre en condiciones aerobias ya que se necesita una elevada concentración de oxígeno para la oxidación del amoníaco [64]. Por ello, la aireación es fundamental en esta etapa del proceso siendo uno de los puntos que encarecen esta técnica. La fase de nitrificación tiene lugar en dos pasos (Figura 2.5): primero, las bacterias amonio oxidantes (AOB) pertenecientes al género *Nitrosomonas*, oxidan el amonio presente en los lixiviados a nitrito. Después las bacterias nitrito oxidantes (NOB) relativas al género *Nitrobacter*, se encargan de transformar ese nitrito a nitrato [65].

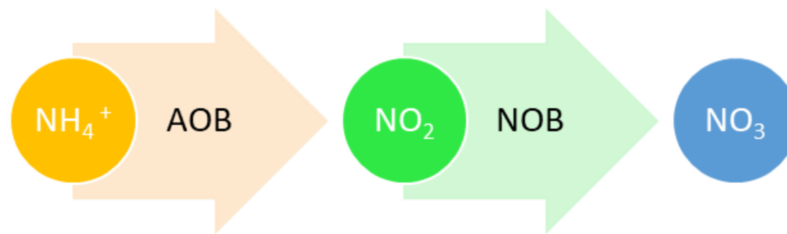


Figura 2. 5. *Proceso de nitrificación mediante bacterias amonio oxidantes (AOB) y bacterias nitrito oxidantes (NOB)*

Desde el punto de vista microbiológico, es necesario que las condiciones de operación en los reactores sean debidamente controladas ya que un desequilibrio en la concentración de los microorganismos podría dificultar seriamente el proceso. Por ejemplo, una elevada concentración de nitritos favorece el crecimiento y la actividad de AOB y disminuye la proliferación y actividad de NOB [66]. Por otro lado, se ha informado de que las bacterias del grupo NOB crecen más lentamente que las AOB, por lo que controlar el tiempo de retención de lodos en los reactores es un factor importante que podría ayudar a disminuir la población de NOB [67]. Algunos estudios también han encontrado que AOB tiene una mayor afinidad por el oxígeno que NOB, lo que significa que la baja concentración de DO podría inhibir el crecimiento y la actividad de NOB [68].

B) Desnitrificación

La siguiente etapa es la desnitrificación, donde el nitrato (formado en la etapa de nitrificación) se transforma, en condiciones de anoxia, en nitrito, luego en óxido nitroso, óxido nítrico y finalmente en nitrógeno gaseoso [64].

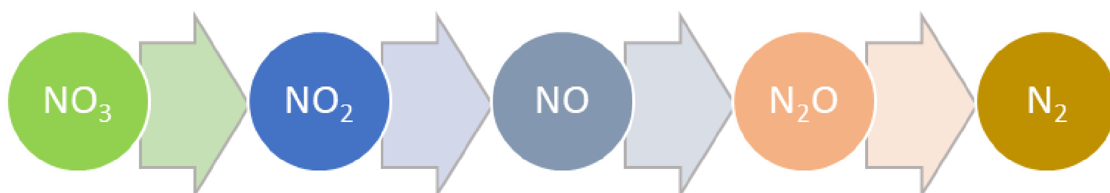


Figura 2. 6. *Proceso de desnitrificación*

Los microorganismos desnitrificantes son heterótrofos y, en condiciones anóxicas, utilizan nitrato o nitrito como aceptor final de electrones [69]. Dado que se necesita materia orgánica (donante de electrones) en este paso, las plantas de tratamiento biológico de lixiviados suelen anteponer el proceso de desnitrificación al de nitrificación, y existir entre ambos, una gran recirculación del material tratado. Algunos de los géneros relacionados con los procesos de desnitrificación son *Thiobacillus*, *Paracoccus*, *Bacillus* y *Pseudomonas* [70].

2.2.3. Tratamientos terciarios de lixiviados

Debido a la composición tan compleja de los lixiviados de vertedero, en ocasiones es necesario un tratamiento terciario para eliminar la materia orgánica que no ha sido degradada durante el tratamiento biológico. Estos tratamientos suelen ser necesarios cuando se emplean lixiviados maduros que poseen una elevada concentración de estructuras fenólicas complejas altamente refractarias [71].

En este sentido se han aplicado tanto tratamientos físicos, químicos y biológicos, siendo necesario en algunos casos la combinación de varios de ellos. Los procesos de adsorción han sido ampliamente utilizados para el tratamiento terciario de lixiviados de vertedero, usando principalmente carbón activo [72,73] u adsorbentes magnéticos [74]. Sin embargo, estas técnicas requieren de la recuperación de los materiales adsorbentes empleados lo que supone un problema.

Las técnicas de oxidación avanzada como la ozonización, la oxidación electroquímica, la oxidación húmeda o los procesos Fenton, han sido ampliamente evaluados para el tratamiento de lixiviados [56,75,76], reportando resultados muy prometedores en cuanto a la eliminación de compuestos refractarios. El mayor inconveniente de estas técnicas es que necesitan una elevada cantidad de energía para su tratamiento. Las condiciones operacionales, así como la edad del lixiviado pueden influir en la efectividad del proceso. En este sentido, Oulego et al. [77] informaron de una menor mineralización y biodegradabilidad final al tratar por oxidación húmeda lixiviados maduros. Otros autores como Anglada et al. [78] han informado de que la previa acidificación del lixiviado a pH ácidos puede favorecer el proceso de oxidación húmeda, ya que reduce el contenido orgánico disuelto y acelera las reacciones de oxidación.

El empleo de métodos de filtración para el tratamiento de lixiviados, generalmente combinados con otros tratamientos fisicoquímicos o biológicos, ha sido evaluado por varios autores [79,80]. Esta tecnología se aplica en la planta de tratamiento de lixiviados de COGERSA (Asturias), que tras el tratamiento biológico depuración biológica a presión mediante nitrificación y desnitrificación acopla a una etapa de ultrafiltración (UF) para la separación del efluente tratado (permeado). En ocasiones, las concentraciones de algunos de los compuestos contenidos en el permeado son superiores a los límites permitidos de vertido, por lo que es necesario aplicar un tratamiento adicional. Concretamente COGERSA aplican un tratamiento de adsorción con carbón activo. El interés por nuevas técnicas que permitan tratar de forma alternativa al permeado ha sido uno de los motivos que ha impulsado las investigaciones del capítulo 4.2 de esta Tesis.

2.3. RESIDUOS AGROPECUARIOS

En los apartados anteriores se ha informado de cómo el incremento de la industrialización y la población global a lo largo de los últimos años ha ocasionado un aumento de las aguas residuales urbanas y de los residuos depositados en vertedero. Estos hechos, también se ven reflejados en el volumen mundial de residuos agropecuarios generados en las actividades agrarias, ganaderas o silvícolas. [81]. Dentro de los residuos agroalimentarios destacan los procedentes del desperdicio alimentario por malas prácticas de producción y consumo, y los procedentes de los descartes generados durante el procesado de los alimentos. En cuanto a los residuos generados por las actividades ganaderas, se encuentran los estiércoles y purines, los residuos zoosanitarios y los subproductos de origen animal no destinados a consumo humano (SANDACH).

2.3.1 Residuos agroalimentarios

Según la Organización de las Naciones Unidas para la Alimentación (FAO) [82], la cantidad de los alimentos que se desperdician per cápita en Europa y América del Norte son de 280 a 300 kg/año, respectivamente.

En estos países, más del 40 % de las pérdidas de alimentos se produce en la venta minorista y el consumo, mientras que en los países que aún están en vías de desarrollo ese porcentaje se corresponde con las etapas de postcosecha y procesamiento. Si analizamos los datos que se muestran en la Figura 2.7, vemos que los consumidores de los países industrializados desperdician volúmenes muy similares a la producción de alimentos neta total del África subsahariana (222 millones de toneladas frente 230 millones de toneladas, respectivamente).

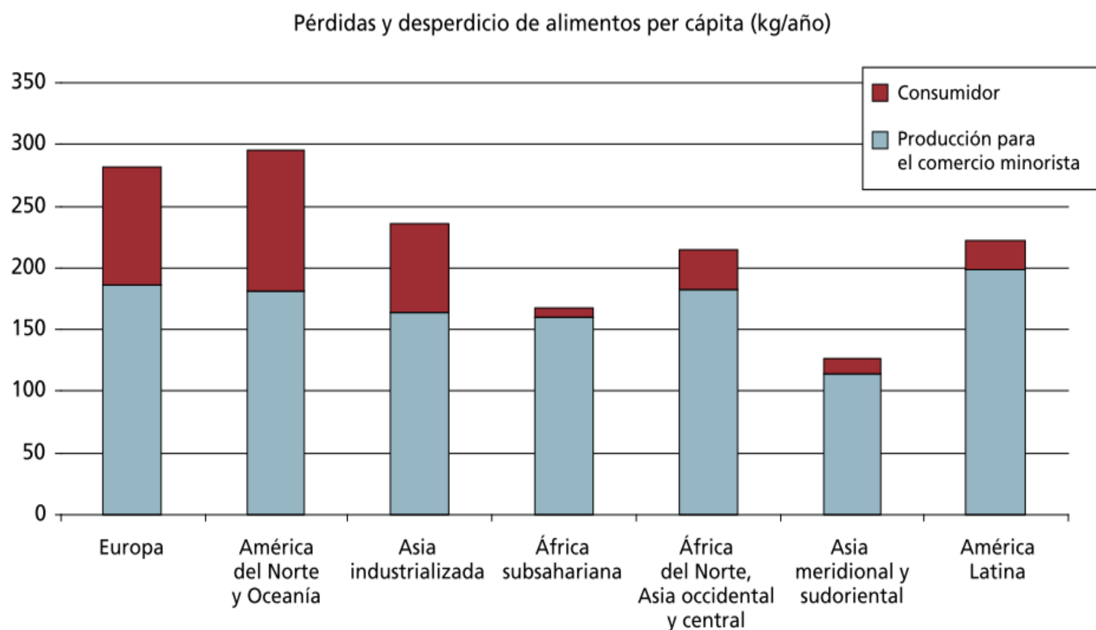


Figura 2. 7. *Pérdidas y desperdicio de alimentos en diferentes países (kg/año) [82]*

Según el Ministerio de Agricultura, Pesca y Alimentación de España (MAPA), en nuestro país se tiraron a la basura más de 1.300 millones de kilos de alimentos sin consumir durante 2020. Son muchos los datos que nos alertan de la gravedad de la situación y de la necesidad de actuar, por ello cada vez cobran más importancia aquellas políticas que tratan de disociar el crecimiento económico de la generación de residuos. En concreto, en Europa, un estudio reciente ha demostrado que las frutas y hortalizas son los grupos de alimentos que presentan mayor cantidad de desperdicio de alimentos en general, con cantidades similares generadas en las etapas primarias de producción y consumo [83].

Recientemente se ha aprobado en España el proyecto de Ley de Prevención de las Pérdidas y el Desperdicio Alimentario, que tiene como objetivo fomentar el aprovechamiento de los residuos agroalimentarios mediante la aplicación de sistemas sostenibles y dirigidos hacia una economía circular. La ley, que consta de 15 artículos repartidos en cinco capítulos, donde se pretende establecer un modelo de buenas prácticas para evitar el desperdicio de alimentos con actuaciones en toda la cadena alimentaria, desde el origen en el propio proceso de cosecha, hasta los hábitos de consumo en los hogares y en la restauración.

Por tanto, la valorización de los residuos agroalimentarios ha captado el interés de las investigaciones más recientes, especialmente el aprovechamiento de los residuos de frutas y hortalizas debido al elevado volumen de residuos y alto contenido en azúcares [84,85].

Otros residuos agroalimentarios que causan un gran impacto ambiental y que se producen en elevadas cantidades en España son los procedentes de la fabricación de aceite de oliva como el alperujo. Este subproducto se genera durante el proceso de extracción del aceite de oliva mediante el sistema de dos fases [86]. En España, esta tecnología se utiliza en aproximadamente el 90% de las almazaras [87]. La aplicación de este sistema de extracción genera unos 800 kg de alperujo por tonelada de aceituna procesada, lo que representa una producción anual de alrededor de cuatro millones de toneladas para la industria aceitera española [86]. El alperujo obtenido es un flujo residual semisólido compuesto por agua vegetal y orujo de oliva con alto contenido de humedad (60%) que aún contiene una cierta cantidad de aceite [88].

En vista al volumen de residuos agroalimentarios y la amplias posibilidades de emplear estos subproductos como materia prima para la obtención de compuestos de interés, no es extraño que la investigación sobre diferentes alternativas de tratamientos y valorización sea el objetivo principal de los estudios más recientes [89,90].

2.3.2. Efluentes ganaderos

Anteriormente se ha comentado que los residuos generados en el sector de la ganadería se pueden clasificar en tres grupos: estiércoles y purines, residuos zoosanitarios y subproductos de origen animal no destinados a consumo humano (SANDACH).

Entre ellos, la producción y la gestión de los purines genera una gran preocupación, especialmente los del sector porcino. La producción de purines en España se incrementó en 2018, un 32% con respecto a 2017, alcanzando una producción total de 163.000 toneladas. Se denomina purín al efluente agrícola resultante de la mezcla de estiércol, orina y agua de limpieza, que se caracteriza por su alta concentración de nitrógeno, fósforo, color y materia orgánica [91,92]. Normalmente, estas aguas residuales se utilizan como biofertilizante, precisamente por su alto contenido en nutrientes. Sin embargo, debido a la alta producción de purines, es necesario gestionar y almacenar una gran cantidad de estas aguas residuales hasta su tratamiento. Problemas ambientales importantes, como la acidificación, la contaminación del suelo, la eutrofización del agua, así como la emisión de gases que incrementan el efecto invernadero, son el resultado de una mala gestión y vertido de este efluente [93].

La industria porcina se ha expandido rápidamente en España, alcanzando actualmente alrededor del 40% de la producción ganadera final [94]. En consecuencia, se ha incrementado el volumen de aguas residuales porcinas y las emisiones de gases de efecto invernadero [95]. Esta situación ha propiciado el desarrollo de diferentes tecnologías para tratar estas aguas residuales con el fin de evitar riesgos ambientales [96].

2.3.3. Tratamientos habituales de los residuos agropecuarios

La necesidad de tratar el exceso de residuos agropecuarios y la posibilidad de obtener productos de interés de su revaloración ha desencadenado una multitud de estudios dentro de esta línea. En la Figura 2.8 se puede observar algunos de los tratamientos habituales para los residuos agroalimentarios, así como los productos de valor añadido resultantes del proceso.

Por ello, varios autores han desarrollado revisiones exhaustivas sobre los métodos de tratamiento más aplicados y los compuestos obtenidos [89,90]. Los exopolisacáridos presentes en los residuos alimentarios puede ser utilizado por los microorganismos que mediante técnicas de fermentación o hidrólisis enzimática son capaces de sintetizar biopolímeros con potenciales aplicaciones en las industrias química, cosmética, alimentaria y farmacéutica [97]. Algunos de los biopolímeros

obtenidos son quitina, goma xantana, colágeno, polihidroxibutiratos (PHB) y polihidroxialcanoatos (PHA).

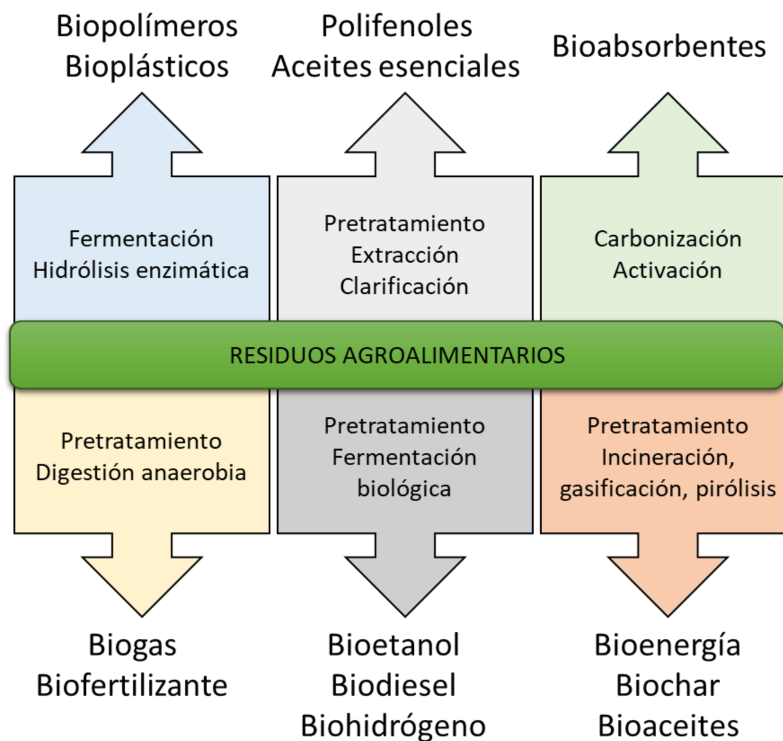


Figura 2. 8. *Tratamientos de los residuos agroalimentarios y productos obtenidos [89].*

Por otro lado, los residuos agroalimentarios, pueden convertirse en carbón activado mediante una carbonización de la biomasa seguida de un proceso de activación que permita obtener un material con alta capacidad de biosorción [98]. Varios autores han informado de resultados óptimos para la eliminación de metales pesados como arsénico, plomo, mercurio, cadmio, cromo, níquel, cobalto, etc., mediante biosorbentes, obtenidos a partir de cáscara de arroz, salvado de trigo, cáscaras de manzana y plátano, etc [99,100].

Así mismo, se informado de la obtención de biocarbón o "biochar", tras someter los residuos agroalimentarios a procesos de pirólisis empleando temperaturas entre los 400ºC – 900ºC. Especialmente el biochar se utiliza como un excelente absorbente para eliminar compuestos químicos orgánicos e inorgánicos de las aguas residuales [101].

Otras tecnologías aplicadas al tratamiento de estos residuos son los procesos biológicos tanto anaerobios que permiten la producción de biogás como aerobias,

fermentando la biomasa residual para obtener bioetanol, biodiesel o biohidrógeno. Esta línea de investigación ha captado el interés de muchos autores debido al agotamiento de los combustibles fósiles y a la demanda creciente de energía [102].

En cuanto a los residuos ganaderos, las técnicas de tratamiento se han centrado principalmente en tres objetivos, obtener compost, obtener biofertilizantes y producir biocombustibles [48,91,103]. En este sentido se han aplicado técnicas de separación como la filtración y la centrifugación para obtener una pasta sólida que pueda someterse a procesos de compostaje [104]. Normalmente estos residuos necesitan someterse a procesos de higienización para evitar la contaminación por microorganismos patógenos [105]. Para la obtención de biofertilizantes, los métodos aplicados se basan en la recuperación del nitrógeno, generalmente mediante procesos de concentración térmica (evaporación al vacío y secado), técnicas de stripping y absorción u obtención de estruvita [106,107].

2.4. RESIDUOS PROCEDENTES DE LA INDUSTRIA PAPELERA

Los residuos procedentes de la industria papelera contemplan mayoritariamente las aguas residuales procedentes del proceso de fabricación de pulpa de celulosa, en concreto el licor negro.

La industria papelera española es uno de los grandes productores europeos de papel y celulosa con 81 plantas de producción distribuidas por toda España. Junto con Francia, es el quinto productor europeo de celulosa y el sexto fabricante de papel de la Unión Europea después de Alemania, Finlandia, Suecia, Italia y Francia [108]. En la Figura 2.9, se muestra el respectivo porcentaje de la producción total de celulosa en los distintos miembros de la Confederación Europea en la Industria del Papel (CEPI), conformada por 18 paises: Austria, Bélgica, República Checa, Finlandia, Francia, Alemania, Hungría, Italia, Países Bajos, Noruega, Polonia, Portugal, Rumania, Eslovaquia, Eslovenia, España, Suecia y Reino Unido.

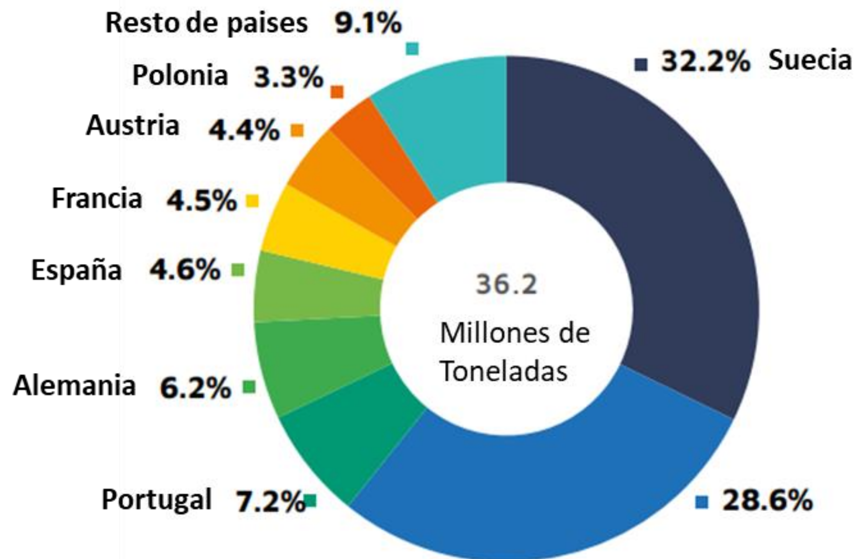


Figura 2. 9. *Producción total de celulosa en los países miembros CEPI en 2020.*

Actualmente el 81% de los residuos de fabricación de la industria papelera se valorizan por distintas vías, según la Memoria de Sostenibilidad de la Industria del Papel [109].

La producción de pulpa y papel conlleva un gran consumo de agua, que depende de las características de la materia prima y del tipo de papel producido. En consecuencia, se producen enormes cantidades de aguas residuales complejas, tóxicas y poco biodegradables, que necesitan ser tratadas. Entre estas aguas residuales, el efluente de licor negro, generado durante el proceso de fabricación de pasta Kraft, se considera la corriente de residuos más problemática [110,111].

El proceso Kraft es el proceso de fabricación de pasta de celulosa más importante en la industria del papel y cartón y el mayor proveedor de lignina. Alrededor de 130 millones de toneladas por año de pulpa Kraft se producen en todo el mundo [112], lo que supone alrededor del 80% de la pulpa química producida, de los cuales entre 50 y 55 millones de toneladas métricas de lignina en forma de licor negro [113].

2.4.1. Proceso de obtención del licor negro

La Figura 2.10 muestra un esquema general del proceso de producción de pulpa y papel mediante proceso Kraft.

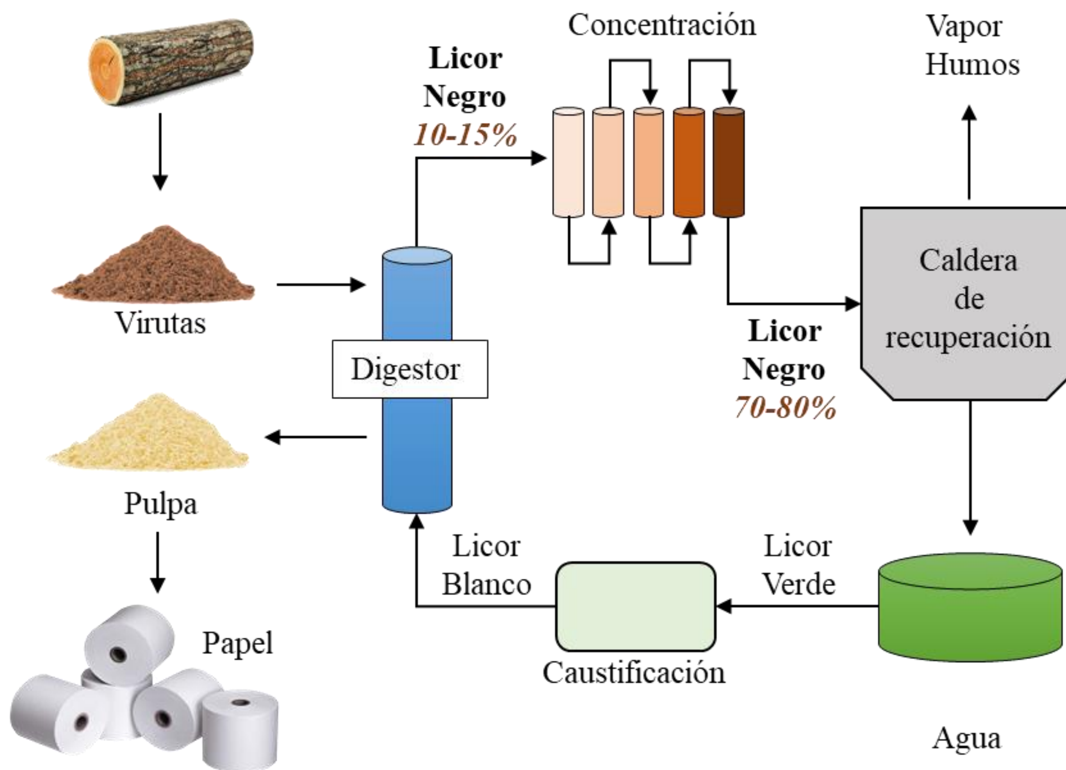


Figura 2. 10. *Esquema del proceso Kraft*

Durante el proceso de fabricación de pasta Kraft, las astillas de madera se digieren a alta temperatura (145ºC – 175ºC) y presión en una solución de hidróxido de sodio (NaOH) y sulfuro de sodio (Na_2S) denominada "licor blanco". Esta solución disocia las fibras de lignina y celulosa, dando una pulpa sólida y un residuo líquido como productos finales (licor negro débil al 10-15%). La pulpa sólida obtenida se lava y continúa con el proceso de fabricación, mientras que el licor negro débil, se mezcla con el residuo líquido del proceso de lavado de la pulpa para formar el licor negro fuerte (75-80%) [114]. Posteriormente, el licor negro fuerte (donde se disuelve la lignina) generalmente se quema en la caldera de recuperación, para recuperar el calor y los productos químicos. Durante la fase de combustión, los compuestos orgánicos disueltos en el licor negro (principalmente lignina) proporcionan calor que se usa para generar

vapor y para convertir el sulfato de sodio en sulfuro de sodio. La gran cantidad de vapor producido en este paso convierte al licor negro en el quinto combustible más importante del mundo

El licor negro es un efluente es una solución de color oscuro y altamente alcalina con alta demanda química de oxígeno y toxicidad, y baja biodegradabilidad [115]. Está compuesto principalmente por lignina, clorofenoles, taninos, ácidos grasos y sales de sodio solubles, entre otros [116]. Por lo tanto, este efluente debe ser tratado adecuadamente antes de ser descargado para evitar daños ambientales adversos en la flora, fauna y cuerpos acuáticos naturales [117].

2.4.2. Tratamientos del licor negro

Uno de los principales tratamientos del licor negro es su concentración y combustión en una caldera. Esto permite emplear la lignina como fuente de energía [118]. Las cenizas obtenidas se utilizan para formar el licor verde mediante la adición de agua. Luego, esta solución se somete a un proceso de caustificación y se convierte en licor blanco, que luego de un proceso de filtración se recircula a la etapa de digestión. Si bien este es el método más empleado, su uso conlleva importantes problemas operacionales y ambientales, es decir, el aumento de la viscosidad del efluente durante las primeras etapas que favorece el taponamiento de tuberías, la formación de depósitos y humos de sales inorgánicas en evaporadores y hornos o la emisión de olores y gases peligrosos [119,120]

La revalorización de estas ligninas es el punto clave para producir pequeños componentes aromáticos que satisfagan la enorme y diversa demanda industrial de este tipo de productos químicos. Las transformaciones catalíticas de la lignina proporcionan rutas para obtener catecoles, quinonas, vainillina, etc., considerados como sustancias químicas potenciales que pueden utilizarse en la industria alimentaria [112]

Se han estudiado otras técnicas fisicoquímicas como la oxidación por aire húmedo, la ozonización, la electrocoagulación o las tecnologías de membranas para reducir su color o su concentración de compuestos recalcitrantes [121–124]. Aunque se ha demostrado que la aplicación de estos métodos es eficaz para eliminar la DQO, muchas desventajas están relacionadas con su uso, principalmente asociadas al alto costo operativo. Además, se ha informado que durante algunos de estos tratamientos

podrían generarse subproductos indeseables como ácidos alifáticos o compuestos fenólicos [125]. Por estos motivos, el interés por buscar métodos alternativos para el tratamiento del licor negro, que permitan la eliminación de contaminantes recalcitrantes de forma económica y ecológica, sigue en su punto máximo.

Es frecuente que los métodos biológicos puedan superar adecuadamente algunos de los inconvenientes de las técnicas fisicoquímicas, ya que permiten la transformación de las sustancias químicas tóxicas en formas menos nocivas [126]. Se han descrito anteriormente técnicas de tratamiento tanto aeróbicas como anaeróbicas para el tratamiento de la industria de la pulpa y el papel, logrando una eliminación parcial de materia orgánica y compuestos fenólicos [127,128]. Sin embargo, la composición de este efluente imposibilita su tratamiento mediante un proceso biológico convencional. La eliminación de contaminantes recalcitrantes del licor no es una tarea fácil para las bacterias, principalmente debido a la presencia de estructuras complejas como la lignina o compuestos de lignina clorada [125]. En este sentido, algunos investigadores se han centrado en la búsqueda de microorganismos adecuados que permitan la degradación de estos compuestos complejos, mejorando la eficacia del proceso de tratamiento biológico [129,130].

2.5. TRATAMIENTO BIOLÓGICO MEDIANTE HONGOS

Los hongos transforman una amplia gama de contaminantes orgánicos. Es por ello que su uso de hongos se ha considerado una de las alternativas más prometedoras en cuanto al tratamiento de efluentes con estructuras fenólicas complejas altamente recalcitrantes [51,52,131]. Su aplicación permite la degradación de estos compuestos desde un punto de vista económico, sostenible y ecológicos. Varios autores han informado de eliminaciones significativas en cuanto al color, la DQO y la presencia de compuestos fenólicos [132,133]. Frente a los métodos biológicos convencionales con bacterias, la biomasa y los subproductos producidos por los hongos durante el tratamiento de residuos presentan una mayor valorización. Los hongos sintetizan enzimas y producen proteínas con gran potencial biotecnológico [134]. Su cultivo utilizando desechos industriales ricos en materia orgánica ha permitido la obtención de varios biocombustibles y bioquímicos de alto valor. Por ello, los hongos se han empleado para el procesamiento de residuos ganaderos y de residuos de las industrias

lácteas, azucareras, textil, papeleras, productoras de aceite de oliva y algodón [135–137].

La mayoría de los trabajos de investigación se han centrado en el empleo de hongos de la podredumbre blanca como *Phanerochaete chrysosporium*, *Pleorotus ostreatus* y *Trametes versicolor*, o en microorganismos pertenecientes al género *Aspergillus*, especialmente con *Aspergillus niger*.

2.5.1. Aspectos generales de los hongos

En el reino Hongos (que se estima que contiene entre 80.000 y 100.000 especies descritas) la mayoría de los degradadores de contaminantes pertenecen a los filos Ascomycota y Basidiomycota [138].

Los hongos tienen mayor resistencia a los compuestos inhibidores que las especies bacterianas y pueden sobrevivir en condiciones adversas como pH bajo, baja temperatura y bajo contenido de nitrógeno [139,140]. La capacidad de sintetizar enzimas oxidativas extracelulares como lignina peroxidasa (LiP), manganeso peroxidasa (MnP) y lacasa (Lac), es una de las principales ventajas del tratamiento de hongos frente a los tratamientos convencionales por bacterias. Estas enzimas, como se verá más adelante, están involucradas en la ruptura de los compuestos refractarios en moléculas más simples que el hongo puede emplear como fuente de nutrientes [141]. Además, debido al sistema de enzimas extracelulares, no se observan limitaciones de difusión del sustrato, que generalmente se encuentran en bacterias [139].

Dentro de los hongos basidiomicetos, los hongos de pudrición blanca han mostrado una amplia capacidad para la degradación de compuestos contaminantes. Especialmente, el hongo *Phanerochaete chrysosporium*. Este microorganismo es un hongo ligninolítico, saprófito y causante de los procesos de pudrición blanca en la madera. Este microorganismo ha sido modelo de estudio para muchos autores debido a su capacidad de degradar lignina y otros compuestos xenobióticos gracias a las enzimas extracelulares que sintetiza, como manganeso peroxidasa (MnP) y lignina peroxidasa (LiP). Varios autores han informado de que la producción de lacasa (Lac) por parte de este microorganismo es muy baja o nula en la mayoría de los tratamientos biológicos. Este microorganismo fue el primer hongo de pudrición blanca en tener secuenciado su genoma completo [142].

Figura 2. 11. *Pudrición blanca producida por Phanerochaete chrysosporium*

Dentro del filo Ascomycota, los microorganismos pertenecientes al género *Aspergillus* han sido ampliamente utilizados en procesos de biorremediación. Estos microorganismos son un grupo complejo de mohos ascomicetos que comprende varios cientos de especies, algunas de las cuales son patógenas para humanos y animales [143]. Muchas especies de *Aspergillus* tienen importantes aplicaciones prácticas en los procesos de fermentación industrial, lo cual es un reflejo de la abundancia de enzimas que *Aspergillus* produce y secreta al medio extracelular, particularmente *Aspergillus oryzae*, *Aspergillus niger* y *Aspergillus nidulans* [144]. Numerosos procesos industriales emplean microorganismos pertenecientes al género *Aspergillus* como biocatalizadores para la producción de enzimas, metabolitos primarios y secundarios, con gran potencial biotecnológico [145]. Dentro del género *Aspergillus*, en el año 2008, se logró aislar de bayas de uva, una nueva especie denominada *Aspergillus uvarum* [146]. La aplicación de este microorganismo en la degradación de estructuras fenólicas complejas apenas ha sido estudiada. Sólo unos pocos estudios recientes hacen mención a su implicación en procesos degradativos de compuestos fenólicos cuando se lleva a cabo un tratamiento fúngico de aguas residuales procedentes de bodegas y almazaras [147–149]. En vista a estos resultados prometedores, parece adecuado seleccionar este microorganismo para futuros estudios de biodegradación, ya que los resultados obtenidos permitirían ampliar las aplicaciones biotecnológicas de esta especie en concreto.

2.5.3. Enzimas extracelulares

La posibilidad de los hongos para degradar los compuestos de naturaleza fenólica como la lignina, los ácidos húmicos, diferentes tintes industriales y fármacos disueltos en aguas residuales, recae principalmente en su capacidad para sintetizar enzimas extracelulares [150]. Entre las enzimas extracelulares involucradas en la degradación de compuestos recalcitrantes, destacan la manganeso peroxidasa (MnP), la lignina peroxidasa (LiP) y la lacasa (Lac).

A) Manganeso peroxidasa

La enzima MnP se descubrió por primera vez en *P. chrysosporium*. La enzima MnP es una glicoproteína cuyo ciclo catalítico se inicia con su oxidación por H_2O_2 o peróxidos orgánicos, transformando el Mn^{2+} a Mn^{3+} , que a su vez puede oxidar una gran cantidad de sustratos fenólicos [151]. Los procesos de reacción se muestran en la Figura 2.12.

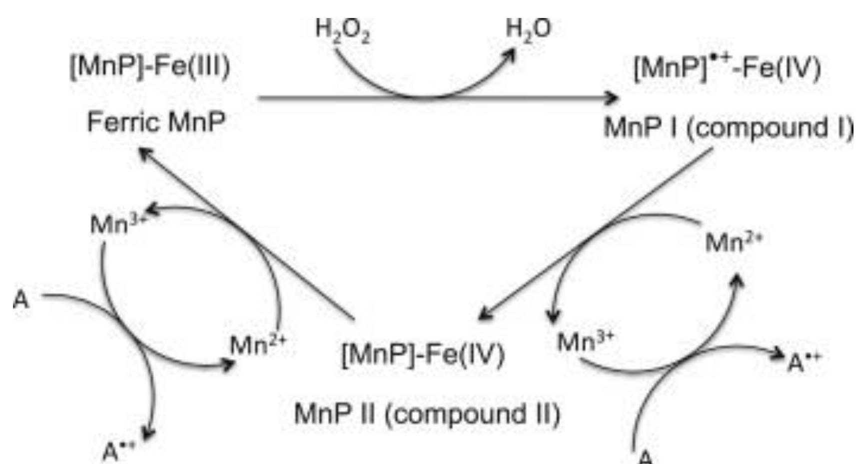


Figura 2. 12. *Ciclo catalítico de MnP*

B) Lignina peroxidasa

Esta enzima está involucrada en la rotura del enlace Cα-Cβ de la lignina mediante la oxidación del hierro hemo gracias al peróxido de hidrogeno. El H_2O_2 oxida al grupo hemo presente en la enzima, el cual oxida a dos compuestos intermedios de la reacción, lo que conlleva la final oxidación de compuestos recalcitrantes, como es el caso del alcohol veratrílico [152]. La enzima LiP puede oxidar anillos aromáticos que están moderadamente activados, mientras que las peroxidasas clásicas actúan solo sobre sustratos aromáticos

fuertemente activados. Sólo la enzima LiP puede oxidar las principales estructuras no fenólicas de la lignina, que constituyen hasta el 90% del polímero.

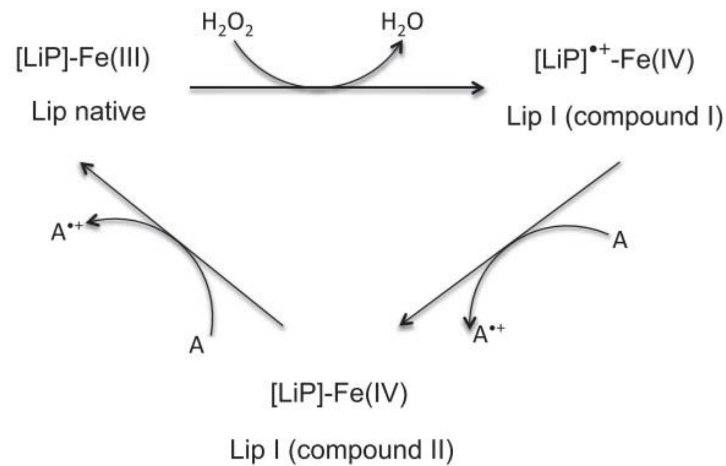


Figura 2. 13. *Reacción catalítica LiP*

C) Lacasa

La lacasa (Lac) es una de las enzimas más importantes, ya que debido a su baja especificidad de sustrato se considera una alternativa atractiva para la aplicación biotecnológica y ambiental. Estas enzimas catalizan la oxidación de varios compuestos fenólicos (fenoles, difenoles, polifenoles, diaminas, aminas aromáticas) y también compuestos no fenólicos en presencia de mediadores, convirtiendo el oxígeno molecular en agua [153]. La lacasa es una enzima común en la naturaleza, encontrándose en plantas, hongos, bacterias, líquenes e insectos. Se ha reportado actividad lacasa tanto en hongos ascomicetos como basidiomicetos y deuteromicetos, siendo particularmente abundante en los hongos de pudrición blanca [154].

2.6. REFERENCIAS

[1] C.S. Liew, N.M. Yunus, B.S. Chidi, M.K. Lam, P.S. Goh, M. Mohamad, J.C. Sin, S.M. Lam, J.W. Lim, S.S. Lam, A review on recent disposal of hazardous sewage sludge via anaerobic digestion and novel composting, J. Hazard. Mater. 423 (2022) 126995. https://doi.org/10.1016/J.JHAZMAT.2021.126995.

[2] Ministerio de Ambiente y Medio Rural y Marino, Caracterización de los lodos de depuradoras generados en España, 2009.

[3] L. Appels, J. Lauwers, J. Degrve, L. Helsen, B. Lievens, K. Willems, J. Van Impe, R. Dewil, Anaerobic digestion in global bio-energy production: Potential and research challenges, Renew. Sustain. Energy Rev. 15 (2011) 4295–4301. https://doi.org/10.1016/J.RSER.2011.07.121.

[4] Yadvika, Santosh, T.R. Sreekrishnan, S. Kohli, V. Rana, Enhancement of biogas production from solid substrates using different techniques—a review, Bioresour. Technol. 95 (2004) 1–10. https://doi.org/10.1016/J.BIORTECH.2004.02.010.

[5] Y. Xu, Y. Lu, L. Zheng, Z. Wang, X. Dai, Perspective on enhancing the anaerobic digestion of waste activated sludge, J. Hazard. Mater. (2019) 121847. https://doi.org/10.1016/J.JHAZMAT.2019.121847.

[6] S.K. Pramanik, F.B. Suja, S.M. Zain, B.K. Pramanik, The anaerobic digestion process of biogas production from food waste: Prospects and constraints, Bioresour. Technol. Reports. 8 (2019) 100310. https://doi.org/10.1016/j.biteb.2019.100310.

[7] Z.S. Lee, S.Y. Chin, J.W. Lim, T. Witoon, C.K. Cheng, Treatment technologies of palm oil mill effluent (POME) and olive mill wastewater (OMW): A brief review, Environ. Technol. Innov. 15 (2019) 100377. https://doi.org/10.1016/j.eti.2019.100377.

[8] S.A. Neshat, M. Mohammadi, G.D. Najafpour, P. Lahijani, Anaerobic co-digestion of animal manures and lignocellulosic residues as a potent approach for sustainable biogas production, Renew. Sustain. Energy Rev. 79 (2017) 308–322. https://doi.org/10.1016/J.RSER.2017.05.137.

[9] R. Karki, W. Chuenchart, K.C. Surendra, S. Shrestha, L. Raskin, S. Sung, A. Hashimoto, S. Kumar Khanal, Anaerobic co-digestion: Current status and perspectives, Bioresour. Technol. 330 (2021) 125001. https://doi.org/10.1016/j.biortech.2021.125001.

[10] S. Wainaina, M.K. Awasthi, S. Sarsaiya, H. Chen, E. Singh, A. Kumar, B. Ravindran, S.K. Awasthi, T. Liu, Y. Duan, S. Kumar, Z. Zhang, M.J. Taherzadeh, Resource recovery and circular economy from organic solid waste using aerobic and anaerobic digestion technologies, Bioresour. Technol. 301 (2020) 122778. https://doi.org/10.1016/J.BIORTECH.2020.122778.

[11] W. Peng, F. Lü, L. Hao, H. Zhang, L. Shao, P. He, Digestate management for high-solid anaerobic digestion of organic wastes: A review, Bioresour. Technol. 297 (2020) 122485. https://doi.org/10.1016/j.biortech.2019.122485.

[12] Y. Zeng, A. De Guardia, P. Dabert, Improving composting as a post-treatment of anaerobic digestate, Bioresour. Technol. 201 (2016) 293–303. https://doi.org/10.1016/j.biortech.2015.11.013.

[13] B.S. Moraes, M. Zaiat, A. Bonomi, Anaerobic digestion of vinasse from sugarcane ethanol production in Brazil: Challenges and perspectives, Renew. Sustain. Energy Rev. 44 (2015) 888–903. https://doi.org/10.1016/J.RSER.2015.01.023.

[14] A. Serrano, J.A. Siles, M.A. Martín, A.F. Chica, F.S. Estévez-Pastor, E. Toro-Baptista, Improvement of anaerobic digestion of sewage sludge through microwave pre-treatment, J. Environ. Manage. 177 (2016) 231–239. https://doi.org/10.1016/J.JENVMAN.2016.03.048.

[15] B. Ruffino, G. Campo, G. Genon, E. Lorenzi, D. Novarino, G. Scibilia, M. Zanetti, Improvement of anaerobic digestion of sewage sludge in a wastewater treatment plant by means of mechanical and thermal pre-treatments: Performance, energy and economical assessment, Bioresour. Technol. 175 (2015) 298–308. https://doi.org/10.1016/J.BIORTECH.2014.10.071.

[16] O. Suárez-Iglesias, J.L. Urrea, P. Oulego, S. Collado, M. Díaz, Valuable compounds from sewage sludge by thermal hydrolysis and wet oxidation. A

review, Sci. Total Environ. 584–585 (2017) 921–934. https://doi.org/10.1016/j.scitotenv.2017.01.140.

[17] J.L. Urrea, M. García, S. Collado, P. Oulego, M. Díaz, Sludge hydrothermal treatments. Oxidising atmosphere effects on biopolymers and physical properties, J. Environ. Manage. 206 (2018) 284–290. https://doi.org/10.1016/j.jenvman.2017.10.043.

[18] MITECO, Gestión de Biorresiduos de competencia municipal. Guía para la implantación de la recogida separa y tratamiento de la fracción orgánica, (2013) 1–355.

[19] K.F. Adekunle, J.A. Okolie, K.F. Adekunle, J.A. Okolie, A Review of Biochemical Process of Anaerobic Digestion, Adv. Biosci. Biotechnol. 6 (2015) 205–212. https://doi.org/10.4236/ABB.2015.63020.

[20] Z.H. Yang, R. Xu, Y. Zheng, T. Chen, L.J. Zhao, M. Li, Characterization of extracellular polymeric substances and microbial diversity in anaerobic co-digestion reactor treated sewage sludge with fat, oil, grease, Bioresour. Technol. 212 (2016) 164–173. https://doi.org/10.1016/j.biortech.2016.04.046.

[21] J.C. Akunna, Anaerobic treatment of brewery wastes, Brew. Microbiol. Manag. Microbes, Ensuring Qual. Valorising Waste. (2015) 407–424. https://doi.org/10.1016/B978-1-78242-331-7.00019-8.

[22] J. Guo, Y. Peng, B.-J. Ni, X. Han, L. Fan, Z. Yuan, Dissecting microbial community structure and methane-producing pathways of a full-scale anaerobic reactor digesting activated sludge from wastewater treatment by metagenomic sequencing, Microb. Cell Fact. 14 (2015) 33. https://doi.org/10.1186/s12934-015-0218-4.

[23] S. Achinas, V. Achinas, G.J.W. Euverink, Microbiology and biochemistry of anaerobic digesters: an overview, Bioreactors. (2020) 17–26. https://doi.org/10.1016/B978-0-12-821264-6.00002-4.

[24] R. Borja, Biogas Production, Compr. Biotechnol. Second Ed. 2 (2011) 785–798. https://doi.org/10.1016/B978-0-08-088504-9.00126-4.

[25] E.M. Fykse, T. Aarskaug, E.H. Madslien, M. Dybwad, Microbial community structure in a full-scale anaerobic treatment plant during start-up and first year of operation revealed by high-throughput 16S rRNA gene amplicon sequencing, Bioresour. Technol. 222 (2016) 380–387. https://doi.org/10.1016/j.biortech.2016.09.118.

[26] M.C. Nelson, M. Morrison, Z. Yu, A meta-analysis of the microbial diversity observed in anaerobic digesters, Bioresour. Technol. 102 (2011) 3730–3739. https://doi.org/10.1016/j.biortech.2010.11.119.

[27] I. V. Yentekakis, G. Goula, Biogas Management: Advanced Utilization for Production of Renewable Energy and Added-value Chemicals, Front. Environ. Sci. 5 (2017) 7. https://doi.org/10.3389/fenvs.2017.00007.

[28] M.U. Khan, J.T.E. Lee, M.A. Bashir, P.D. Dissanayake, Y.S. Ok, Y.W. Tong, M.A. Shariati, S. Wu, B.K. Ahring, Current status of biogas upgrading for direct biomethane use: A review, Renew. Sustain. Energy Rev. 149 (2021) 111343. https://doi.org/10.1016/j.rser.2021.111343.

[29] C. Moya, R. Santiago, D. Hospital-Benito, J. Lemus, J. Palomar, Design of biogas upgrading processes based on ionic liquids, Chem. Eng. J. 428 (2022) 132103. https://doi.org/10.1016/J.CEJ.2021.132103.

[30] A. Carranza-Abaid, R.R. Wanderley, H.K. Knuutila, J.P. Jakobsen, Analysis and selection of optimal solvent-based technologies for biogas upgrading, Fuel. 303 (2021) 121327. https://doi.org/10.1016/J.FUEL.2021.121327.

[31] R. Augelletti, S. Galli, P. Gislon, M. Granati, G. Monteleone, M.A. Murmura, M.C. Annesini, Biogas upgrading through CO2 removal by chemical absorption in an amine organic solution: Physical and technical assessment, simulation and experimental validation, Biomass and Bioenergy. 141 (2020) 105729. https://doi.org/10.1016/J.BIOMBIOE.2020.105729.

[32] W. Ahmad, S. Sethupathi, G. Kanadasan, L.C. Lau, R. Kanthasamy, A review on the removal of hydrogen sulfide from biogas by adsorption using sorbents derived from waste, Rev. Chem. Eng. 37 (2021) 407–431. https://doi.org/10.1515/REVCE-2018-

0048/MACHINEREADABLECITATION/RIS.

[33] T. Montanari, E. Finocchio, I. Bozzano, G. Garuti, A. Giordano, C. Pistarino, G. Busca, Purification of landfill biogases from siloxanes by adsorption: A study of silica and 13X zeolite adsorbents on hexamethylcyclotrisiloxane separation, Chem. Eng. J. 165 (2010) 859–863. https://doi.org/10.1016/J.CEJ.2010.10.032.

[34] A. Shokri, Synthesize and characterization of polysulfone membrane for the separation of hydrogen sulfide from natural gas, Surfaces and Interfaces. 25 (2021) 101233. https://doi.org/10.1016/J.SURFIN.2021.101233.

[35] E. Tilahun, E. Sahinkaya, B. Çalli, A hybrid membrane gas absorption and bio-oxidation process for the removal of hydrogen sulfide from biogas, Int. Biodeterior. Biodegradation. 127 (2018) 69–76. https://doi.org/10.1016/J.IBIOD.2017.11.015.

[36] M. Li, Z. Zhu, M. Zhou, X. Jie, L. Wang, G. Kang, Y. Cao, Removal of CO2 from biogas by membrane contactor using PTFE hollow fibers with smaller diameter, J. Memb. Sci. 627 (2021) 119232. https://doi.org/10.1016/J.MEMSCI.2021.119232.

[37] H. Huynh Nhut, V. Le Thi Thanh, L. Tran Le, Removal of H2S in biogas using biotrickling filter: Recent development, Process Saf. Environ. Prot. 144 (2020) 297–309. https://doi.org/10.1016/J.PSEP.2020.07.011.

[38] P. San-Valero, J.M. Penya-roja, F. Javier Álvarez-Hornos, G. Buitrón, C. Gabaldón, G. Quijano, Fully aerobic bioscrubber for the desulfurization of H2S-rich biogas, Fuel. 241 (2019) 884–891. https://doi.org/10.1016/J.FUEL.2018.12.098.

[39] S. Le Borgne, G. Baquerizo, Microbial Ecology of Biofiltration Units Used for the Desulfurization of Biogas, ChemEngineering 2019, Vol. 3, Page 72. 3 (2019) 72. https://doi.org/10.3390/CHEMENGINEERING3030072.

[40] L. Wu, W. Wei, L. Song, M. Woźniak-Karczewska, Ł. Chrzanowski, B.J. Ni, Upgrading biogas produced in anaerobic digestion: Biological removal and bioconversion of CO2 in biogas, Renew. Sustain. Energy Rev. 150 (2021)

111448. https://doi.org/10.1016/J.RSER.2021.111448.

[41] A. Naquash, M.A. Qyyum, J. Haider, A. Bokhari, H. Lim, M. Lee, State-of-the-art assessment of cryogenic technologies for biogas upgrading: Energy, economic, and environmental perspectives, Renew. Sustain. Energy Rev. 154 (2022) 111826. https://doi.org/10.1016/J.RSER.2021.111826.

[42] G. Piechota, Removal of siloxanes from biogas upgraded to biomethane by Cryogenic Temperature Condensation System, J. Clean. Prod. 308 (2021) 127404. https://doi.org/10.1016/J.JCLEPRO.2021.127404.

[43] W. Peng, A. Pivato, Sustainable Management of Digestate from the Organic Fraction of Municipal Solid Waste and Food Waste Under the Concepts of Back to Earth Alternatives and Circular Economy, Waste and Biomass Valorization. 10 (2019) 465–481. https://doi.org/10.1007/s12649-017-0071-2.

[44] E. Monfet, G. Aubry, A.A. Ramirez, Nutrient removal and recovery from digestate: a review of the technology, Biofuels. 9 (2018) 247–262. https://doi.org/10.1080/17597269.2017.1336348.

[45] Mohanakrishnan Logan and Chettiyappan Visvanathan, M. Logan, C. Visvanathan, Management strategies for anaerobic digestate of organic fraction of municipal solid waste: Current status and future prospects, Waste Manag. Res. 37 (2019) 27–39. https://doi.org/10.1177/0734242X18816793.

[46] B. Qi, X. Jiang, H. Wang, J. Li, Q. Zhao, R. Li, W. Wang, Resource recovery from liquid digestate of swine wastewater by an ultrafiltration membrane bioreactor (UF-MBR) and reverse osmosis (RO) process, Environ. Technol. Innov. 24 (2021) 101830. https://doi.org/10.1016/J.ETI.2021.101830.

[47] M. Vondra, V. Máša, P. Bobák, The energy performance of vacuum evaporators for liquid digestate treatment in biogas plants, Energy. 146 (2018) 141–155. https://doi.org/10.1016/J.ENERGY.2017.06.135.

[48] D. Lorick, B. Macura, M. Ahlström, A. Grimvall, R. Harder, Effectiveness of struvite precipitation and ammonia stripping for recovery of phosphorus and nitrogen from anaerobic digestate: a systematic review, Environ. Evid. 9 (2020)

1–20. https://doi.org/10.1186/S13750-020-00211-X/TABLES/5.

[49] M.K. Doula, J.L. Moreno-Ortego, F. Tinivella, V.J. Inglezakis, A. Sarris, K. Komnitsas, Olive mill waste: recent advances for the sustainable development of olive oil industry, in: Olive Mill Waste, Elsevier, 2017: pp. 29–56. https://doi.org/10.1016/B978-0-12-805314-0.00002-9.

[50] A.I. Díaz, A. Laca, M. Díaz, Fungal treatment of an effluent from sewage sludge digestion to remove recalcitrant organic matter, Biochem. Eng. J. 172 (2021) 108056. https://doi.org/10.1016/j.bej.2021.108056.

[51] S. Collado, P. Oulego, O. Suárez-Iglesias, M. Díaz, Leachates and natural organic matter. A review of their biotreatment using fungi, Waste Manag. 96 (2019) 108–120. https://doi.org/10.1016/J.WASMAN.2019.07.018.

[52] S. Collado, P. Oulego, O. Suárez-Iglesias, M. Díaz, Biodegradation of dissolved humic substances by fungi, Appl. Microbiol. Biotechnol. 102 (2018) 3497–3511. https://doi.org/10.1007/s00253-018-8851-6.

[53] C. Romio, M. Vedel, W. Kofoed, H.B. Møller, P. Lohani, A. Siciliano, Digestate Post-Treatment Strategies for Additional Biogas Recovery: A Review, Sustain. 2021, Vol. 13, Page 9295. 13 (2021) 9295. https://doi.org/10.3390/SU13169295.

[54] Instituto Nacional de Estadística de España, España en cifras 2019, 2019.

[55] B.K. Tripathy, G. Ramesh, A. Debnath, M. Kumar, Mature landfill leachate treatment using sonolytic-persulfate/hydrogen peroxide oxidation: optimization of process parameters, Ultrason. Sonochem. (2019). https://doi.org/10.1016/J.ULTSONCH.2019.01.036.

[56] P. Oulego, S. Collado, A. Laca, M. Díaz, Tertiary treatment of biologically pre-treated landfill leachates by non-catalytic wet oxidation, Chem. Eng. J. 273 (2015) 647–655. https://doi.org/10.1016/j.cej.2015.03.089.

[57] D. Yu, Y. Pei, Z. Ji, X. He, Z. Yao, A review on the landfill leachate treatment technologies and application prospects of three-dimensional electrode technology, Chemosphere. (2021) 132895.

https://doi.org/10.1016/J.CHEMOSPHERE.2021.132895.

[58] E.R. Bandala, A. Liu, B. Wijesiri, A.B. Zeidman, A. Goonetilleke, Emerging materials and technologies for landfill leachate treatment: A critical review, Environ. Pollut. 291 (2021) 118133. https://doi.org/10.1016/J.ENVPOL.2021.118133.

[59] C. Teng, K. Zhou, C. Peng, W. Chen, Characterization and treatment of landfill leachate: A review, Water Res. 203 (2021) 117525. https://doi.org/10.1016/J.WATRES.2021.117525.

[60] Y.M.N.S. Ismail, N. Ngadi, M.H. Hassim, M.J. Kamaruddin, A. Johari, M.A.A. Aziz, Preparation of activated carbon from oil palm empty fruit bunch by physical activation for treatment of landfill leachate, IOP Conf. Ser. Mater. Sci. Eng. 458 (2018) 012036. https://doi.org/10.1088/1757-899X/458/1/012036.

[61] Y. Long, J. Xu, D. Shen, Y. Du, H. Feng, Effective removal of contaminants in landfill leachate membrane concentrates by coagulation, Chemosphere. 167 (2017) 512–519. https://doi.org/10.1016/J.CHEMOSPHERE.2016.10.016.

[62] P. Oulego, S. Collado, A. Laca, M. Díaz, Impact of leachate composition on the advanced oxidation treatment, Water Res. 88 (2016) 389–402. https://doi.org/10.1016/j.watres.2015.09.048.

[63] S. Renou, J.G. Givaudan, S. Poulain, F. Dirassouyan, P. Moulin, Landfill leachate treatment: Review and opportunity, J. Hazard. Mater. 150 (2008) 468–493. https://doi.org/10.1016/j.jhazmat.2007.09.077.

[64] G.P. Robertson, P.M. Groffman, Nitrogen transformations, 4th ed., Elsevier Inc., 2006. https://doi.org/10.1016/b978-0-12-415955-6.00014-1.

[65] Y. Yang, Y. Liu, T. Yang, Y. Lv, Characterization of a microbial consortium capable of heterotrophic nitrifying under wide C / N range and its potential application in phenolic and coking wastewater, Biochem. Eng. J. 120 (2017) 33–40. https://doi.org/10.1016/j.bej.2016.12.008.

[66] V. Poot, M. Hoekstra, M.A.A. Geleijnse, M.C.M. van Loosdrecht, J. Pérez,

Effects of the residual ammonium concentration on NOB repression during partial nitritation with granular sludge, Water Res. 106 (2016) 518–530. https://doi.org/10.1016/J.WATRES.2016.10.028.

[67] P. Regmi, M.W. Miller, B. Holgate, R. Bunce, H. Park, K. Chandran, B. Wett, S. Murthy, C.B. Bott, Control of aeration, aerobic SRT and COD input for mainstream nitritation/denitritation, Water Res. 57 (2014) 162–171. https://doi.org/10.1016/J.WATRES.2014.03.035.

[68] L. Miao, G. Yang, T. Tao, Y. Peng, Recent advances in nitrogen removal from landfill leachate using biological treatments – A review, J. Environ. Manage. 235 (2019) 178–185. https://doi.org/10.1016/J.JENVMAN.2019.01.057.

[69] S.J. Ergas, V. Aponte-Morales, Biological Nitrogen Removal, Elsevier Ltd., 2014. https://doi.org/10.1016/B978-0-12-382182-9.00047-5.

[70] D.A. Martens, Denitrification, Elsevier Ltd, 2005. https://doi.org/10.1016/B0-12-348530-4/00138-7.

[71] P. Wijekoon, P.A. Koliyabandara, A.T. Cooray, S.S. Lam, B.C.L. Athapattu, M. Vithanage, Progress and prospects in mitigation of landfill leachate pollution: Risk, pollution potential, treatment and challenges, J. Hazard. Mater. 421 (2022) 126627. https://doi.org/10.1016/J.JHAZMAT.2021.126627.

[72] E. Marañón, L. Castrillón, Y. Fernández-Nava, A. Fernández-Méndez, A. Fernández-Sánchez, Tertiary treatment of landfill leachates by adsorption, Waste Manag. Res. 27 (2009) 527–533. https://doi.org/10.1177/0734242X08096900.

[73] K.Y. Foo, B.H. Hameed, An overview of landfill leachate treatment via activated carbon adsorption process, J. Hazard. Mater. 171 (2009) 54–60. https://doi.org/10.1016/J.JHAZMAT.2009.06.038.

[74] M.A.M. Reshadi, A. Bazargan, G. McKay, A review of the application of adsorbents for landfill leachate treatment: Focus on magnetic adsorption, Sci. Total Environ. 731 (2020) 138863. https://doi.org/10.1016/J.SCITOTENV.2020.138863.

[75] Q. Zhou, W. Li, T. Hua, Removal of organic matter from landfill leachate by advanced oxidation processes: A review, Int. J. Chem. Eng. (2010). https://doi.org/10.1155/2010/270532.

[76] P. Mandal, B.K. Dubey, A.K. Gupta, Review on landfill leachate treatment by electrochemical oxidation: Drawbacks, challenges and future scope, Waste Manag. 69 (2017) 250–273. https://doi.org/10.1016/J.WASMAN.2017.08.034.

[77] P. Oulego, S. Collado, A. Laca, M. Díaz, Impact of leachate composition on the advanced oxidation treatment, Water Res. 88 (2016) 389–402. https://doi.org/10.1016/J.WATRES.2015.09.048.

[78] Á. Anglada, A. Urtiaga, I. Ortiz, D. Mantzavinos, E. Diamadopoulos, Treatment of municipal landfill leachate by catalytic wet air oxidation: Assessment of the role of operating parameters by factorial design, Waste Manag. 31 (2011) 1833–1840. https://doi.org/10.1016/J.WASMAN.2011.03.023.

[79] M. Saleem, A. Spagni, L. Alibardi, A. Bertucco, M.C. Lavagnolo, Assessment of dynamic membrane filtration for biological treatment of old landfill leachate, J. Environ. Manage. 213 (2018) 27–35. https://doi.org/10.1016/J.JENVMAN.2018.02.057.

[80] A.R. Esfahani, L. Zhai, A.H.M.A. Sadmani, Removing heavy metals from landfill leachate using electrospun polyelectrolyte fiber mat-laminated ultrafiltration membrane, J. Environ. Chem. Eng. 9 (2021) 105355. https://doi.org/10.1016/J.JECE.2021.105355.

[81] S. Corrado, S. Sala, Food waste accounting along global and European food supply chains: State of the art and outlook, Waste Manag. 79 (2018) 120–131. https://doi.org/10.1016/J.WASMAN.2018.07.032.

[82] J. Gustavsson, C. Cederberg, U. Sonesson, R. Van Oteerdijk, A. Meybeck, FAO, Pérdidas y desperdicio de alimentos en el mundo, FAO. (2012).

[83] C. Caldeira, V. De Laurentiis, S. Corrado, F. van Holsteijn, S. Sala, Quantification of food waste per product group along the food supply chain in the European Union: a mass flow analysis, Resour. Conserv. Recycl. 149 (2019)

479–488. https://doi.org/10.1016/J.RESCONREC.2019.06.011.

[84] A. Iqbal, P. Schulz, S.S.H. Rizvi, Valorization of bioactive compounds in fruit pomace from agro-fruit industries: Present Insights and future challenges, Food Biosci. 44 (2021) 101384. https://doi.org/10.1016/J.FBIO.2021.101384.

[85] A.G. Erman, T. Sar, G. Seker, M.Y. Akbas, Bioethanol production from fruit processing wastes, J. Biotechnol. 256 (2017) S41. https://doi.org/10.1016/J.JBIOTEC.2017.06.685.

[86] J. Alburquerque, Agrochemical characterisation of “alperujo”, a solid by-product of the two-phase centrifugation method for olive oil extraction, Bioresour. Technol. 91 (2004) 195–200. https://doi.org/10.1016/S0960-8524(03)00177-9.

[87] S. Dermeche, M. Nadour, C. Larroche, F. Moulti-Mati, P. Michaud, Olive mill wastes: Biochemical characterizations and valorization strategies, Process Biochem. 48 (2013) 1532–1552. https://doi.org/10.1016/j.procbio.2013.07.010.

[88] I. Sampedro, E. Aranda, G. Rodríguez-Guti Errez, A. Lama-Mu~ Noz, J.A. Ocampo, J. Fern Andez-Bola~, I. García-Romera, Effect of a New Thermal Treatment in Combination with Saprobic Fungal Incubation on the Phytotoxicity Level of Alperujo, J. Agric. Food Chem. 59 (2011) 3239–3245. https://doi.org/10.1021/jf2003305.

[89] A. Nayak, B. Bhushan, An overview of the recent trends on the waste valorization techniques for food wastes, J. Environ. Manage. 233 (2019) 352–370. https://doi.org/10.1016/J.JENVMAN.2018.12.041.

[90] B. Socas-Rodríguez, G. Álvarez-Rivera, A. Valdés, E. Ibáñez, A. Cifuentes, Food by-products and food wastes: are they safe enough for their valorization?, Trends Food Sci. Technol. 114 (2021) 133–147. https://doi.org/10.1016/J.TIFS.2021.05.002.

[91] Z. Luo, D. Wang, J. Yang, W. Zeng, The effect of using pig manure as an internal carbon source in a traditional piggery wastewater treatment system for biological denitrification, Ecol. Eng. 143 (2020) 105638. https://doi.org/10.1016/j.ecoleng.2019.105638.

[92] C.A. Sepúlveda-Muñoz, I. de Godos, D. Puyol, R. Muñoz, A systematic optimization of piggery wastewater treatment with purple phototrophic bacteria, Chemosphere. 253 (2020). https://doi.org/10.1016/j.chemosphere.2020.126621.

[93] Z. Yuan, X. Pan, T. Chen, X. Liu, Y. Zhang, S. Jiang, H. Sheng, L. Zhang, Evaluating environmental impacts of pig slurry treatment technologies with a life-cycle perspective, J. Clean. Prod. 188 (2018) 840–850. https://doi.org/10.1016/j.jclepro.2018.04.021.

[94] MAPA, “EL SECTOR DE LA CARNE DE CERDO EN CIFRAS: Principales indicadores económicos”, Subdirección General de Producciones Ganaderas y Cinegéticas, Dirección General de Producciones y Mercados Agrarios., 2020. https://cpage.mpr.gob.es/.

[95] F.X. Philippe, B. Nicks, Review on greenhouse gas emissions from pig houses: Production of carbon dioxide, methane and nitrous oxide by animals and manure, Agric. Ecosyst. Environ. 199 (2015) 10–25. https://doi.org/10.1016/J.AGEE.2014.08.015.

[96] W. Antezana, C. De Blas, P. García-Rebollar, C. Rodríguez, A. Beccaccia, P. Ferrer, A. Cerisuelo, V. Moset, F. Estellés, M. Cambra-López, S. Calvet, Composition, potential emissions and agricultural value of pig slurry from Spanish commercial farms, Nutr. Cycl. Agroecosystems. 104 (2016) 159–173. https://doi.org/10.1007/S10705-016-9764-3/TABLES/5.

[97] G. Sharmila, C. Muthukumaran, N.M. Kumar, V.M. Sivakumar, M. Thirumarimurugan, Food waste valorization for biopolymer production, Curr. Dev. Biotechnol. Bioeng. Resour. Recover. from Wastes. (2020) 233–249. https://doi.org/10.1016/B978-0-444-64321-6.00012-4.

[98] L.S. Oliveira, A.S. Franca, Food Science and Technology: New Research. Low cost adsorbents from agri-food wastes, Nova Sciences Publishers, Inc., 2008.

[99] F. Ahmad, S. Zaidi, Potential Use of Agro/Food Wastes as Biosorbents in the Removal of Heavy Metals, Emerg. Contam. (2020). https://doi.org/10.5772/INTECHOPEN.94175.

[100] E. Matei, M. Râpă, A.M. Predescu, A.A. Țurcanu, R. Vidu, C. Predescu, C. Bobirica, L. Bobirica, C. Orbeci, Valorization of Agri-Food Wastes as Sustainable Eco-Materials for Wastewater Treatment: Current State and New Perspectives, Mater. 2021, Vol. 14, Page 4581. 14 (2021) 4581. https://doi.org/10.3390/MA14164581.

[101] R. Yukesh Kannah, J. Merrylin, T. Poornima Devi, S. Kavitha, P. Sivashanmugam, G. Kumar, J. Rajesh Banu, Food waste valorization: Biofuels and value added product recovery, Bioresour. Technol. Reports. 11 (2020) 100524. https://doi.org/10.1016/J.BITEB.2020.100524.

[102] C. Gong, A. Singh, P. Singh, A. Singh, Anaerobic Digestion of Agri-Food Wastes for Generating Biofuels, Indian J. Microbiol. 2021 614. 61 (2021) 427–440. https://doi.org/10.1007/S12088-021-00977-9.

[103] J.A. Sáez, R. Clemente, M.Á. Bustamante, D. Yañez, M.P. Bernal, Evaluation of the slurry management strategy and the integration of the composting technology in a pig farm – Agronomical and environmental implications, J. Environ. Manage. 192 (2017) 57–67. https://doi.org/10.1016/j.jenvman.2017.01.040.

[104] D. Ronga, L. Setti, C. Salvarani, R. De Leo, E. Bedin, A. Pulvirenti, J. Milc, N. Pecchioni, E. Francia, Effects of solid and liquid digestate for hydroponic baby leaf lettuce (Lactuca sativa L.) cultivation, Sci. Hortic. (Amsterdam). 244 (2019) 172–181. https://doi.org/10.1016/J.SCIENTA.2018.09.037.

[105] J. Venglovsky, N. Sasakova, I. Placha, Pathogens and antibiotic residues in animal manures and hygienic and ecological risks related to subsequent land application, Bioresour. Technol. 100 (2009) 5386–5391. https://doi.org/10.1016/J.BIORTECH.2009.03.068.

[106] A. Bonmatí, X. Flotats, Air stripping of ammonia from pig slurry: Characterisation and feasibility as a pre- or post-treatment to mesophilic anaerobic digestion, Waste Manag. 23 (2003) 261–272. https://doi.org/10.1016/S0956-053X(02)00144-7.

[107] A. Makara, Z. Kowalski, Selection of pig manure management strategies: Case study of Polish farms, J. Clean. Prod. 172 (2018) 187–195.

https://doi.org/10.1016/J.JCLEPRO.2017.10.095.

[108] ASPAPEL, Agenda Sectorial Industria Papelera, 2018. http://www.aspapel.es/sites/default/files/publicaciones/doc_513_agenda_sectorial_industria_papelera_0.pdf.

[109] Asociación española de fabricantes de pasta papel y cartón, Memoria de sostenibilidad 2020. La bioindustria circular del papel y su descarbonización., 2020. http://www.aspapel.es/sites/default/files/adjuntos/doc_738_publicacion_actualizacion_m_sostenibilidad_2020.pdf.

[110] M.A. Hubbe, J.R. Metts, D. Hermosilla, M.A. Blanco, L. Yerushalmi, F. Haghighat, P. Lindholm-Lehto, Z. Khodaparast, M. Kamali, A. Elliott, Wastewater treatment and reclamation: A review of pulp and paper industry practices and opportunities, BioResources. 11 (2016) 7953–8091. https://doi.org/10.15376/biores.11.3.hubbe.

[111] R. Toczyłowska-Mamińska, Limits and perspectives of pulp and paper industry wastewater treatment – A review, Renew. Sustain. Energy Rev. 78 (2017) 764–772. https://doi.org/10.1016/j.rser.2017.05.021.

[112] J. Lauwaert, I. Stals, C.S. Lancefield, W. Deschaumes, D. Depuydt, B. Vanlerberghe, T. Devlamynck, P.C.A. Bruijnincx, A. Verberckmoes, Pilot scale recovery of lignin from black liquor and advanced characterization of the final product, Sep. Purif. Technol. 221 (2019) 226–235. https://doi.org/10.1016/j.seppur.2019.03.081.

[113] N. Mahmood, Z. Yuan, J. Schmidt, C. Xu, Depolymerization of lignins and their applications for the preparation of polyols and rigid polyurethane foams: A review, Renew. Sustain. Energy Rev. 60 (2016) 317–329. https://doi.org/10.1016/j.rser.2016.01.037.

[114] D. Pokhrel, T. Viraraghavan, Treatment of pulp and paper mill wastewater - A review, Sci. Total Environ. 333 (2004) 37–58. https://doi.org/10.1016/j.scitotenv.2004.05.017.

[115] L. Pola, S. Collado, P. Oulego, P. Calvo, M. Díaz, Characterisation of the wet oxidation of black liquor for its integration in Kraft paper mills, Chem. Eng. J. 405 (2021) 126610. https://doi.org/10.1016/j.cej.2020.126610.

[116] E. Nikolskaya, P. Janhunen, M. Haapalainen, Y. Hiltunen, Solids Content of Black Liquor Measured by Online Time-Domain NMR, Appl. Sci. 9 (2019) 2169. https://doi.org/10.3390/app9102169.

[117] G. Adhikari, K.G. Bhattacharyya, Impact of pulp and paper mill effluents and solid wastes on soil mineralogical and physicochemical properties, Environ. Monit. Assess. 187 (2015). https://doi.org/10.1007/s10661-015-4330-z.

[118] M. Kamali, Z. Khodaparast, Review on recent developments on pulp and paper mill wastewater treatment, Ecotoxicol. Environ. Saf. 114 (2015) 326–342. https://doi.org/10.1016/j.ecoenv.2014.05.005.

[119] T. Kinnarinen, M. Golmaei, E. Jernström, A. Häkkinen, Separation, treatment and utilization of inorganic residues of chemical pulp mills, J. Clean. Prod. 133 (2016) 953–964. https://doi.org/10.1016/j.jclepro.2016.06.024.

[120] Z. Al-Kaabi, R. Pradhan, N. Thevathasan, A. Gordon, Y.W. Chiang, P. Arku, A. Dutta, Ash removal from various spent liquors by oxidation process for bio-carbon production, J. Environ. Chem. Eng. 8 (2020) 103520. https://doi.org/10.1016/j.jece.2019.103520.

[121] E.C.L. Coimbra, A.H. Mounteer, A.L.V. do Carmo, M.J.F. Michielsen, L.A. Tótola, J.P.F. Guerino, J.G.A.N. Gonçalves, P.R. da Silva, Electrocoagulation of kraft pulp bleaching filtrates to improve biotreatability, Process Saf. Environ. Prot. 147 (2021) 346–355. https://doi.org/10.1016/j.psep.2020.09.039.

[122] J.P. Ribeiro, C.C. Marques, I. Portugal, M.I. Nunes, AOX removal from pulp and paper wastewater by Fenton and photo-Fenton processes: A real case-study, in: Energy Reports, Elsevier Ltd, 2020: pp. 770–775. https://doi.org/10.1016/j.egyr.2019.09.068.

[123] L. Bijan, M. Mohseni, Using ozone to reduce recalcitrant compounds and to enhance biodegradability of pulp and paper effluents, Water Sci. Technol. 50

(2004) 173–182. https://doi.org/10.2166/wst.2004.0190.

[124] M. Efgan Kibar, S. Veli, A. Arslan, S. Ketizmen, A. Nilgün Akin, Catalytic Wet Air Oxidation of Pulp and Paper Industry Wastewater 1, J. Water Chem. Technol. 41 (2019) 36–43. https://doi.org/10.3103/S1063455X19010065.

[125] S.S. Hassan, G.A. Williams, A.K. Jaiswal, Emerging technologies for the pretreatment of lignocellulosic biomass, Bioresour. Technol. 262 (2018) 310–318. https://doi.org/10.1016/j.biortech.2018.04.099.

[126] S. Madan, P. Sachan, U. Singh, A review on bioremediation of pulp and paper mill effluent – An alternative to conventional remedial technologies, J. Appl. Nat. Sci. 10 (2018) 367–374. https://doi.org/10.31018/jans.v10i1.1632.

[127] H. Vashi, O.T. Iorhemen, J.H. Tay, Degradation of industrial tannin and lignin from pulp mill effluent by aerobic granular sludge technology, J. Water Process Eng. 26 (2018) 38–45. https://doi.org/10.1016/j.jwpe.2018.09.002.

[128] A. Brink, C.M. Sheridan, K.G. Harding, A kinetic study of a mesophilic aerobic moving bed biofilm reactor (MBBR) treating paper and pulp mill effluents: The impact of phenols on biodegradation rates, J. Water Process Eng. 19 (2017) 35–41. https://doi.org/10.1016/j.jwpe.2017.07.003.

[129] R. Hooda, N.K. Bhardwaj, P. Singh, Screening and Identification of Ligninolytic Bacteria for the Treatment of Pulp and Paper Mill Effluent, Water, Air, Soil Pollut. 226 (2015) 305. https://doi.org/10.1007/s11270-015-2535-y.

[130] D.M. Brown, J. Pawlak, A.M. Grunden, Bacterial valorization of pulp and paper industry process streams and waste, Appl. Microbiol. Biotechnol. 105 (2021) 1345–1363. https://doi.org/10.1007/s00253-021-11107-2.

[131] S. Rodríguez-Couto, Industrial and environmental applications of white-rot fungi, Mycosphere. 8 (2017) 456–466. https://doi.org/10.5943/mycosphere/8/3/7.

[132] J.A. Mir-Tutusaus, R. Baccar, G. Caminal, M. Sarrà, Can white-rot fungi be a real wastewater treatment alternative for organic micropollutants removal? A review, Water Res. 138 (2018) 137–151.

https://doi.org/10.1016/j.watres.2018.02.056.

[133] A.I. Díaz, M. Ibañez, A. Laca, M. Díaz, Biodegradation of Olive Mill Effluent by White-Rot Fungi, Appl. Sci. 11 (2021) 9930. https://doi.org/10.3390/app11219930.

[134] R. Zhuo, F. Fan, A comprehensive insight into the application of white rot fungi and their lignocellulolytic enzymes in the removal of organic pollutants, Sci. Total Environ. 778 (2021) 146132. https://doi.org/10.1016/j.scitotenv.2021.146132.

[135] A. Zerva, G.I. Zervakis, P. Christakopoulos, E. Topakas, Degradation of olive mill wastewater by the induced extracellular ligninolytic enzymes of two wood-rot fungi, J. Environ. Manage. 203 (2017) 791–798. https://doi.org/10.1016/J.JENVMAN.2016.02.042.

[136] E. Rouches, I. Herpoël-Gimbert, J.P. Steyer, H. Carrere, Improvement of anaerobic degradation by white-rot fungi pretreatment of lignocellulosic biomass: A review, Renew. Sustain. Energy Rev. 59 (2016) 179–198. https://doi.org/10.1016/j.rser.2015.12.317.

[137] D. Rajwar, J.P.N. Rai, Kraft black liquor decolorization by fungi isolated from contaminated pulp and paper mill sludge, Int. J. Recent Sci. Res. 6 (2015) 7770–7775.

[138] R. Deshmukh, A.A. Khardenavis, H.J. Purohit, Diverse Metabolic Capacities of Fungi for Bioremediation, Indian J. Microbiol. 56 (2016) 247–264. https://doi.org/10.1007/s12088-016-0584-6.

[139] A.F. Ariste, R.A. Batista-García, V.K. Vaidyanathan, N. Raman, V.K. Vaithyanathan, J.L. Folch-Mallol, S.A. Jackson, A.D.W. Dobson, H. Cabana, Mycoremediation of phenols and polycyclic aromatic hydrocarbons from a biorefinery wastewater and concomitant production of lignin modifying enzymes, J. Clean. Prod. 253 (2020) 119810. https://doi.org/10.1016/j.jclepro.2019.119810.

[140] S. Sankaran, S.K. Khanal, N. Jasti, B. Jin, A.L. Pometto, J.H. Van Leeuwen, Use

of filamentous fungi for wastewater treatment and production of high value fungal byproducts: A review, Crit. Rev. Environ. Sci. Technol. 40 (2010) 400–449. https://doi.org/10.1080/10643380802278943.

[141] D. Gao, L. Du, J. Yang, W.M. Wu, H. Liang, A critical review of the application of white rot fungus to environmental pollution control, Crit. Rev. Biotechnol. 30 (2010) 70–77. https://doi.org/10.3109/07388550903427272.

[142] D. Martinez, L.F. Larrondo, N. Putnam, M.D. Sollewijn Gelpke, K. Huang, J. Chapman, K.G. Helfenbein, P. Ramaiya, J.C. Detter, F. Larimer, P.M. Coutinho, B. Henrissat, R. Berka, D. Cullen, D. Rokhsar, Genome sequence of the lignocellulose degrading fungus Phanerochaete chrysosporium strain RP78, Nat. Biotechnol. 22 (2004) 695–700. https://doi.org/10.1038/nbt967.

[143] M. Dixit, H. Liu, J. Luo, P. Shukla, Effluents detoxification from pulp and paper industry using microbial engineering and advanced oxidation techniques, J. Hazard. Mater. 398 (2020) 122998. https://doi.org/10.1016/j.jhazmat.2020.122998.

[144] G. Marinho, B.C.A. Barbosa, K. Rodrigues, M. Aquino, L. Pereira, Potential of the filamentous fungus Aspergillus niger AN 400 to degrade Atrazine in wastewaters, Biocatal. Agric. Biotechnol. 9 (2017) 162–167. https://doi.org/10.1016/j.bcab.2016.12.013.

[145] F. Lu, K. Ping, L. Wen, W. Zhao, Z. Wang, J. Chu, Y. Zhuang, Enhancing gluconic acid production by controlling the morphology of Aspergillus niger in submerged fermentation, Process Biochem. 50 (2015) 1342–1348. https://doi.org/10.1016/j.procbio.2015.04.010.

[146] G. Perrone, J. Varga, A. Susca, J.C. Frisvad, G. Stea, S. Kocsubé, B. Tóth, Z. Kozakiewicz, R.A. Samson, J. Nos Varga, A. Susca, J.C. Frisvad, G. Stea, S. Ndor Kocsubé, B. Ta Tó, Z. Kozakiewicz, R.A. Samson, Aspergillus uvarum sp. nov., an uniseriate black Aspergillus species isolated from grapes in Europe, Int. J. Syst. Evol. Microbiol. 58 (2008) 1032–1039. https://doi.org/10.1099/ijs.0.65463-0.

[147] J.M. Salgado, L. Abrunhosa, A. Venâncio, J.M. Domínguez, I. Belo, Screening

of winery and olive mill wastes for lignocellulolytic enzyme production from Aspergillus species by solid-state fermentation, Biomass Convers. Biorefinery. 4 (2014) 201–209. https://doi.org/10.1007/s13399-013-0100-8.

[148] J.M. Salgado, L. Abrunhosa, A. Venâncio, J.M. Domínguez, I. Belo, Combined bioremediation and enzyme production by Aspergillus sp. in olive mill and winery wastewaters, Int. Biodeterior. Biodegrad. 110 (2016) 16–23. https://doi.org/10.1016/j.ibiod.2015.12.011.

[149] J.J. Salgado, L. Abrunhosa, A. Venâ, J. Josémanuel Domínguez, I. Belo, Enhancing the Bioconversion of Winery and Olive Mill Waste Mixtures into Lignocellulolytic Enzymes and Animal Feed by Aspergillus uvarum Using a Packed-Bed Bioreactor, (2015). https://doi.org/10.1021/acs.jafc.5b02131.

[150] F. Spina, V. Tigini, A. Romagnolo, G.C. Varese, Bioremediation of landfill leachate with fungi: Autochthonous vs. allochthonous strains, Life. 8 (2018). https://doi.org/10.3390/life8030027.

[151] V. Faraco, C. Pezzella, A. Miele, P. Giardina, G. Sannia, Bio-remediation of colored industrial wastewaters by the white-rot fungi Phanerochaete chrysosporium and Pleurotus ostreatus and their enzymes, Biodegradation. 20 (2009) 209–220. https://doi.org/10.1007/s10532-008-9214-2.

[152] S. Rodríguez Couto, I. Rivela, M.R. Muñoz, A. Sanromán, Ligninolytic enzyme production and the ability of decolourisation of Poly R-478 in packed-bed bioreactors by Phanerochaete chrysosporium, Bioprocess Eng. 23 (2000) 287–293. https://doi.org/10.1007/PL00009126.

[153] K. Pakshirajan, P. Radhika, Enzymatic Decolourization of Textile Dyeing Wastewater by the White Rot Fungus Phanerochaete Chrysosporium, Text. Light Ind. Sci. Technol. 2 (2013) 42–48. www.tlist-journal.org.

[154] O. Benzina, D. Daâssi, H. Zouari-Mechichi, F. Frikha, S. Woodward, L. Belbahri, S. Rodriguez-Couto, T. Mechichi, Decolorization and detoxification of two textile industry effluents by the laccase/1-hydroxybenzotriazole system, Environ. Sci. Pollut. Res. 20 (2013) 5177–5187. https://doi.org/10.1007/s11356-013-1491-6.

Capítulo 3.

MATERIAL Y MÉTODOS

3.1. MATERIAS PRIMAS

3.1.1. Licor de biometanización

El licor de digestión anaerobia o de biometanización (ADL) es un efluente procedente de la digestión anaerobia de lodos de depuradora. Tras el tratamiento biológico de digestión, el digestato obtenido se somete a un proceso de centrifugación, que da como resultado una fracción sólida y una fracción líquida. Generalmente, la fracción sólida se deriva a procesos de compostaje, mientras que la fracción líquida, se mantiene en un decantador durante un periodo aproximado de 2 días. El efluente obtenido tras el proceso de decantación, es lo que se conoce como licor de biometanización o licor de digestión anaerobia [1]. A continuación, en la Figura 3.1 se muestra un esquema del proceso.

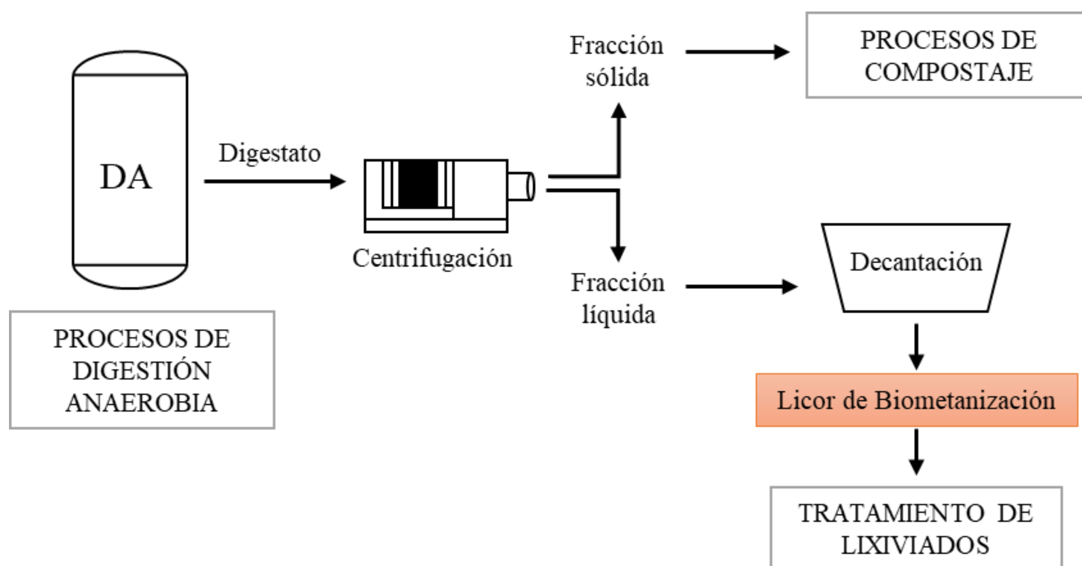


Figura 3.1. *Proceso de obtención del licor de biometanización*

Las muestras empleadas en esta Tesis fueron suministradas por el Consorcio para la Gestión de los Residuos Sólidos de Asturias (COGERSA, S.A.U.). En concreto, el efluente fue recogido de la planta de biometanización localizada en el Centro Integrado de Tratamiento de Residuos. La Tabla 3.1 muestra las principales características fisicoquímicas del efluente. El rango de valores de los parámetros mostrados corresponde a varias muestras de licor de biometanización analizadas durante un periodo de 12 meses.

Dado que este efluente fue empleado para todas las investigaciones recogidas en el Capítulo 4.1, también se muestra la caracterización concreta del efluente en apartado de "*Material and Methods*" de cada investigación.

Tabla 3.1. *Caracterización del licor de biometanización*

Parámetro	Valor
pH	7.96 – 8.26
DQO total (mg O_2/L)	15179 - 16802
DQO soluble (mg O_2/L)	7205 - 8098
COT soluble (mg/L)	3218 - 3788
DBO_5 soluble (mg O_2/L)	2135 - 2960
Color (CN)	0.899 – 1.275
AOSC	0.580 – 0.904
NH_4^+ (mg/L)	3812 - 4166

3.1.2. Permeado de ultrafiltración

El permeado de ultrafiltración o permeado de lixiviado es un residuo procedente del tratamiento combinado (biológico y fisicoquímico) de lixiviados de vertedero [2]. En concreto, las muestras de empleadas en esta Tesis Doctoral fueron recogidas de la depuradora de lixiviados de vertedero situada en el Centro de Tratamiento de Residuos de Asturias (COGERSA, S.A.U.), que basa el proceso de tratamiento en la tecnología BIOMEMBRAT. En este tipo de plantas de tratamiento, tras los procesos biológicos de desnitrificación y nitrificación de los lixiviados de vertedero, donde se reduce el contenido en amonio y materia orgánica biodegradable del lixiviado, el efluente tratado se somete a un proceso posterior de filtración por membranas. Dichas membranas poseen una superficie 280 m^2 y un tamaño de poro de 0,02 µm. Como productos finales se obtienen el agua tratada (permeado de ultrafiltración) y los lodos biológicos [3]. Las investigaciones realizadas con este residuo, cuya composición fisicoquímica se muestra en la Tabla 3.2, se describen en el Capítulo 4.2.2.

Tabla 3.2. *Caracterización del permeado de ultrafiltración*

Parámetro	Valor
pH	6,78 ± 0,11
Conductividad (µS/cm)	11723 ± 453
DQO soluble (mg O_2/L)	1265 ± 6
DBO_5 soluble (mg O_2/L)	28 ± 3
Biodegradabilidad (BI)	0,02 ± 0,003
Amonio (mg/L)	21 ± 3
Nitratos (mg/L)	469 ± 79
Nitritos (mg/L)	1,5 ± 0,3
Fosfatos (mg/L)	9 ± 2
Sólidos Totales (mg/L)	6,8 ± 0, 1

3.1.3. Descartes alimentarios

Los tratamientos fisicoquímicos de los residuos agroalimentarios se llevaron a cabo empleando desechos procedentes de la venta de hortalizas y verduras, en concreto patata, tomate y pimiento verde (Fig. 3.2).

Figura 3.2. *Residuos agroalimentarios empleados*

Los residuos fueron abastecidos por un mercado local y su selección se realizó teniendo en cuenta tres factores:

i) **El volumen de residuos generados:** los tres residuos empleados representaron en torno al 40% del total de residuos del supermercado local que suministró las materias primas.

ii) **La complejidad de los residuos:** Alto contenido en carbohidratos complejos que necesitan hidrolizarse.

iii) **La disponibilidad de los residuos:** no son productos de temporada por lo que están disponibles durante todo el año.

La composición nutricional de cada uno de residuos empleados se muestra seguidamente en la Tabla 3.3. Esta información también puede consultarse para cada ensayo específico recogido en el apartado 4.3.1 de esta Tesis.

Tabla 3.3. *Composición nutricional de los vegetales empleados como sustrato. Los valores aportados corresponden a 100 g de residuo fresco.*

	Tomate	Pimiento	Patata
Carbohidratos (g)*	3.9	4.6	17
Lípidos (g)*	0.2	0.2	0.1
Proteínas (g)*	0.9	0.9	2.0
Agua (g)**	93.0	93.5	63.5
Vitaminas (mg)*	15.7[1]	133[2]	21.5[3]
Minerales (mg)*	272[1]	218[2]	521[3]

*Valores medios adaptados de USDA (United States Department of Agriculture)

**Valores medios de específicos de los residuos empleados

[1]Vitaminas (A, B_1, B_3, B_6, C, E, K) / Minerales (Mg, Mn, P, K)

[2]Vitaminas (A, B_1, B_2, B_3, B_6, C, E, K) / Minerales (Ca, Fe, Mg, P, K, Na, Zn)

[3]Vitaminas (B_1, B_2, B_3, B_6, C) / Minerales (Ca, Fe, Mg, P, K, Na)

3.1.4. Licor negro

Las muestras de licor negro empleadas en este trabajo fueron facilitadas por ENCE, una empresa fabricante de papel ubicada en Navia, Asturias (España). El residuo fue obtenido durante el proceso de fabricación de pasta Kraft, tras la etapa de cocción de madera de *Eucalytus* [4].

En la Tabla 3.4, se detalla la caracterización inicial del licor negro empleado para llevar a cabo los ensayos de biodegradación descritos en el Capítulo 4.4. De nuevo, en el apartado "*Material and Methods*", de la sección 4.4.1, se ha detallado la composición específica del efluente para cada tratamiento en particular.

Tabla 3.4. *Caracterización del licor negro.*

Parámetro	Valor
pH	12.9 ± 0.02
DQO total (mg O_2/L)	146540 ± 115
DBO_5 total (mg O_2/L)	12356 ± 29
Biodegradabilidad (BI)	0.080 ± 0.001
Azúcares reductores solubles (mg/L)	10028 ± 152
Compuestos fenólicos solubles (mg/L)	16425 ± 102
Color (CN)	113 ± 8
Materia seca (%)	12.5 ± 0.1

3.1.5. Alperujo

El alperujo es un subproducto agroalimentario procedente de la industria del aceite de oliva que se genera durante la obtención del aceite de oliva mediante un sistema de extracción de dos fases [5]. Como puede observarse en la Figura 3.3, se trata de un residuo semisólido con alto contenido en humedad (65-70%), compuesto por restos de hueso y piel de la aceituna triturada, y que aún contiene cierta cantidad de aceite [6].

Figura 3.3. *Residuo de Alperujo*

El alperujo utilizado en esta Tesis Doctoral fue facilitado por una fábrica de aceite de oliva ubicada en Sevilla, España. La composición fisicoquímica de este residuo se detalla en la Tabla 3.5.

Tabla 3.5. *Caracterización del residuo de Alperujo*

Parámetro	Valor
pH	4.6 ± 0.01
DQO soluble (mg O_2/L)	4854 ± 19
BOD_5 soluble (mg O_2/L)	408 ± 14
Biodegradabilidad (B.I.)	0.080 ± 0.003
Azúcares reductores (mg/L)	578 ± 24
Compuestos fenólicos totales (mg/L)	134 ± 4
Color (CN)	1.60 ± 0.04
Sólidos suspendidos totales (mg/L)	2475 ± 21
Sólidos suspendidos fijos (mg/L)	375 ± 12
Sólidos suspendidos volátiles (mg/L)	2100 ± 28

3.1.6. Aguas residuales porcinas

En este trabajo se han realizado estudios de biodegradación con dos tipos de aguas residuales porcinas diferentes. Por un lado, se emplearon aguas residuales porcinas correspondientes a la mezcla de las deyecciones (heces y orina) de los cerdos, con las aguas de lavado procedentes de las pocilgas. En concreto, las muestras empleadas fueron recolectadas de una granja de cerdos ubicada en Salamanca (España). Las características principales de este efluente se muestran en la Tabla 3.6.

Tabla 3.6. *Caracterización de los purines de cerdo*

Parámetro	Muestra 1
DQO total (mg O_2/L)	23569-16780
DQO soluble (mg O_2/L)	3916- 3639
DBO_5 soluble (mg O_2/L)	724 - 672
Biodegradabilidad	0.19 – 0.18
Nitrógeno total (mg N/L)	3631 - 2134
pH	8.6 – 8.2
Color (CN)	2.41 – 2.24

Por otro lado, se realizaron estudios de biodegradación empleando como material de partida dos efluentes procedentes de la digestión anaerobia de purines. La diferencia entre ambos digestatos fue en el tipo de reactor anaerobio empleado durante el pretratamiento de digestión. En la Tabla 3.7, se muestra la caracterización fisicoquímica de ambos digestatos. El digestato A, se obtuvo a partir de un proceso de digestión anaerobia convencional mientras que el digestato B, se obtuvo a partir de un reactor anaeróbico al cual fue acoplada una membrana de captación de amoniaco. Una información más detallada de este proceso puede consultarse en los trabajos desarrollados por González-García et al. [7] y Riaño et al. [8].

Tabla 3.7. *Caracterización de los efluentes procedentes de la digestión anaerobia de purines de cerdo*

Parámetro	Digestato A	Digestato B
pH	7.58	8.03
DQO total (mg O_2/L)	5362	4125
DQO soluble (mg O_2/L)	3246	2852
DBO_5 soluble (mg O_2/L)	213	234
Biodegradabilidad	0.07	0.08
Color (CN)	2.122	2.106

3.2. METODOLOGÍA EXPERIMENTAL

La metodología empleada en esta Tesis Doctoral puede clasificarse en tres grupos: las técnicas aplicadas para la caracterización microbiológica de los efluentes, los métodos de tratamiento fisicoquímicos y, por último, los métodos de tratamiento biológico con hongos. Seguidamente, se describen detalladamente cada una de ellas:

3.2.1. Caracterización microbiológica

Para la caracterización microbiológica de las muestras evaluadas en los apartados 4.1 y 4.2 (licor de biometanización y permeado de ultrafiltración), se aplicaron técnicas de secuenciación de nueva generación (NGS), basadas en la secuenciación masiva del gen ARNr 16S, empleando el sistema Ion Torrent PGM de secuenciación en masa.

Este proceso consta de 4 etapas: extracción del ADN, amplificación del ADN, construcción de librerías de ADN y secuenciación del ADN. A continuación, se desarrolla cada una de ellas:

3.2.1.1. Extracción del ADN

La extracción del ADN se realizó a partir de la fracción sólida de los diferentes efluentes evaluados, por lo que las muestras fueron sometidas a un pretratamiento físico de centrifugación. Para ello, 160 ml de muestra se centrifugaron durante 20 minutos a

13000 g (Kubota 6500 High Speed Refrigerated Centrifuge). El sobrenadante fue descartado mientras que la fracción sólida se conservó para extraer el ADN.

Para la extracción de ADN se empleó el kit Power Biofilm DNA Isolation Kit (MoBio Laboratories, Inc., Carlsbad, CA). Esta etapa se desarrolló siguiendo las instrucciones técnicas del kit, salvo en el caso del volumen de buffer BF3 añadido. Este buffer se emplea para disminuir los procesos de inhibición durante la extracción del ADN. Su cantidad ha de incrementarse cuando el color de las muestras es muy elevado o cuando dichas muestras contienen una elevada carga de compuestos inhibidores. Dada la complejidad y la coloración de las muestras evaluadas en esta Tesis Doctoral se añadieron 200 µL de BF3 en vez de 100 µL, como se recomienda en el protocolo.

Finalizado el proceso de extracción, el ADN se concentró usando el kit Concentrator Plus Vacufuge® (Eppendorf, Germany). La concentración de ADN se determinó mediante espectrofotometría, empleando el espectrofotómetro BioPhotometer Plus (Eppendorf, Germany), con la finalidad de asegurar que la concentración de ADN fuese óptima para continuar con el proceso de amplificación.

3.2.1.2. Amplificación del ADN

La amplificación del ADN se llevó a cabo mediante la reacción en cadena de la polimerasa (PCR) empleando el kit Ion 16S Metagenomics Kit (Ion Torrent, Life Technologies). Este kit incluye material para amplificar por un lado las regiones V2-4-8, y por otro las regiones V3-6, 7-9 del gen 16S.

El proceso de amplificación se desarrolló en un termociclador de acuerdo con las condiciones descritas en la Tabla 3.8.

Los fragmentos de ADN resultantes de la amplificación fueron purificados usando el kit Agencourt AMPure XP Kit (Beckman Coulter, USA). Finalmente, los amplicones se cuantificaron empleando el kit dsDNA HS Assay Kit (Invitrogen, USA) y usando el fluorómetro Qubit® 2.0 Fluorometer.

Tabla 3.8. *Condiciones de la PCR para la amplificación del ADN.*

Fase	Temperatura (ºC)	Tiempo
Calentamiento	95	10 min
Desnaturalización*	95	30 seg
Alineamiento	58	30 seg
Extensión	72	30 min
Elongación	72	7 min
Conservación	4	20 min

*25 ciclos de desnaturalización

3.2.1.3. Construcción de librerías de ADN

La preparación de las librerías de ADN consiste en la fragmentación del ADN obtenido en la fase de purificación con la finalidad de obtener fragmentos más pequeños de hasta 150 pb. La fragmentación del ADN amplificado se llevó a cabo mediante el kit Ion Plus Fragment Library Kit (AB Library Builder™), mientras que para la construcción de las librerías se empleó el kit PGM ™ Hi-Q ™ OT2. Cada librería se corresponde con una colección diferente de fragmentos de ADN a secuenciar y es única para cada muestra.

3.2.1.4. Secuenciación del ADN

Finalmente, las muestras se secuenciaron empleando técnicas de secuenciación masiva mediante el kit PGM ™ Hi-Q ™ y el kit de chip Ion 318 ™ v2.

3.2.2. Tratamientos fisicoquímicos

Según el residuo tratado inicialmente, los métodos fisicoquímicos empleados en esta Tesis pueden clasificarse en dos grupos. Por un lado, se encuentran las técnicas evaluadas como tratamientos de post-anaerobiosis, cuando el residuo tratado corresponde al licor de biometanización, mientras que, por otro lado, están las técnicas empleadas como pretratamiento de residuos agroalimentarios alimentarios. Estos tratamientos han sido aplicados en las investigaciones recogidas en el apartado 4.1.2 y 4.3.1 de este trabajo, respectivamente. Esta metodología puede consultarse de forma específica para cada residuo en la sección "*Material and Methods*", de cada apartado.

3.2.2.1. Técnicas fisicoquímicas post-anaerobiosis

Se evaluaron 4 tratamientos diferentes: ultrasonidos, ozonización, hidrólisis y oxidación húmeda. En todos los casos, después del tratamiento, las muestras se almacenaron a 4ºC durante 24 h antes de ser analizadas. La metodología empleada se describe a continuación:

a) Ultrasonidos

Los ensayos con ultrasonidos se llevaron a cabo en un homogeneizador ultrasónico (BANDELIN SONOPULS HD 2070) equipado con una sonda de ultrasonidos VS 70, a una frecuencia de 20 kHz. Se realizaron experimentos empleando dos amplitudes de trabajo distintas, 50% y 90%, y se tomaron muestras tras 5, 10, 15, 30 y 45 minutos de sonicación. La amplitud de sonicación del 100% es equivalente a 80 μm de amplitud. Los ensayos se realizaron en condiciones isotérmicas a 40ºC, manteniendo la temperatura constante mediante un baño de agua. Todos los ensayos se realizaron en matraces Erlenmeyer de vidrio de 250 mL de capacidad con 100 mL de muestra.

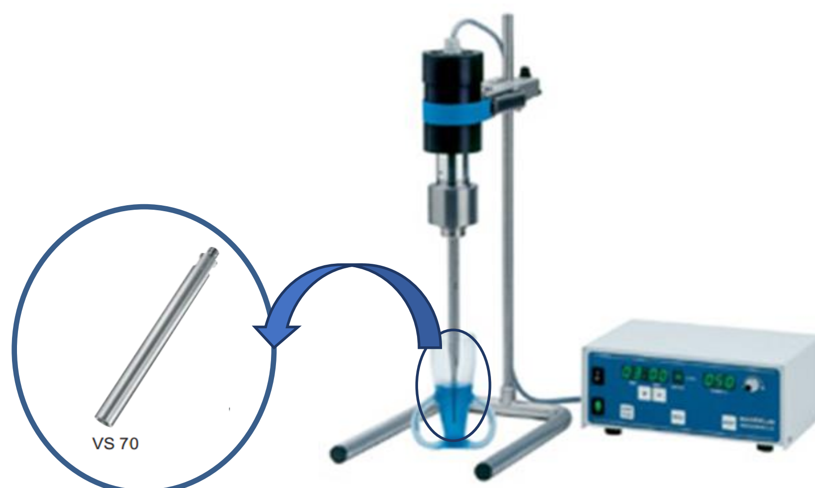


Figura 3.4. *Equipo de ultrasonidos BANDELIN SONOPULS HD 2070 con sonda VS 70.*

b) Ozonización

El proceso de ozonización se realizó introduciendo 300 mL de muestra en un reactor cilíndrico agitado con una capacidad de 1 L. Para estos experimentos se burbujeó a través del reactor un flujo de ozono de 12 g O_3/h. El ozono se generó a partir de oxígeno de grado industrial utilizando un generador de ozono (ZonoSistem).

Los ensayos se realizaron en condiciones isotérmicas a 25ºC y se tomaron muestras periódicamente durante 8 h. Además, con la finalidad de estudiar el efecto de los sólidos presentes en la muestra sobre la efectividad del tratamiento, se realizaron ensayos adicionales en las mismas condiciones, pero empleando la muestra sin sólidos. Estos sólidos se eliminaron mediante centrifugación a 20ºC y 13000 g durante 15 min.

Figura 3.5. *Equipo de ozonización*

c) Hidrólisis

Los experimentos de hidrólisis se llevaron a cabo en un reactor semidiscontinuo de 1 L de capacidad (Parr T316SS), cargado con un volumen de 700 mL de muestra, garantizando así unas condiciones operativas seguras. Además, en base a experiencias previas, se adicionó a la muestra 20 µL de antiespumante 289 (Sigma-Aldrich) para evitar la formación de espumas durante el ensayo.

A continuación, el reactor se presurizó y se calentó en función de las condiciones de trabajo deseadas (160ºC, 180ºC o 200ºC). En todos los casos, la

velocidad de agitación en el reactor se ajustó a 350 rpm y la presión se proporcionó mediante nitrógeno comprimido suministrado al reactor con un flujo de 1400 mL/min. Este flujo fue regulado mediante un controlador de flujo másico electrónico (Brooks). El nitrógeno se burbujeó a través del depósito de agua para su saturación con agua antes de ser empleado en el recipiente de reacción. La presión se mantuvo constante a 6,0 MPa por medio de un controlador de contrapresión instalado al final de la línea de gas. Se tomaron muestras periódicamente durante 8 h.

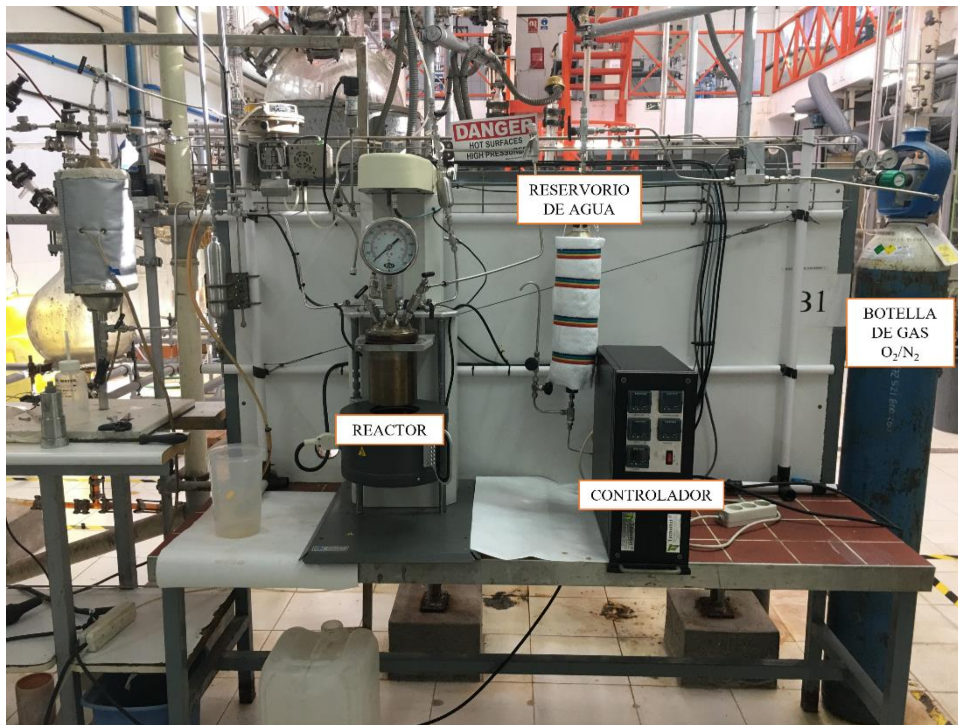


Figura 3.6. *Equipo de hidrólisis y oxidación húmeda.*

d) Oxidación húmeda

El equipo y el procedimiento experimental empleado para los ensayos de oxidación húmeda fueron los mismos que se usaron en los experimentos de hidrólisis, descritos en el apartado anterior, con la única diferencia de que se usó un gas oxidante (oxígeno) en lugar de nitrógeno.

3.2.2.2. Técnicas fisicoquímicas como pretratamiento de residuos alimentarios

Estas técnicas fisicoquímicas fueron empleadas para el tratamiento de los residuos agroalimentarios procedentes de la venta de verduras y hortalizas, en concreto patata, tomate y pimiento verde (Apartado 4.3.1). En este caso, los residuos se lavaron inicialmente con agua destilada y se conservaron a 4ºC por un periodo máximo de tres días hasta el momento del tratamiento.

Se evaluaron diferentes técnicas de hidrólisis: térmica, ácida, enzimática, y una hidrólisis mixta termo-enzimática. Todos los ensayos se llevaron a cabo en botellas Pyrex de 250 mL. Para cada residuo se evaluó el tratamiento llevando a cabo experimentos por triplicado y utilizando diferentes lotes de cada residuo.

En todos los casos, el hidrolizado final obtenido se centrifugó durante 5 min a 5000 rpm y 20ºC (Centrífuga Refrigerada de Alta Velocidad Kubota 6500). Finalmente, la fracción solida obtenida fue desechada, mientras que, el sobrenadante (fracción líquida) se ajustó a pH 6,5-7 con NaOH 6 M o HCl 1 M y conservó hasta su análisis a -20ºC [9].

A continuación, se describe de forma detallada cada uno de los tratamientos hidrolíticos aplicados a los residuos agroalimentarios:

a) Hidrólisis térmica

Se evaluaron dos tratamientos térmicos diferentes con la finalidad de conocer el efecto de un secado previo del material de partida sobre la efectividad del tratamiento.

- **Tratamiento 1:** Se llevó a cabo un tratamiento térmico en base a los resultados reportados por Del Campo et al. [10] con algunas modificaciones. En primer lugar, se trituraron 100 g de muestra en fragmentos menores a 2 cm y se secaron a 55ºC durante 24 h en una incubadora (Heidolph Unimax 2010). Una vez seco, la muestra se trituró en un robot de cocina (Moulinex Minirobot D81) obteniendo un tamaño de partícula por debajo de 2 mm. Posteriormente, se introdujo la muestra triturada en botellas Pyrex de 250 mL y se añadió agua destilada en una relación de 5% (p/v). Esta mezcla fue sometida a hidrólisis empleando una autoclave a 110ºC y 1.5 atm durante 5 min.

- **Tratamiento 2:** Para este tratamiento se siguió el protocolo descrito por Correa et al. [11]. En este caso, 10 g del residuo de partida se mezclaron con agua destilada en una relación 1:1 (p/v). Esta mezcla se homogeneizó y se introdujo en botellas Pyrex de 250 mL, para someterse posteriormente a un tratamiento de hidrólisis en una autoclave a 135ºC y 3 atm durante 5 min.

b) Hidrólisis ácida

La lignina es el componente más recalcitrante de la pared celular vegetal y cuanto mayor es la proporción de lignina, mayor es la resistencia a la degradación química y enzimática [12]. Este compuesto actúa como una barrera física que protege a los polisacáridos de la acción enzimática, por lo que la eliminación de la lignina aumenta la eficacia de las enzimas al eliminar los sitios de adsorción no productivos y al aumentar el acceso a celulosa y hemicelulosa [13,14].

Con el objetivo de eliminar la lignina de las muestras antes de la hidrólisis ácida, se trituraron 10 g de cada muestra empleando un robot de cocina (Moulinex Minirobot D81) hasta obtener un tamaño de partícula inferior a 2 mm. A continuación, se añadió a la muestra un volumen de 20 mL de NaOH 0.1N y se mantuvo la reacción durante 15 min. Pasado este tiempo, se adicionó 0.8 g de $CaSO_4$ y se mantuvo la muestra a temperatura ambiente durante 3 h.

Finalmente, la muestra se tamizó, con la finalidad de separar la fracción sólida correspondiente al material deslignificado, de la fracción líquida. La fracción líquida, fue desechada mientras que el material sólido fue sometido al tratamiento de hidrólisis ácida. En esta etapa, la muestra se mezcló con H_2SO_4 al 5% en una relación 2:1 (p/v) y se hidrolizó empleando una autoclave a 125ºC y 2 atm durante 15 min.

c) Hidrólisis enzimática

Finalmente se llevó a cabo una hidrólisis enzimática de los residuos agroalimentarios. En este caso, el primer paso consistió en la deslignificación de las muestras como se describe en el apartado anterior (hidrólisis ácida). En este caso, a la fracción sólida deslignificada se le adicionó agua destilada en una relación de 6% (p/v).

La mezcla se calentó en un baño de agua a 75ºC durante 5 min, ajustando previamente el pH a 4.5. Pasado ese tiempo, las muestras se mantuvieron en reposo a temperatura ambiente para su enfriamiento. Es importante remarcar que antes de la hidrólisis enzimática, la muestra debe encontrarse a temperatura ambiente ya que una tempera excesivamente alta inactivaría las enzimas.

Para llevar a cabo la hidrólisis enzimática, se empleó una combinación de enzimas de la siguiente manera: 83 μL de celulasa de *Trichoderma reesei* (C2730) (actividad enzimática ≥ 700 unidades de beta-glucanasa/g, densidad 1,10-1,30 g/ml), 50 μL de α-amilasa de *Aspergillus oryzae* (A8220) (actividad enzimática ≥ 800 unidades de alfa amilasa fúngica/g, densidad 1,10-1,30 g/ml) y 8 μL de amiloglucosidasa de *Aspergillus niger* (A7095) (actividad enzimática ≥ 260 unidades/ml, densidad 1,2 g/mL).

Posteriormente, las muestras se incubaron a 60ºC durante 60 min sin agitación. Para detener la reacción enzimática, las muestras calentaron a 95ºC durante 5 min en un baño de agua.

La mezcla de enzimas fue seleccionada de acuerdo al contenido en polímeros complejos de los residuos vegetales, considerando principalmente el almidón en la patata y la celulosa en el caso del tomate y del pimiento [15]. La celulasa cataliza la descomposición de la celulosa en glucosa, celobiosa y polímeros de glucosa superior. La α-amilasa y la amiloglucosidasa hidrolizan los enlaces α-(1,4)-glucosídicos del almidón en glucosa [16]. El pH y las condiciones de incubación se establecieron con el objetivo de que fueran precisos para todas las enzimas empleadas [17].

d) Hidrólisis térmico-enzimática

En base a los resultados obtenidos, se ensayó un tratamiento que combinaba la hidrólisis térmica y enzimática. Para este proceso, la deslignificación de las muestras se llevó a cabo tal y como se describe anteriormente en apartado de "*Hidrólisis ácida*".

Una vez que se obtuvo el material deslignificado, se mezcló el residuo sólido con agua destilada en una relación 1:1 (p/v) y se realizó el primer tratamiento de hidrólisis mediante una autoclave a 135ºC y 3 atm durante 5 min. Finalizada la etapa de hidrólisis térmica, se dejó enfriar la muestra a temperatura ambiente, y se ajustó el pH a

4,5. A continuación, se realizó el segundo tratamiento de hidrólisis, esta vez enzimático, desarrollándose con las mismas condiciones que las descritas en el apartado anterior de "*Hidrólisis enzimática*".

3.2.3. Tratamientos biológicos

En cuanto a los métodos biológicos aplicados en esta Tesis Doctoral, se han ensayado diferentes tratamientos de biorremediación, tanto en continuo como en discontinuo, con los hongos *Phanerochaete chrysosporium* Burdsall 1974 (cepas CECT 2798 y MUM 95.01) y *Aspergillus uvarum* MUM 08.01.

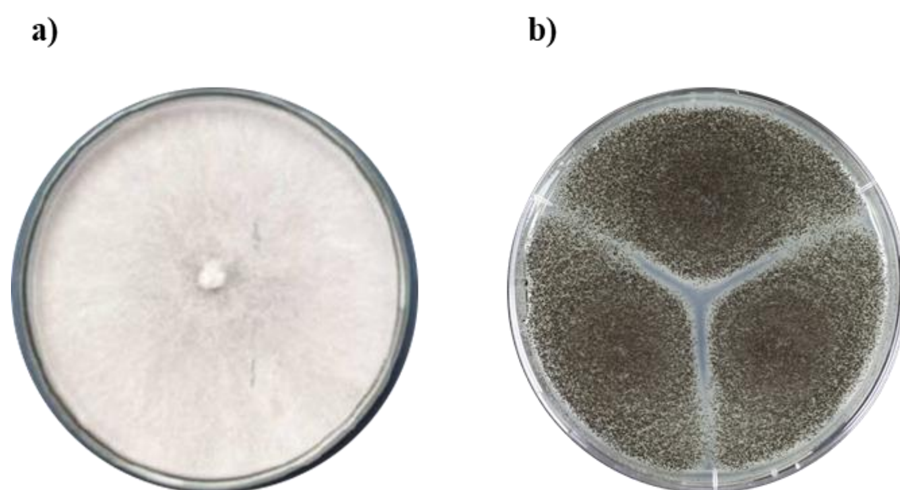


Figura 3.7. *Phanerochaete chrysosporium y Asperillus uvarum.*

En todos los casos, los tratamientos se llevaron a cabo empleando el hongo en forma de pellets. Por lo tanto, se describen inicialmente las técnicas de cultivo y obtención de los pellets fúngicos, que fueron aplicadas de igual manera en todos los tratamientos.

3.2.3.1. Obtención de los pellets de Phanerochaete chrysosporium

Todas las cepas del hongo se adquirieron liofilizadas y fueron conservadas a oscuridad y 4ºC hasta su recuperación. El primer paso fue la recuperación de las cepas liofilizadas. Este proceso, al igual que la obtención de los pellets fúngicos, se realizó en una cabina de flujo laminar, para garantizar las condiciones asépticas durante todo el

proceso. Todo el material empleado, así como las soluciones y medios de cultivo, fue esterilizado en una autoclave durante 20 min a 121ºC.

En cuanto a la recuperación de las cepas liofilizadas, el liófilo se resuspendió en 0.3 mL de medio de extracto de malta (VWR Chemicals BDH). Esta suspensión se mantuvo en reposo dentro de la campana laminar durante un tiempo de 30 minutos, para asegurar la óptima hidratación del liófilo.

Después, 100 µL de la suspensión se inocularon una placa de Petri con 25 mL de extracto de malta con agar 1.5% (p/v. Posteriormente, la placa fue incubada a 26ºC durante 7 días. Antes de utilizar el hongo para obtención de los pellets fúngicos fue necesario realizar tres subcultivos en placas Petri de extracto de malta con agar 1.5% (p/v), con el fin de asegurar la correcta activación del hongo y por tanto un crecimiento óptimo del mismo.

La obtención de los pellets fúngicos se llevó a cabo empleando la metodología descrita por Blánquez et al. [18], con algunas modificaciones. Un diagrama del proceso se muestra en la Figura 3.8.

1. En un matraz Erlenmeyer de 500 mL, se añadieron 150 mL de caldo de extracto de malta, previamente esterilizado durante 10 min a 115ºC. A continuación, el medio de cultivo se inoculó con 5 cilindros de 1 cm de diámetro extraídos de la zona de crecimiento activo con *P.chysosporium*. Este matraz se incubó a 26ºC y 135 rpm durante 6 días, observando un crecimiento alrededor de los cilindros incubados.
2. Mediante un tamiz con un tamaño de 1 mm, se recuperaron los cilindros previamente inoculados. El sobrenadante fue desechado mientras que los cilindros cubiertos de micelio se conservaron para la siguiente fase.
3. La biomasa obtenida se resuspendió en NaCl al 0.8% (p/v) en una proporción de 1:3 (p/v) y se homogeneizó empleando un homogeneizador (Heidolph Silent Crusher) durante 5 min a 11000 rpm, obteniendo la suspensión micelial.
4. Posteriormente, en un matraz Erlenmeyer de 1 L de capacidad con 250 mL de medio de extracto de malta esterilizado, se inocularon 600 µL de la suspensión micelial. El matraz se incubó a 26ºC y 135 rpm durante 6 días.

5. Después del periodo de incubación se obtuvieron los pellets fúngicos de *P. chrysosporium*. Estos pellets se recuperaron del medio de cultivo siguiendo el mismo protocolo que se indica en el punto 2.
6. Los pellets fúngicos obtenidos se sumergieron en una solución estéril de NaCl al 0,8% (p/v) y se conservaron en nevera a 4ºC hasta su uso.

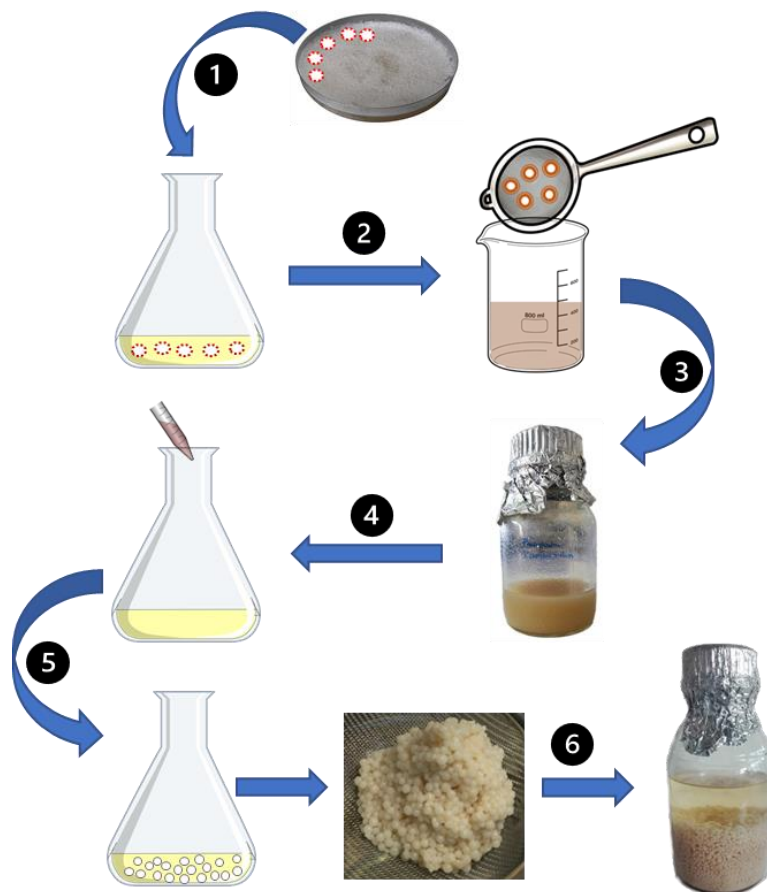


Figura 3.8. *Proceso de obtención de los pellets fúngicos de P. chrysosporium*

3.2.3.2. Obtención de los pellets de Aspergillus uvarum

Los tratamientos de biorremediación con hongos ascomicetos del género *Aspergillus*, se llevaron a cabo con *Aspergillus uvarum* MUM 08.01. La cepa liofilizada fue adquirida de la Micoteca de la Universidad de Minho (MUM), situada en Braga, Portugal. En este caso la recuperación del liófilo se realizó creciendo el hongo en extracto de malta con agar 2% (p/v) durante 7 días a 25ºC.

Para obtener los pellets fúngicos se realizó un frotis de una pequeña porción de biomasa fúngica de la parte de crecimiento activo de la placa de Petri con un asa de

siembra, y se inoculó en un matraz Erlenmeyer de 250 mL con 150 mL de medio estéril Czapek-Yeast, cuya composición fue la siguiente: 1.3 g/L de K_2HPO_4, 5 g/L de extracto de levadura, 30 g/L of sacarosa y 5 mL de concentrado Czapeck). El concentrado Czapeck consistió en 300 g/L de $NaNO_3$, 50 g/L de KCl, 50 g/L de $MgSO_4 \cdot 7H_2O$, 1 g/L de $FeSO_4 \cdot 7H_2O$, 1 g/L de $ZnSO_4 \cdot 7H_2O$ y 0.5 g/L de $CuSO_4 \cdot 5H_2O$. Después de 6 días se obtuvieron los pellets fúngicos de *A. uvarum*, que fueron recuperados y conservados siguiendo el mismo procedimiento que en el caso de *P. chrysosporium*.

3.2.3.3. Ensayos de biodegradación

El tipo de tratamiento realizado, así como las diferentes cepas empleadas se detallan en la Tabla 3.9.

Tabla 3.9. *Tratamientos de biorremediación con hongos.*

Residuo	Tratamiento	Cepa
Licor de biometanización	Reactor en continuo y discontinuo	*P. chrysosporium* CECT 2798
Permeado de ultrafiltración	Reactor en discontinuo	*P. chrysosporium* CECT 2798
Alperujo	Reactor en discontinuo	*P. chrysosporium* CECT 2798
Purines	Reactor en discontinuo	*P. chrysosporium* CECT 2798
Licor Negro	Reactor en discontinuo	*P. chrysosporium* MUM 95.01 y *Aspergillus Uvarum* MUM 08.01.

a) Ensayos de biodegradación discontinuos

Los tratamientos discontinuos se llevaron a cabo en matraces Erlenmeyer, manteniendo siempre una relación 1:5 entre el volumen de muestra empleada y la capacidad del matraz. En todos los casos, las muestras se inocularon con 3 g/L (peso seco) de hongo en forma de pellets. Las muestras inoculadas se incubaron a una temperatura de 25ºC o 26ºC, y una agitación orbital de 135 rpm o 150 rpm, en función del tratamiento. Además, se evaluó la efectividad del proceso de biodegradación tras la incorporación de fuentes externas de carbono (glucosa) y nitrógeno (tartrato de amonio), así como la influencia de la variación del pH durante los tratamientos. Las condiciones

operacionales específicas de cada ensayo pueden consultarse en la sección de "*Material and Methods*" incluida en cada apartado del Capítulo 4.

b) Ensayos de biodegradación continuos

Para las pruebas en continuo, se realizó la puesta en marcha de un biorreactor fúngico, evaluando diferentes condiciones operativas. Los experimentos continuos se llevaron a cabo en un biorreactor encamisado de 1,5 L de capacidad cargado con 1,25 L de muestra suplementada con 3 g/L de glucosa e inoculado con 3 g/L (peso seco) de hongo, en concreto de *P. chrysosporium*. Durante el tratamiento, el pH se mantuvo entre 5,6 y 6,5 añadiendo NaOH 0,5 M o HCl 0,5 M utilizando una bureta de pH (CRISON, PH-BURETTE 24 1S). La temperatura se fijó en 26ºC y los pellets fúngicos se mantuvieron fluidizados mediante pulsos de aire generados por una electroválvula. La electroválvula fue controlada por un temporizador cíclico (60 segundos abierta, 30 segundos cerrada) con un flujo de aire de 36 L/h. Se utilizaron piedras difusoras de burbuja fina para asegurar la homogeneidad y evitar el estrés mecánico de la biomasa. A la salida del reactor, se colocó un tamiz de malla de 1 mm para evitar la pérdida de gránulos de hongos. Al final de cada experimento, el reactor se vació y se volvió a llenar con muestra nueva. Se probaron tres tiempos de retención hidráulica (HRT) diferentes (104, 208 y 240 horas).

3.3. MÉTODOS ANALÍTICOS

Una vez realizados los ensayos de caracterización microbiológica y degradación de los efluentes, se deben caracterizar las muestras finales con el fin de determinar la efectividad de los diferentes procesos de tratamiento aplicados. En esta sección se detallan principalmente las técnicas analíticas empleadas para el análisis de los resultados y la caracterización de los efluentes tratados.

3.3.1. Análisis taxonómico

Los resultados obtenidos mediante las técnicas de secuenciación masiva se analizaron utilizando el software Ion Reporter™ de Life Technologies. Este software permite obtener una clasificación taxonómica de los microorganismos presentes en las muestras en rangos de filo, clase, orden, familia, género y especie.

Para asegurar que la clasificación taxonómica es correcta, el software coteja los datos de secuenciación genómica obtenidos con la información almacenada en dos bases de datos de referencia de ARNr, "Premium Curated MicroSEQ ID 16S" y "Curated Greengenes". Ambas bases de datos están curadas, lo que quiere decir que han sido minuciosamente revisadas por diferentes expertos para que la información sea fiable. Además, el software permite aplicar distintos criterios de restricción en el momento de contrastar las secuencias obtenidas con las bases de datos. En este sentido, cuanto más estrictos sean estos criterios, mayor será el nivel de coincidencia entre los datos y la información almacenada en las bases de datos.

Para la obtención de los resultados de esta Tesis Doctoral se aplicaron los siguientes criterios de restricción:

- Longitud de las lecturas: 150 pb.
- Cobertura mínima de alineación: 90%.
- Número mínimo de lecturas: 10.
- Nivel de coincidencia para determinar género: 97%.
- Nivel de coincidencia para determinar especie: 99%.

Adicionalmente, la información correspondiente a cada una de las muestras analizadas fue tratada reflejando en los resultados exclusivamente aquellas poblaciones bacterianas que representaron más de un 10% del microbiota total. Este criterio fue seleccionado con la finalidad de dar una visión más general de los microorganismos presentes, así como para destacar la microbiología más abundante que pudiese interferir en los procesos de operación de la planta.

3.3.2. Análisis de parámetros fisicoquímicos

3.3.2.1. Demanda química de oxígeno (DQO)

La materia orgánica presente en las muestras se determinó en términos de DQO, ampliamente utilizado como medida de contaminantes en aguas residuales y aguas naturales. Se define la DQO como el contenido de materia orgánica que es susceptible de ser oxidado en presencia de un oxidante químico fuerte. La cantidad de materia orgánica oxidada se expresa en términos de su equivalencia en oxígeno (mg O_2/L).

Para su determinación se empleó el método del dicromato 5220 según los métodos estándar para el análisis de agua y aguas residuales [19]. En este proceso, la muestra se somete a una digestión con dicromato potásico, en medio fuertemente ácido, a temperatura de 150ºC durante 2 horas. Además, a la reacción se añade también sulfato de mercurio y sulfato de plata con la finalidad de evitar la interferencia de los cloruros y mejorar la oxidación de los compuestos alifáticos, respectivamente. Una vez finalizado el proceso de digestión, las muestras se midieron espectrofotométricamente a 600 nm utilizando un espectrofotómetro DR2500 (Hach Company). La concentración de DQO se determinó a través de una curva de calibración preparada con patrones de ftalato a distintas concentraciones.

Este método también se empleó para el análisis de la DQO soluble (DQOs) con la única diferencia de que, en este caso, la muestra se centrifugó previamente a 13000 g durante 15 min para eliminar las partículas sólidas, empleando una centrífuga refrigerada de alta velocidad (Kubota 6500).

En algunos casos, la concentración de DQO en las muestras fue determinada empleando el kit comercial Hach Lange LCK 514. Para asegurar que los datos obtenidos por ambos métodos fuesen comparables, se midieron varias veces las mismas muestras empleando ambos métodos. En todos los casos, la diferencia entre los valores obtenidos fue menor del 5%.

3.3.2.2. Demanda biológica de oxígeno (DBO)

La DBO se define como la cantidad de oxígeno que los microorganismos consumen para degradar, únicamente por acción bioquímica aerobia, la materia orgánica degradable presente en un agua residual. En esta Tesis, la cantidad de DBO presente en las muestras se determinó mediante respirometría manométrica (Lovibond® Water Testing BD 600), por el método Wanburg. Mediante esta técnica, la DBO se determina midiendo directamente la variación de presión en un volumen constante que se produce como consecuencia del consumo de oxígeno por parte de la población bacteriana al degradar la muestra. Al igual que la DQO, su concentración se expresa en términos de mg O_2/L.

En concreto, la determinación de este parámetro se llevó a cabo midiendo la DBO_5 de las muestras, de acuerdo al métodos estándar 5210B para el análisis de agua y

aguas residuales [19]. En este caso, se mide el oxígeno consumido en un período de 5 días. El procedimiento se llevó a cabo ajustando previamente las muestras a un pH entorno a 6.5-7. Además, dada la complejidad de las muestras evaluadas y la posible presencia de organismos nitrificantes, se adicionó un inhibidor de la nitrificación para evitar interferencias entre la demanda de nitrógeno y las pruebas de DBO. Si no se usa un químico inhibidor, la demanda de oxígeno medida es la suma de las demandas de nitrógeno y carbonáceas.

3.3.2.3. Carbono orgánico total (TOC)

El análisis del carbono orgánico total (COT) se realizó con un analizador de COT (Shimadzu TOC-VCSH, Japón).

3.3.2.4. Nitrógeno

La presencia de nitrógeno en las muestras se determinó en los Servicios Científico Técnicos de la Universidad de Oviedo, mediante un autoanalizador de flujo segmentado, (SKALAR SAN PLUS), basado en reacciones colorimétricas. Este equipo permite cuantificar la concentración de nitrógeno en forma de nitritos, nitratos, amoniaco, nitrógeno inorgánico total, nitrógeno orgánico disuelto y nitrógeno total.

3.3.2.5. Carbohidratos

a) Carbohidratos totales

Para determinar la cantidad de carbohidratos totales, se empleó el método del fenol-sulfúrico, basado en la metodología descrita por DuBois et al. [20], con algunas modificaciones [21]: 0,5 mL de fenol al 5% (Sigma Aldrich) y 2,5 mL de H_2SO_4 al 96% (Panreac) se añadieron a 1 mL de muestra. La reacción se mantuvo a temperatura ambiente durante 1 h, asegurando el enfriamiento de las muestras. Finalmente, se midió la absorbancia de las muestras a 492 nm. Las mediciones se realizaron con un espectrofotómetro UV (ThermoScientific Heλios γ). La concentración de azúcares reductores se determinó a través de una curva de calibración preparada con patrones de glucosa a distintas concentraciones.

b) Carbohidratos reductores

Los azúcares reductores son aquellos cuyo grupo carbonilo no está involucrado en la unión de monosacáridos para la formación de oligosacáridos o polisacáridos y por tanto conservan el carácter reductor de la molécula, siendo directamente fermentables.

La concentración de azúcares reductores se determinó mediante el método del ácido 3,5-dinitrosalicílico (DNS) desarrollado por Miller [22]. Para este procedimiento, se añadieron 0,5 mL de reactivo DNS a 0,5 mL de muestra. La mezcla se agitó en un vórtex y se incubó en un baño de agua hirviendo durante 5 min. Inmediatamente después, las muestras se introdujeron en hielo para detener la reacción de oxidación por contraste térmico. Finalmente, se adicionaron 5 mL de agua destilada a los tubos de reacción y se midió la absorbancia de las muestras a 540 nm. Los análisis se realizaron con un espectrofotómetro UV (ThermoScientific Heλios γ). De la misma forma que en el caso de los carbohidratos totales, la concentración de azúcares reductores se determinó a través de una curva de calibración empleando glucosa como patrón.

3.3.2.6. Inhibidores de la fermentación

Los diferentes pretratamientos de hidrólisis aplicados a los residuos agroalimentarios pueden provocar la formación de compuestos secundarios que dificulten el proceso fermentativo posterior. Entre estos compuestos se incluyen el hidroximetilfurfural (HMF), el furfural y el ácido acético. La concentración de estos inhibidores se determinó mediante cromatografía líquida de alta resolución (HPLC) utilizando el cromatógrafo Agilent 1200 (Agilent Technologies).

La concentración de ácido acético se determinó empleando una columna ICSep ICE-ION-300 (Transgenomic) acoplada a un detector de índice de refracción (RID). La fase móvil empleada fue ácido sulfúrico (0,450 mM, pH 3,1) a un flujo de 0,3 mL/min con la temperatura de la columna ajustada a 75 ºC [23].

Para determinar el HMF y el furfural, se siguió la metodología descrita por Abu-Bakar et al. [24] y De Andrade et al. [25], con algunas modificaciones. Así pues, para la determinación del furfural se utilizó una columna Gemini-NX 5µm C18 110A (Phenomenex) acoplada a un sistema de detección por matriz de diodos (DAD). Se empleó como fase móvil una mezcla de acetonitrilo y agua (20:80) con un flujo de 1

mL/min, fijando la detección final de la señal UV en 260 nm. En cambio, para el análisis del HMF, la fase móvil fue metanol/agua (10:90) y la detección de UV se fijó a 285 nm. Los límites de detección del equipo para el HMF y el furfural fueron de 1 ppm, mientras que para el ácido acético fue de 5 ppm.

En todos los casos, el análisis de los datos se realizó con el software ChemStation (Agilent Technologies). Todos los compuestos se determinaron empleando como referencia estándares analíticos externos (Sigma-Aldrich).

3.3.2.7. Compuestos fenólicos totales

Los compuestos fenólicos totales se determinaron por el método de Folin-Ciocalteu en condiciones de oscuridad, según Moussi et al. [26], utilizando el ácido gálico como estándar. En este procedimiento, se mezclaron 400 µL de muestra con 3 mL de reactivo Folin-Ciocalteu (previamente diluido 1:10 con agua destilada). Esta mezcla se mantuvo a 22 °C durante 5 minutos. A continuación, se añadieron 3 mL de bicarbonato sódico (NaHCO36 g/100 mL) y la muestra se incubó de nuevo a 22 °C durante 90 min. Tras la incubación, se midió la absorbancia a 725 nm.

3.3.2.8. Actividad enzimática.

Para el análisis de las actividades enzimáticas lacasa (Lac), lignina peroxidasa (LiP) y manganeso peroxidasa (MnP) se siguió la metodología descrita por Lisboa et al. [27].

Para la determinación de la actividad Lac, la mezcla de reacción fue: 0,8 mL de ABTS (0,03 % v/v), 0,1 mL de tampón de acetato de sodio (0,1M, pH 5,0) y 0,1 mL de muestra. La oxidación del ABTS se midió a 420 nm con un coeficiente de extinción molar (ε) de 3,6 × 104 $M^{-1}cm^{-1}$.

La actividad LiP se evaluó mezclando 1 mL de solución tampón de tartrato sódico (125 mM, pH 3,0), 500 µL de alcohol veratrílico (10 mM), 500 µL de peróxido de hidrógeno (2 mM) y 500 µL de muestra. La producción de veratraldehído se midió a 310 nm (ε = 9,3 × 103 M -1cm -1).

Finalmente, la medida de la actividad de la MnP se llevó a cabo mezclando 500 µl de muestra, 100 µl de rojo de fenol (0,01% p/v), 100 µl de lactato de sodio (250

mM), 200 µl de albúmina bovina (0,5% p/v), 50 µl de sulfato de manganeso (2 mM), 50 µl de peróxido de hidrógeno (2 mM) y 1,0 mL de tampón de succinato de sodio (20 mM, pH 4,0). La actividad del MnP se determinó a 610 nm ($\varepsilon = 2,2 \times 103\ M^{-1}cm^{-1}$).

Todas las reacciones se realizaron a 30ºC durante 5 minutos y se detuvieron añadiendo 40 µL de NaOH 2 N. Una unidad de enzima (U) se definió como la liberación de 1 µmol de producto formado por minuto en las condiciones del ensayo.

3.3.2.9. Color

El cambio de color en las muestras se determinó mediante el índice de color (IC), que se define según la ecuación (1) [28].

$$IC=\frac{SAC_{436}^2+SAC_{525}^2+SAC_{620}^2}{SAC_{436}+SAC_{525}+SAC_{620}} \qquad \text{Ecuación (1)}$$

En esta ecuación, se define SAC como el coeficiente de absorbancia espectral, que se obtiene del cociente del valor de absorbancia a una longitud de onda determinada, entre el espesor de la celda o cubeta. Lo habitual es que el espesor de la cubeta sea de 1 cm, de forma que los valores de SAC sean iguales a los valores de absorbancia. Como blanco para la medida de la absorbancia se utilizó agua destilada.

3.3.2.9. Sólidos

La medida de los sólidos totales se determinó en base a los métodos estándar para el análisis de agua y aguas residuales [19]. La concentración de sólidos totales nos indica la cantidad de materias disueltas y en suspensión que tiene el efluente. Para su medida, la muestra se sometió a incubó durante al menos 1 h en una estufa a 105 °C, con la finalidad de evaporar el agua. Después, tras el enfriamiento de la muestra en un desecador, se pesó la muestra. El ciclo de evaporación, enfriamiento, desecación y pesaje se repitió hasta obtener un peso constante de la muestra.

3.3.3. Cálculo de parámetros adicionales

3.3.3.1. Índice de biodegradabilidad (IB)

La biodegradabilidad de las muestras se ha expresado mediante la relación DBO_5/DQO. Este parámetro indica la cantidad de materia orgánica biodegradable en relación con la cantidad de materia orgánica total presente en la muestra.

3.3.3.2. DQO no soluble

Se entiende como DQO no soluble a la diferencia entre la DQO total y la DQO soluble de las muestras. Por tanto, para su calculo se empleó la Ecuación 2.

$$\mathrm{nsCOD} = \mathrm{tCOD} - \mathrm{sCOD} \qquad \mathrm{Eq}\ (2)$$

3.3.3.3. Estado medio de oxidación de los átomos de carbono (AOSC)

El estado medio de oxidación de los átomos de carbono (AOSC) es un parámetro descrito por Vogel et al. [29], que permite evaluar el grado de oxidación de las muestras después de diferentes tratamientos. Los valores de AOSC deben estar siempre entre el rango 4 y - 4. Los valores bajos de AOSC indican que la oxidación no es completa, mientras que los valores altos indican un estado de oxidación alto de los compuestos orgánicos [30]. Para determinar el valor de AOSC se empleó la Ecuación 4:

$$\mathrm{AOSC} = 4 - 1{,}5 * \left(\frac{\mathrm{COD}}{\mathrm{TOC}}\right) \qquad \mathrm{Ecuación}\ (4)$$

3.4. REFERENCIAS

[1] A.I. Díaz, P. Oulego, S. Collado, A. Laca, J.M. González, M. Díaz, Impact of anaerobic digestion and centrifugation/decanting processes in bacterial communities fractions, J. Biosci. Bioeng. 126 (2018) 742–749. https://doi.org/10.1016/J.JBIOSC.2018.05.024.

[2] P. Oulego, S. Collado, A. Laca, M. Díaz, Tertiary treatment of biologically pre-treated landfill leachates by non-catalytic wet oxidation, Chem. Eng. J. 273 (2015) 647–655. https://doi.org/10.1016/j.cej.2015.03.089.

[3] A.I. Díaz, P. Oulego, A. Laca, J.M. González, M. Díaz, Metagenomic Analysis of Bacterial Communities from a Nitrification–Denitrification Treatment of Landfill Leachates, CLEAN – Soil, Air, Water. 47 (2019) 1900156. https://doi.org/10.1002/clen.201900156.

[4] L. Pola, S. Collado, P. Oulego, P. Calvo, M. Díaz, Characterisation of the wet oxidation of black liquor for its integration in Kraft paper mills, Chem. Eng. J. 405 (2021) 126610. https://doi.org/10.1016/j.cej.2020.126610.

[5] V. Borroni, M.T. González, A.A. Carelli, Bioproduction of carotenoid compounds using two-phase olive mill waste as the substrate, Process Biochem. 54 (2017) 128–134. https://doi.org/10.1016/j.procbio.2017.01.003.

[6] I. Sampedro, E. Aranda, G. Rodríguez-Guti Errez, A. Lama-Mu~ Noz, J.A. Ocampo, J. Fern Andez-Bola~, I. García-Romera, Effect of a New Thermal Treatment in Combination with Saprobic Fungal Incubation on the Phytotoxicity Level of Alperujo, J. Agric. Food Chem. 59 (2011) 3239–3245. https://doi.org/10.1021/jf2003305.

[7] I. González-García, B. Riaño, B. Molinuevo-Salces, M.B. Vanotti, M.C. García-González, Improved anaerobic digestion of swine manure by simultaneous ammonia recovery using gas-permeable membranes, Water Res. 190 (2021). https://doi.org/10.1016/j.watres.2020.116789.

[8] B. Riaño, B. Molinuevo-Salces, M.B. Vanotti, M.C. García-González, Application of gas-permeable membranes for-semi-continuous ammonia

recovery from swine manure, Environ. - MDPI. 6 (2019). https://doi.org/10.3390/environments6030032.

[9] J. Monsalve G., V. Medina de Perez, A. Ruiz Colorado, Ethanol Producction of Banana Shell and Cassava Starch, Dyna. 73 (2006) 21–27.

[10] I. del Campo, I. Alegría, M. Zazpe, M. Echeverría, I. Echeverría, Diluted acid hydrolysis pretreatment of agri-food wastes for bioethanol production, Ind. Crops Prod. 24 (2006) 214–221. https://doi.org/10.1016/J.INDCROP.2006.06.014.

[11] J.F. Correa, Á.A. Ruiz, G. Garrote, Estudio del tratamiento hidrotérmico de fruta de banano, Afinidad. 69 (2012) 191–196.

[12] M.J. Taherzadeh, K. Karimi, Pretreatment of Lignocellulosic Wastes to Improve Ethanol and Biogas Production: A Review, Int. J. Mol. Sci. 2008, Vol. 9, Pages 1621-1651. 9 (2008) 1621–1651. https://doi.org/10.3390/IJMS9091621.

[13] P. Kumar, D.M. Barrett, M.J. Delwiche, P. Stroeve, Methods for Pretreatment of Lignocellulosic Biomass for Efficient Hydrolysis and Biofuel Production, Ind. Eng. Chem. Res. 48 (2009) 3713–3729. https://doi.org/10.1021/IE801542G.

[14] R. Sun, X. Song, R. Sun, J. Jiang, Effect of lignin content on enzymatic hydrolysis of furfural residues, BioResources. 6 (2011) 317–328. https://doi.org/10.15376/biores.6.1.317-328.

[15] J.S. Van Dyk, R. Gama, D. Morrison, S. Swart, B.I. Pletschke, Food processing waste: Problems, current management and prospects for utilisation of the lignocellulose component through enzyme synergistic degradation, Renew. Sustain. Energy Rev. 26 (2013) 521–531. https://doi.org/10.1016/J.RSER.2013.06.016.

[16] A. Dura, W. Błaszczak, C.M. Rosell, Functionality of porous starch obtained by amylase or amyloglucosidase treatments, Carbohydr. Polym. 101 (2014) 837–845. https://doi.org/10.1016/J.CARBPOL.2013.10.013.

[17] E. Uçkun Kiran, Y. Liu, Bioethanol production from mixed food waste by an effective enzymatic pretreatment, Fuel. 159 (2015) 463–469.

https://doi.org/10.1016/J.FUEL.2015.06.101.

[18] P. Blánquez, N. Casas, X. Font, X. Gabarrell, M. Sarrà, G. Caminal, T. Vicent, Mechanism of textile metal dye biotransformation by Trametes versicolor, Water Res. 38 (2004) 2166–2172. https://doi.org/10.1016/j.watres.2004.01.019.

[19] B. R.B., E. A.D., R. E.W., Standard Methods for Examination of Water and Wastewater (23th ed.), Am. Water Work. Assoc. (AWWA, WEF APHA). (2017).

[20] M. DuBois, K.A. Gilles, J.K. Hamilton, P.A. Rebers, F. Smith, Colorimetric Method for Determination of Sugars and Related Substances, Anal. Chem. 28 (1956) 350–356. https://doi.org/10.1021/ac60111a017.

[21] M.B. Hall, Efficacy of reducing sugar and phenol–sulfuric acid assays for analysis of soluble carbohydrates in feedstuffs, Anim. Feed Sci. Technol. 185 (2013) 94–100. https://doi.org/10.1016/J.ANIFEEDSCI.2013.06.008.

[22] G.L. Miller, Use of Dinitrosalicylic Acid Reagent for Determination of Reducing Sugar, Anal. Chem. 31 (1959) 426–428. https://doi.org/10.1021/ac60147a030.

[23] S. Alonso, M. Herrero, M. Rendueles, M. Díaz, Physiological heterogeneity in Lactobacillus casei fermentations on residual yoghurt whey, Process Biochem. 49 (2014) 732–739. https://doi.org/10.1016/J.PROCBIO.2014.01.033.

[24] N.B. Abu-Bakar, A. Makahleh, B. Saad, Vortex-assisted liquid–liquid microextraction coupled with high performance liquid chromatography for the determination of furfurals and patulin in fruit juices, Talanta. 120 (2014) 47–54. https://doi.org/10.1016/J.TALANTA.2013.11.081.

[25] J.K. De Andrade, E. Komatsu, H. Perreault, Y.R. Torres, M.R. Da Rosa, M.L. Felsner, In house validation from direct determination of 5-hydroxymethyl-2-furfural (HMF) in Brazilian corn and cane syrups samples by HPLC–UV, Food Chem. 190 (2016) 481–486. https://doi.org/10.1016/J.FOODCHEM.2015.05.131.

[26] K. Moussi, B. Nayak, L.B. Perkins, F. Dahmoune, K. Madani, M. Chibane, HPLC-DAD profile of phenolic compounds and antioxidant activity of leaves

extract of Rhamnus alaternus L., Ind. Crops Prod. 74 (2015) 858–866. https://doi.org/10.1016/j.indcrop.2015.06.015.

[27] D.S. Lisboa, C. Santos, R.N. Barbosa, O. Magalhães, L.M. Paiva, K.A. Moreira, N. Lima, C.M. Souza-Motta, Requalification of a Brazilian Trichoderma collection and screening of its capability to decolourise real textile effluent, Int. J. Environ. Res. Public Health. 14 (2017) 1–16. https://doi.org/10.3390/ijerph14040373.

[28] J.L. Urrea, S. Collado, P. Oulego, M. Díaz, Wet oxidation of the structural sludge fractions, J. Clean. Prod. 168 (2017) 1163–1170. https://doi.org/10.1016/j.jclepro.2017.09.033.

[29] F. Vogel, J. Harf, A. Hug, P.R. von Rohr, F.Â. De, R. Vogel, J. Harf, A. Hug, P. Rudolf, V. Rohr, The mean oxidation number of carbon (MOC) - A useful concept for describing oxidation processes, Pergamon, 2000. https://doi.org/10.1016/S0043-1354(00)00029-4.

[30] A. Garg, A. Mishra, Wet oxidation-an option for enhancing biodegradability of leachate derived from municipal solid waste (msw) landfill, Ind. Eng. Chem. Res. 49 (2010) 5575–5582. https://doi.org/10.1021/ie100003q.

Capítulo 4.

RESULTADOS Y DISCUSIÓN

4.1. TRATAMIENTOS POST-ANAEROBIOSIS

En este apartado se recogen todas las investigaciones relacionadas con la caracterización y el tratamiento del licor de biometanización. Como se describió anteriormente en el Capítulo 3, este efluente se corresponde con la fracción líquida resultante de la centrifugación del digestato procedente de la digestión anaerobia de lodos. Por tanto, es un efluente altamente recalcitrante que contiene, además, una concentración de amonio que supera los parámetros de diseño de la depuradora localizada en COGERSA, incrementando de manera importante en el consumo de metanol durante el proceso de desnitrificación.

A lo largo de esta sección, se ha investigado la composición fisicoquímica y biológica del licor de biometanización. Para ello, en el inicialmente en el apartado **4.1.1** se ha llevado a cabo la caracterización microbiológica de varios puntos del tratamiento de digestión anaerobia. Estos resultados permiten conocer de forma detallada el proceso de biometanización y cómo los parámetros operacionales influyen en la distribución de los microorganismos. En el apartado **4.1.2**, se han ensayado diferentes técnicas fisicoquímicas de tratamiento, incluyendo ultrasonidos, ozonización, hidrólisis y oxidación húmeda, con el fin de reducir la materia orgánica recalcitrante y acondicionar las características del licor a las condiciones de la depuradora, reduciendo los costes operacionales ocasionados por su incorporación. Finalmente, en el apartado **4.1.3**, se ha evaluado el empleo de hongos de pudrición blanca, específicamente el hongo *P. chrysosporium*, para el tratamiento biológico, tanto discontinuo como continuo, del licor de biometanización. Los resultados mostraron una alternativa prometedora de tratamiento y permitieron conocer el efecto sinérgico de la aplicación de los hongos frente al efecto degradativo del microbiota presente en el licor de biometanización.

Los resultados obtenidos en esta etapa de investigación se recogen en tres publicaciones, cuya cita se detalla al inicio de cada apartado.

4.1.1. Impact of anaerobic digestion and centrifugation/decanting processes in bacterial communities fractions

Ana Isabel Díaz[1], Paula Oulego[1], Sergio Collado[1], Adriana Laca[1], J. Manuel González[2] and Mario Díaz[1*]

[1]Department of Chemical and Environmental Engineering. University of Oviedo. C/ Julián Clavería, s/n, E-33006, Oviedo, Asturias, Spain.

[*]E-mail: mariodiaz@uniovi.es Phone: +34 985 10 34 39; Fax: +34 985 10 34 34

[2] R&D, COGERSA SAU. C/ La Zoreda, s/n, E-33697, Gijón, Asturias, Spain.

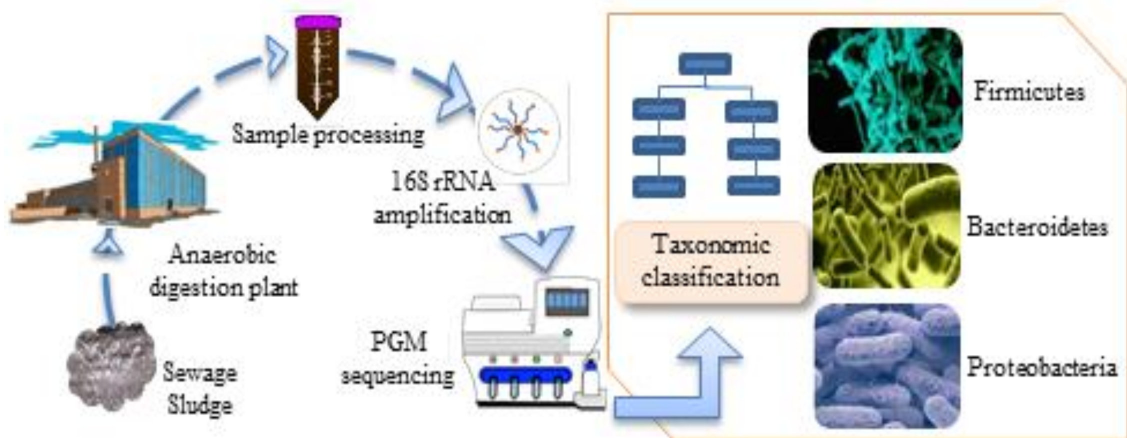


Figura 4. 1. *Resumen gráfico del trabajo 1*

*Artículo publicado en la revista "**Journal of Bioscience and Bioengineering**"

Cita: Ana Isabel Díaz, Paula Oulego, Sergio Collado, Adriana Laca, José Manuel González, Mario Díaz, *Impact of anaerobic digestion and centrifugation/decanting processes in bacterial communities fractions*, Journal of Bioscience and Bioengineering, Volume 126, Issue 6, **2018**, Pages 742-749, ISSN 1389-1723, https://doi.org/10.1016/j.jbiosc.2018.05.024.

ABSTRACT

Sewage sludge can be treated by anaerobic processes that frequently are followed by physical separation processes. In this work, a high-throughput sequencing technology, based on variation in the bacterial 16S rRNA gene, has been used to characterise the bacterial populations present in samples taken from different points of an industrial anaerobic digestion process fed with sewage sludge. Relative abundances of phyla and classes throughout the biological process and the subsequent separation steps were determined.

Results revealed that the *Bacteroidetes*, *Firmicutes* and *Proteobacteria* phyla were the most representative. However, significant changes in relative abundance were detected along treatments, showing the influence of operational parameters on the distribution of microorganisms throughout the process. After anaerobic digestion, phylum *Firmicutes* doubled its relative abundance, which seems to indicate that the anaerobic conditions and the nutrients favoured its growth, in contrast to other phyla that almost disappeared. After centrifugation, *Proteobacteria* went preferentially to the solid phase, in contrast to *Firmicutes* which was the dominant phylum in the liquid phase. After decanting the liquid phase during 14 h, an important growth of *Proteobacteria*, *Spirochaetes* and *Tenericutes* was detected. At class level, only significantly changes were observed for *Proteobacteria* classes being *α-proteobacteria* dominant in the digestate, while *γ-proteobacteria* was the majority since this point to the final steps. To know the changes on the kind and abundance of microbial populations throughout the anaerobic and separation processes is very important to understand how the facilities design and operation conditions can influence over the efficiencies of next biological treatments.

Keywords: 16S rRNA gene; anaerobic digestion; PGM sequencing; bacterial community; separation processes; sewage sludge.

1. INTRODUCTION

The anaerobic processes take place in four stages: hydrolysis, acidogenesis, acetogenesis and methanogenesis. The microorganisms of two biological domains, Bacteria and Archaea, carry out interdependent and complex biological reactions during the process. Bacteria microorganisms produce enzymes which hydrolyse polymers to monomers. These are subsequently converted by acidogenic and acetogenic bacteria to H_2 and volatile fatty acids (1). The Archaea domain is mainly involved in the last stage, forming methane by the reduction of CO_2 or by the decarboxylation of acetate. Microorganism within this domain can also utilize a limited number of other substrates, such as methanol, methylamines and formate, to produce methane (2).

Anaerobic digestion is the most widely employed method for sewage sludge treatment, and since the last years, it is an attractive technology for processing various organic wastes produced in urban, industrial, and agricultural settings (3). In this process, a large fraction of the organic matter, which is degraded by a complex community of microorganisms, is broken down into carbon dioxide (CO_2) and methane (CH_4). Such decomposition occurs in the absence of oxygen and two main final products can be distinguished: digestate and biogas, which is considered a product of high added value to be used as biofuel in a sustainable and environmentally friendly way (4). After the anaerobic digestion, the digestate can be treated to remove liquid fraction and simultaneously concentrate nutrients, so a solid–liquid phase separation is usually carried out prior to any further post-treatment. The solid fraction mixed with vegetable wastes, is mainly used in composting processes to be employed as a fertilizer (5).

Regarding the liquid fraction, depending on substrates used and final disposal, a biological post-treatment could be necessary to reduce its toxicity and nitrogen content. Microorganisms play a main role in anaerobic digestion treatments, so it is essential to characterize the microbiota at each stage of the process in order to detecting the core functional groups responsible of anaerobic degradation and key for the further post-treatment. The substrate characteristics and operational parameters are determining factors for the stability of the microbial communities.

Previous studies describing the microbial communities in anaerobic processes were based primarily on classical methods, such as terminal restriction fragment length polymorphism (T-RFLP), denaturing gradient gel electrophoresis (DGGE), single-strand conformation polymorphism (SSCP), or Sanger sequencing of clone libraries (3, 6).

Just fear years ago, the next generation of sequencing (NGS) based on 16S rRNA gene sequencing has been applied to characterize anaerobic digestion processes. This technology allows the generation of thousands to millions of short sequencing reads for massive studies of genes and a higher taxonomic resolution (7). Most of such works investigated only a few hypervariable regions, mainly V3, V4 and V5, so the phylogenetic and taxonomic information obtained was limited. In this study, 7 of the 9 hypervariable regions of the 16S rRNA gene have been sequenced to obtain extensive phylogenetic information by using the Ion Torrent PGM system of mass sequencing.

The microbiota present in anaerobic processes has been studied by several authors as Chen et al (8) which studied the microbiota present in a anaerobic digestion of pharmaceutical wastewater and Zhou et al (9) which showed the variation of the microbial community in thermophilic anaerobic digestion of pig manure mixed with different ratios of rice straw.

However, as far as we know, the evolution of the microbial population throughout the anaerobic digestion of sludge and the subsequent separation processes, i.e. centrifugation and decanting, have not been previously studied. Obviously, changes in microbiota distribution, especially in their relative abundance and activity, are ultimately reflected in the reactor performance and the efficiency of following biological treatments, where the bacteria also play an important role.

In particular, the aims of this work were:

i) To characterize the bacterial population in the anaerobic digestion process and compare the results with the microbiota present in raw material.

ii) To study the distribution of the bacterial communities between the separated fractions to determine the effect of the separation processes as centrifugation and decanting.

2. MATERIALS AND METHODS

2.1. Plant operation parameters and sample description

The samples used in this study were taken from the anaerobic digestion plant sited in Serín (Asturias, Spain). Anaerobic digestion process was conducted with sewage sludge, which was used as raw material. Previously to the digestion, the sewage sludge was received in two concrete pits, each with an operational capacity of 175 m^3. These pits are fitted with moving floors from which the sludge was pumped to the anaerobic digestion system. The raw material consists of a mixture of sewage sludge and recirculated process water, in a ratio of 1: 1.2 (v/v) respectively. This raw leachate was treated by means of a wet treatment for 60 minutes. This treatment was carried out in order to disintegrate the biodegradable waste for improving the subsequent digestion process and removing the non-biodegradable contaminants, such as the heavy fraction (stones, large bones, glass, batteries and metal objects) and the light one (textiles, wood, plastic sheets and thread, etc.). The mixture was sent to the Grit Removal System (GRS) consisting of three hydro-cyclones connected in cascade for removing any sand or impurities that might remain in the suspension. All impurities removed were sent to the reject container. Finally, it was stored in a 1000 m^3 capacity tank to enable continuous feeding of the digester.

Anaerobic digestion process was carried out in an anaerobic completely stirred tank digester with capacity of 3000 m^3, under mesophilic conditions (36 ± 0.6ºC). The digester was heated up by means of external heat exchangers. Proper mixing system inside the digester was achieved by injecting part of the produced biogas through an air-cooled rotary vane compressor.

After anaerobic digestion process, biogas and digestate was obtained. The biogas was extracted and leaded to the general biogas network of the landfill. The digestate was separated in solid and liquid phase using an industrial centrifuge at 3000 rpm. The

percentage of solid and liquid phase obtained was around 20% and 80% of total, respectively. For this process, it was necessary the addition of a polyelectrolyte (Chemipol CZ-600) in a concentration of 0.35 % (v/v). The solid phase was sent to a composting plant and the liquid phase was decanted for 14 hours in an open tank volume of 80 m^3, before being sent to the biological subsequent treatment. A flow diagram of the treatment plant is shown in Figure 4.2.

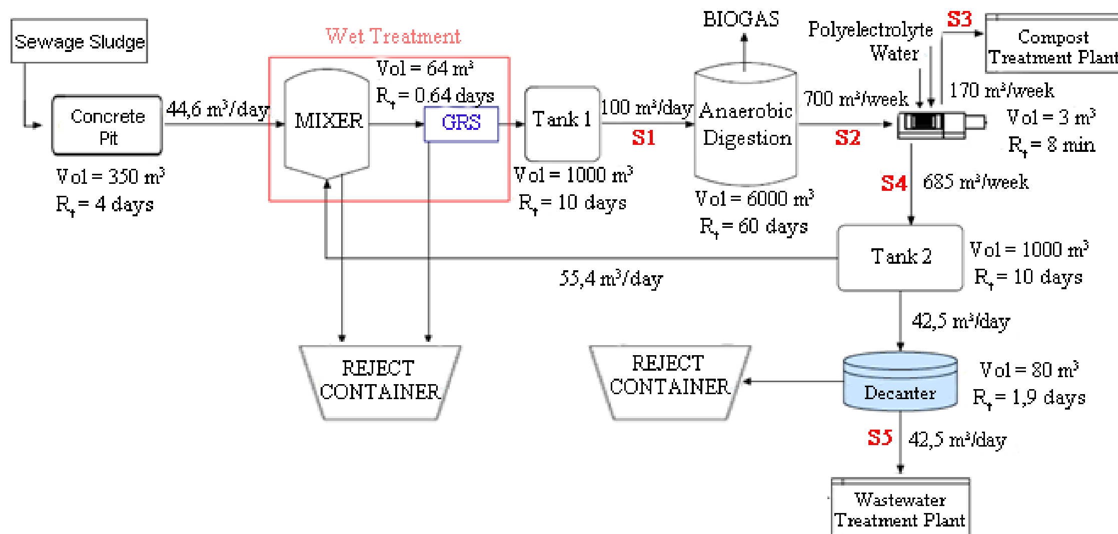


Figura 4. 2. *Flow diagram of anaerobic digestion plant.*

2.2. Sample processing and DNA extraction

Different samples were collected in the anaerobic digestion plant in order to be analysed microbiologically. Such samples were the following: the feed to the anaerobic digester (S1), the digestate (S2), the solid fraction (S3) and the liquid fraction (S4) obtained after centrifugation and the liquor obtained after decanting (S5). Detailed information for each sample is shown in Table 4.1.

In all cases, 160 ml of each of the samples were centrifuged for 20 minutes at 13000g. The supernatant was discarded, and the solid fraction was preserved for DNA extraction.

DNA extraction was performed with the Power Biofilm DNA Isolation Kit (MoBio Laboratories, Inc., Carlsbad, CA), which is specific for leachate samples. The

extraction was carried out with 0.25 g of wet sludge (solid fraction) according to the manufacturer's instructions. The only modification was the addition of 200 µl of solution BF3 due to the excessive colour of the samples (recommended in the protocol of the kit).

Extracted DNA was concentrated using Concentrator Plus Vacufuge® (Eppendorf, Germany) and the concentrations were determined through the BioPhotometer Plus (Eppendorf, Germany), ensuring that the amount of DNA was high enough to continue the process.

2.3. DNA amplification and purification

For DNA amplification, Ion 16S Metagenomics Kit (Ion Torrent, Life Technologies) was employed. This kit simultaneously examines 7 of the 9 hypervariable regions in the bacterial 16S rRNA gene, using one primer for the V2-4-8 regions and another primer for V3-6 and V7-9. This method has a wide range of uses, including the characterization of bacterial populations, taxonomical analysis, and species identification.

The DNA samples and the primers with their respective linkers were amplified by PCR reaction, which was performed in several steps: i) initial heating at 95 °C for 10 minutes, ii) 25 cycles of denaturation at 95 °C for 30 seconds, iii) alignment at 58 °C for 30 seconds, iv) extension at 72 °C for 30 minutes seconds, and v) elongation at 72 ° C for 7 minutes. Next, a preservation step at 4 °C for 20 minutes was carried out. The resulting products were purified using the Agencourt AMPure XP Kit (Beckman Coulter, USA) and finally the 16S rRNA amplicons were quantified through Qubit® 2.0 Fluorometer using dsDNA HS Assay Kit (Invitrogen, USA).

Tabla 4. 1. *Characteristics of the samples analysed. The average values correspond to four samples taken along 2016. The ± value correspond to the standard deviation.*

Parameters						
Samples	**pH (ud.)**	**COD (mg/L)**	**NH_4 (mg/L)**	**BOD_5 (mg/L)**	**TS (mg/L)**	**Moisture (%)**
S1	6.99 ± 0.26	N/A	N/A	N/A	81193 ± 7360	92 ± 0.69
S2	8.13 ± 0.07	56375 ± 1431	5501 ± 421	3750 ± 395	63670 ± 1422	94 ± 0.13
S3	8.21 ± 0.38	67475 ± 13530	17274 ± 1125	N/A	223125 ± 11442	79 ± 1.26
S4	8.39 ± 0.17	10405 ± 4269	4385 ± 428	1525 ± 540	10347 ± 2724	99 ± 0.33
S5	8.42 ± 0.09	10600 ± 4988	3989 ± 177	2735 ± 1572	10198 ± 3544	99 ± 0.30

2.4. Library construction and sequence analysis

Once the DNA was purified, the sequencing step was performed. First, the library was prepared using the Ion Plus Fragment Library Kit (AB Library Builder™). During this process, the fragmentation of the DNA obtained in the purification phase was carried out in order to obtain smaller fragments of up to 150 base pairs (bp). For the library construction, each fragment of the DNA obtained was coupled to a marker or barcode and two adapters. Each library corresponds to a different collection of DNA fragments to be sequenced and is unique to each sample. Construction of the library was conducted using the PGM™ Hi-Q™ OT2 Kit. Subsequently, the samples were sequenced using the PGM™ Hi-Q™ Sequencing Ion Kit and the Ion 318™ Chip Kit v2, which has a minimum capacity of 4 million readings.

The final results obtained were analysed with Life Technologies Ion Reporter™ Software, which provided the final sequencing results. The restriction criteria applied, was as follows: i) read length filter: 150 bp, ii) minimum alignment coverage: 90%, iii) read abundance filter: 10, iv) genus cut off (level of coincidence to determine gender): 97%, and v) species cut off (level of coincidence to determine species): 99%.

3. RESULTS AND DISCUSSION

PGM sequencing and the amplification of hypervariable regions of 16S rRNA allowed us to obtain a detailed taxonomic bacterial classification. Twenty-one different phyla were identified in the samples. The classification of the microorganisms up to specie level is shown in the Supplementary Material, which includes from Fig.5.1.S1 to Fig.5.1.S5. Taxonomic classification is represented by Krona plot, which depicts different bacteria taxonomic levels in concentric circles, from subspecies in the outermost circle to the bacteria kingdom in the innermost circle. The relative abundance of bacterial phyla was estimated in each sample as the percentage of mapped reads.

The phyla *Firmicutes*, *Bacteroidetes* and *Proteobacteria* were the more representative in all the samples. Nelson et al (10) and Sundberg et al (11) found in samples taken from anaerobic processes the cited phyla and also *Chloroflexi* and *Actinobacteria*.

In this work, *Actinobacteria*, and *Chloroflexi* were detected, but with relative abundances below 15% and 1%, respectively, in contrast to data reported in other anaerobic digestion processes (12, 13). Finally, other phyla as *Synergistetes* and *Thermodesulfobacteria* were also detected in all analysed samples, although in low concentrations.

3.1. Anaerobic digestion process

The leachate fed to the digester (S1) and the digestate (S2) were analysed. Fig. 4.3.A, presents the relative abundance of the different phyla in the initial leachate (S1) fed to the anaerobic digester. In sample S1, that contains the pretreated sludge mixed with the recirculation, *Firmicutes* was the majority phylum representing 41% of the total abundance. Its presence has been reported by several authors as one of the most abundant phyla in anaerobic digestion processes (14, 15, 16). During the hydrolysis, the complex molecules are broken down into simpler ones. This phylum is related to the production of extracellular enzymes with cellulases, lipases or proteases (17). Therefore, a high relative abundance of this phylum in S1 is an advantage for the subsequent anaerobic digestion process. The recirculation of the clarified during the leachate treatment is an important aspect that can influence significantly in the relative abundance of each phylum in the anaerobic reactor. In this case, *Firmicutes* is present in the recirculation (S4, see Fig.4.4.B), contributing to its high relative abundance in S1. Zamanzadeh et al (12) studied the microbial communities present in mesophilic and thermophilic anaerobic digesters, with and without recirculation. The study showed that phylum *Firmicutes* was dominant in processes with recirculation and the phylum *Chloroflexi* was dominant in processes without recirculation. This last phylum was identified in this work with a relative abundance lower than 1% in all samples.

The phyla *Bacteroidetes, Proteobacteria* and *Spirochaetes*, which are associated with fermentative metabolism of macromolecular organic compounds, represent 19%, 15% and 15% of the total abundance in S1, respectively (18). The phylum *Bacteroidetes* is composed of gram-negative bacteria which are specialist for the degradation of high molecular weight organic matter to acetic and propionic acid (19). This phylum has been frequently detected in anaerobic reactors loaded with sludge, vegetal biomass or mixed organic residues (20, 21).

Proteobacteria and *Spirochaetes* phyla contain anaerobic and aerobic bacteria, and they are associated with hydrolysis, acidogenesis and acetogenesis reactions in anaerobic digestion processes (22). For this reason, the presence of *Bacteroidetes* and *Proteobacteria* phyla in S1 sample is crucial to favour the acidogenesis, the second stage of anaerobic digestion. Other phyla as *Tenericutes, Actinobacteria* and *Synergistetes* do not exceed 5% of the total abundance which is in accordance to results obtained in other studies for similar samples (15, 23).

Fig.4.3.B, presents the relative abundance of the different phyla in the digestate after anaerobic digestion (S2). Results showed that an important variation occurs in the microbiota during the process. The phylum *Firmicutes* increases severely reaching 83% of total abundance. On the opposite, *Bacteroidetes* and *Proteobacteria* decreased drastically below 10%. In the case of *Spirochaetes*, almost the disappearance of the phylum occurred during the anaerobic process.

The majority of microorganisms who belong to *Firmicutes* phylum are mesophilic, so its growth was favoured at 36ºC. Its predominance in the S2 sample showed a correct functioning of the digester. The presence of *Bacteroidetes* phylum is also closely related with operating temperature, decreasing their relative abundance with the increase in operating temperature (24). *Proteobacteria* and *Spirochaetes* were reduced in the digestate because of the absence of oxygen during the anaerobic process (1).

With respect to the classes, five different classes were detected within the phylum *Proteobacteria* in S1 and S2: *δ-proteobacteria*, *ε-proteobacteria*, *α-proteobacteria*, *β-proteobacteria*, and *γ-proteobacteria* (see Fig.4.3)

After anaerobic digestion, in the digestate (S2), the *α-proteobacteria* class significantly increased their relative abundance reaching values higher than 50%, while *β-proteobacteria* and *γ-proteobacteria* classes decreased.

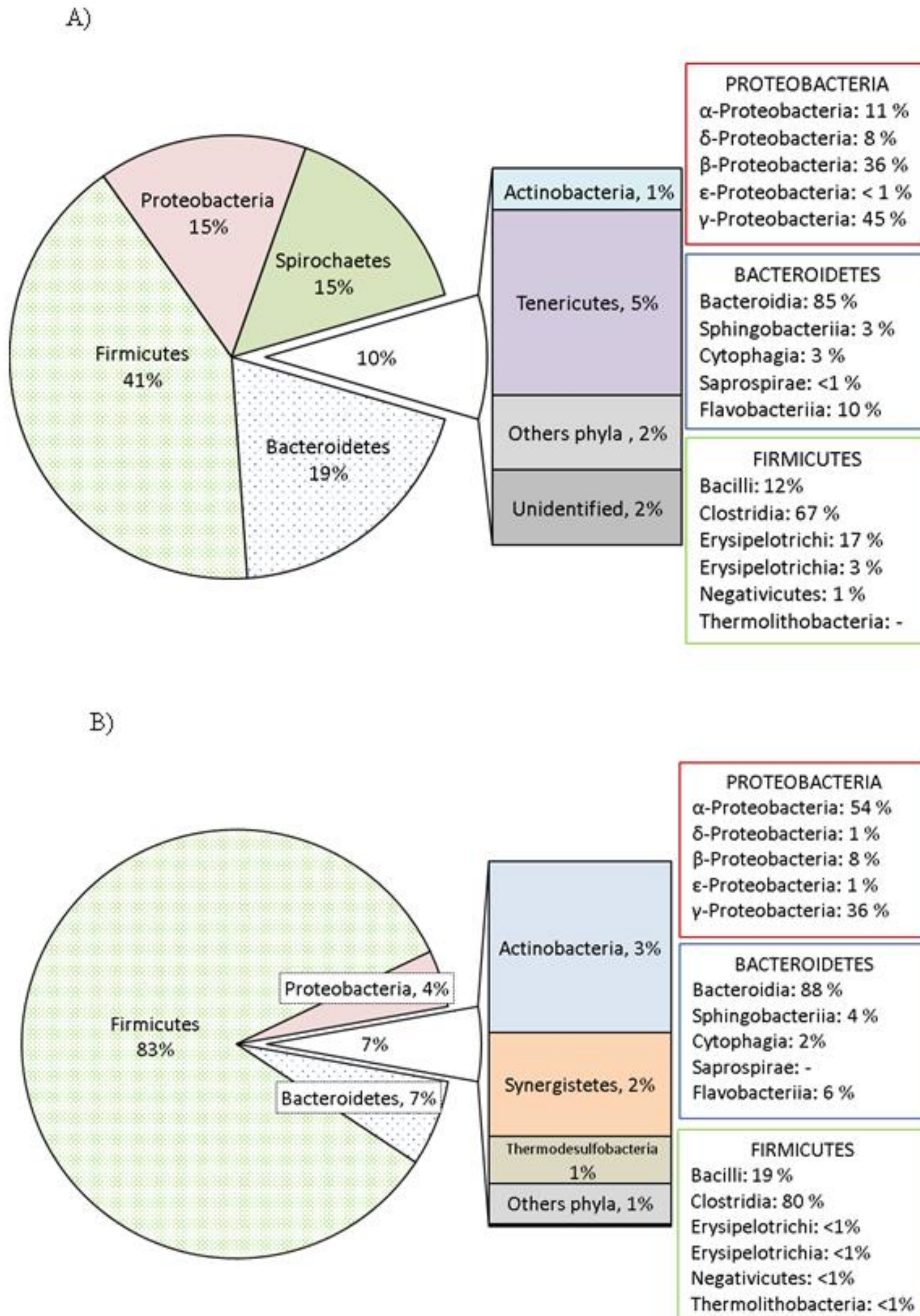


Figura 4. 3. *Relative abundance for the phyla detected in the initial sludge (A) and digestate (B) samples (S1 and S2, respectively). In boxes are shown the relative abundance obtained for the classes within Firmicutes, Bacteroidetes and Proteobacteria phyla.*

The *α-proteobacteria class* include mainly fermentative anaerobic bacteria, while *β-proteobacteria* and *γ-proteobacteria* classes contain aerobic bacteria, so the anaerobic conditions limited the viability of these microorganisms. Previous studies described *α-proteobacteria* as predominant class in mesophilic digesters while *γ-proteobacteria* was dominant in thermophilic digesters (11). Pervin et al (25) detected *β-proteobacteria* as dominant class in a thermophilic reactor, especially genus *Comamonas* that can metabolise a wide variety of organic acids.

For *Firmicutes* and *Bacteroidetes* phyla, no significant changes were detected in the distribution of the classes after anaerobic treatment (Fig.4.3.B).

Within *Firmicutes* phyla in S1 sample, *Clostridia* was the most abundant class, represented mainly for the *Clostridial* order. Bacteria of the genus *Clostridium* are strict anaerobes, typical in biogas plants, which have the capacity of producing hydrogen (26). Many species of *Clostridia* class had been defined as typical anaerobic cellulolytic bacteria. Areas of strong degradation in cellulose structures with these bacteria occupying these depressions had been reported (27). Michalke et al (28) have reported that microorganisms belonging to *Clostridia* class and sulphate-reducing bacteria are producers of trimethylarsines and small amounts of arsine in sewage sludge digestion. Guo et al (29) have described that this class performed the acidogenic process at the second stage and produced VFA, CO_2 and H_2.

Jaenicke et al (30) carried out metagenomic studies during the anaerobic digestion of a mixture of maize silage, green rye, and chicken manure. Results showed that *Clostridia* populations were in syntrophic association with hydrogenotrophic methanogens. For this reason, the presence of this class supposes an advantage to maintain efficient biogas production.

For the digestate (S2), the *Bacilli* and *Clostridia* classes represented 19% and 80% respectively; increasing their relative abundance compared with other classes as *Erysipelotrichi*, *Erysipelotrichia* and *Negativicutes* which have hydrolytic properties. The class *Bacilli*, was mainly represented by the order *Lactobacillales*, where the family of the *Carnobacteriaceae*, which contains bacterial species for decomposing fat and carbohydrate, supposed more than 90% (31).

For *Bacteroidetes* phylum, the class *Bacteroidia* was the most representative in both samples (S1 and S2), previously reported as the most abundant in mesophilic reactors. This class play an important role in hydrolyzing and fermenting organic materials and producing organic acids, CO_2 and H_2 during the anaerobic digestion process (29). The relative abundance found for other classes as *Flavobacteria* and *Sphingobacteria* were lower in comparison with other studies that reported these classes as dominant (32).

3.2. Separation process I: Centrifugation

The data obtained for the solid phase (S3) and the clarified (S4) resulting after the centrifugation process are shown in Fig.4.4.

With respect to the centrifuge feed (S2), in the solid phase (S3), the abundance of the phylum *Firmicutes* decreased to a value of 57%. On the opposite, the phylum *Proteobacteria* increased its abundance reaching a value of 31%. Therefore, it can be concluded that bacteria belonging to the phylum *Proteobacteria* have a greater tendency to be removed by centrifugation.

Yi et al (33) studied the effect of total solids concentration on microbial communities involved anaerobic digestion processes of food waste and, in agreement with our results, reported an increase in *Proteobacteria* abundance for higher solid concentrations.

After the centrifugation process, sample S3 is mixed with vegetable wastes and sent to a composting process. *Proteobacteria* and *Firmicutes*, between others, are able to synthesize enzymes that catalyse the hydrolysis of plant polymers (34). For this reason, the high abundance of these phyla in S3 is an advantage for the subsequent composting process.

In the clarified (S4) important variations were not observed compared to the digestate (S2). It should be noted that the majority of *Firmicutes* microorganisms from the digestate were preserved in the liquid fraction after the centrifugation process.

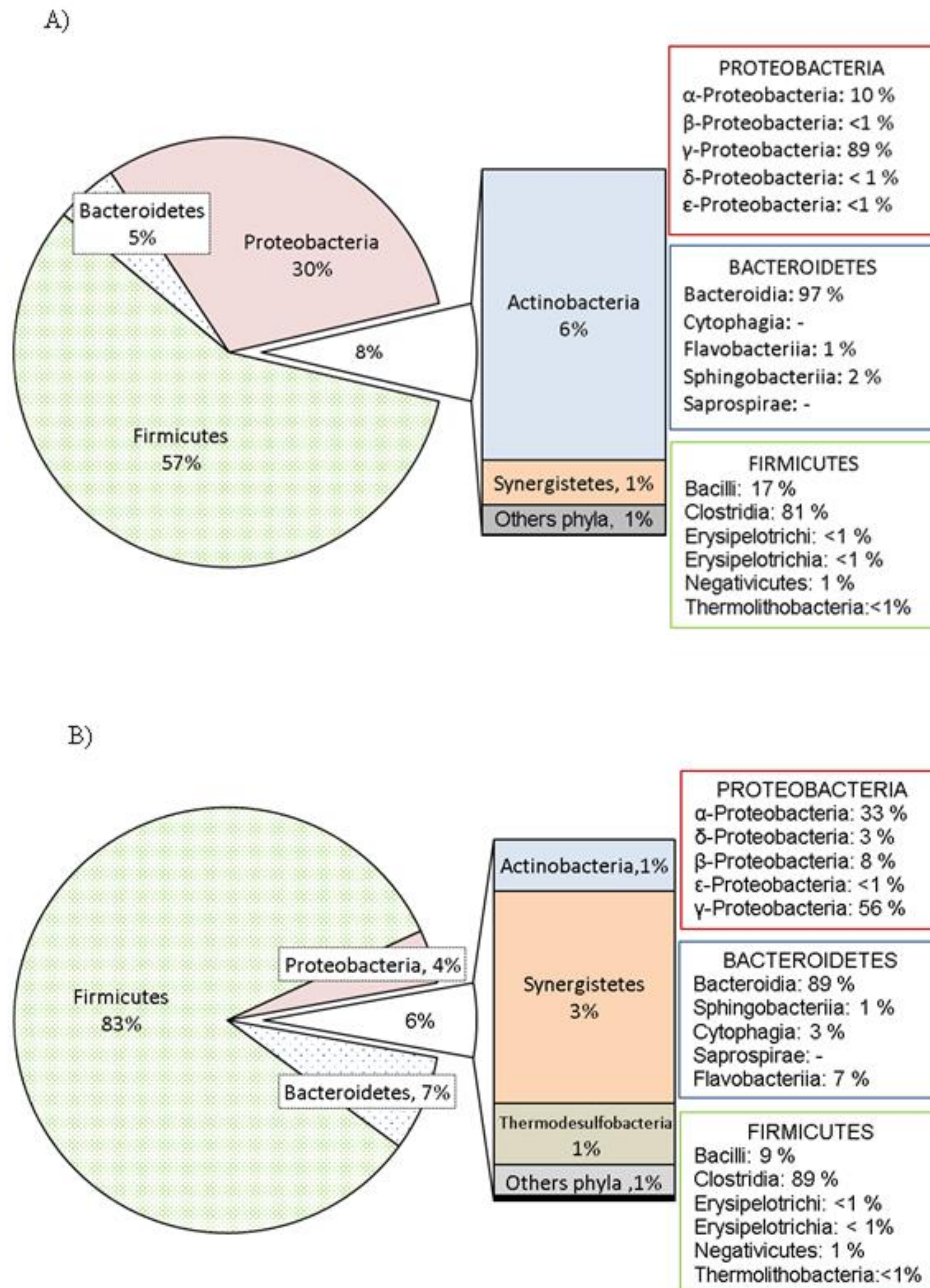


Figura 4. 4. *Relative abundance for the phyla detected in the solid phase (A) and clarified (B) samples (S3 and S4, respectively). In boxes are shown the relative abundance obtained for the classes within Firmicutes, Bacteroidetes and Proteobacteria phyla*

Gao et al (35) studied the microbial populations in a bioreactor fed with sludge mixed with sewage from cat food and they reported high relative abundance of the phylum *Firmicutes* in the bioreactor suspension, as occurs here in the clarified (S4). This fact, together with the recirculation of sample S4 during the sewage sludge treatment, contributes to a correct operation of the anaerobic reactor.

With respect to the classes, in the solid phase (S3), *γ-proteobacteria* represented the 90% of relative abundance, within *Proteobacteria* phylum, being the predominant families *Pseudomonadaceae* and *Xanthomonadaceae*. This class showed a greater ability to be removed by centrifugation than the *β-proteobacteria* and the *α-proteobacteria* classes whose abundances were higher in the clarified (S4). This fact may be due to a lower interaction of the *β-proteobacteria* and the *α-proteobacteria* with the polyelectrolyte added for the centrifugation process and a lower tendency to the form flocs.

De Gannes et al (36) studied microbiota diversity in composts employing 454-pyrosequencing, and they observed that *γ-proteobacteria* was especially abundant in the mesophilic stage, whereas during thermophilic and mature stages *α-proteobacteria* and *γ-proteobacteria* classes were more abundant. The high relative abundance of *γ-proteobacteria* class in S3 sample, may be important in favour of the initial phase of composting.

With respect to *Firmicutes* phylum, 6 different classes were detected in solid phase and clarified samples (S3 and S4), i.e. *Bacilli, Clostridia, Erysipelotrichi, Erysipelotrichia, Negativicutes* and *Thermolithobacteria* (Fig.4.4). Significant differences in the relative abundances were not observed compared with digestate. The class *Clostridia* was again the most representative and, as previously commented, these bacteria are known to metabolize relatively recalcitrant materials such as cellulose and lignin. In addition, species of *Bacilli* are known to secrete catabolic enzymes, such as proteases (37). Therefore, the presence of these classes in the S3 sample is convenient for the subsequent composting process.

Similarly to *Firmicutes* phylum, the distribution of the classes within phylum *Bacteroidetes* was not affected by the centrifugation process and *Bacteroidia* was again the predominant class.

3.3. Separation process II: Decanting

The results obtain for the decanted supernatant (S5) are shown in Fig. 4.5 After the decanting process a great variety of phyla were detected, whose distribution varies significantly in comparison with the liquid before decanting (S4).

The phylum *Firmicutes* was reduced to 37% of total abundance. On the contrary, other phyla such as *Bacteroidetes*, *Proteobacteria*, *Spirochaetes* or *Tenericutes* increase their total abundance reaching values of 10%, 37%, 8% and 6% respectively.

To understand this change, it is important to take into consideration that the decanting process takes place into an open tank during 14 h as previously commented, in S4, *Clostridia* was the class most abundant and these bacteria are strict anaerobes, thus it is expectable that, during decanting step, its concentration decrease, being replaced for other aerobic or facultative microorganisms.

The composition of relative abundance for the classes belonging to *Bacteroidetes* and *Firmicutes* phyla were almost not affected by the decanting being again *Bacteroidia* and *Clostridia* classes the most representative respectively. These classes contain well-known fermentative bacteria which are closely involved in degradation of organic materials and volatile fatty acids. Their presence is potentially associated with hydrolysis in wastewater treatment plant (38).

The phylum *Proteobacteria* was the most affected by decantation, with an increase of the *γ-proteobacteria* class and a drastic decrease of the *α-proteobacteria* class. The *α-proteobacteria* class is mostly anaerobic microorganisms, so the decanting process in an open tank compromises their survival. On the contrary, the class of *γ-proteobacteria* includes groups of aerobic bacteria that can grow during this process, as *Pseudomonas*. The high relative abundance of *γ-proteobacteria* is important since these microorganisms together with *β-proteobacteria* class, as *Nitrosomonas* bacteria, play a crucial role in biological treatment processes as nitrification-denitrification (38), which is the final destination of the decanted product.

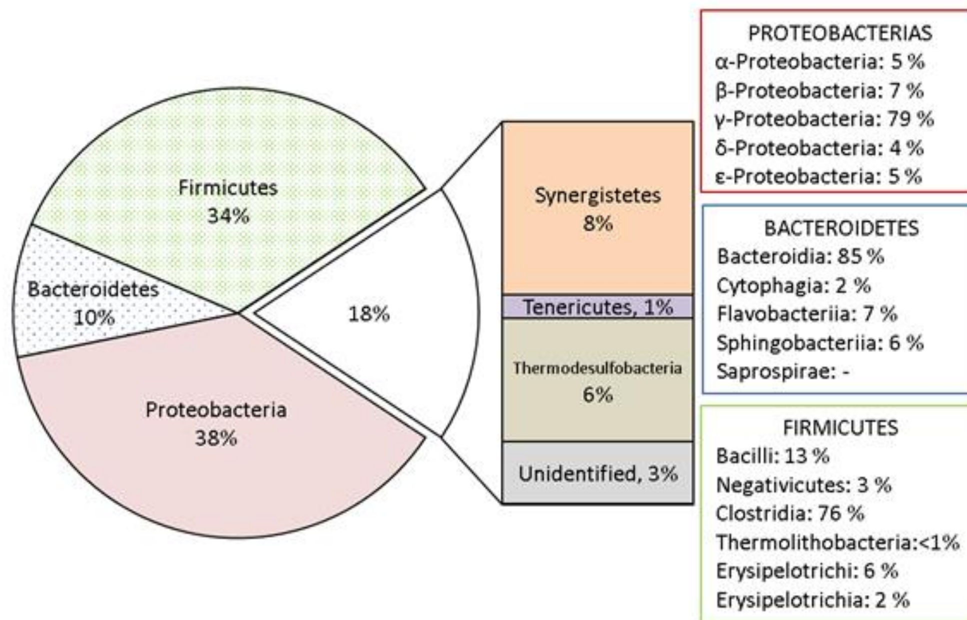


Figura 4. 5. *Relative abundance for de phyla detected in the decanted product (S5). In boxes are shown the relative abundance obtained for the classes within Firmicutes, Bacteroidetes and Proteobacteria phyla.*

In conclusion, the study indicated that the methodology employed, based on PGM sequencing and the amplification of all variable regions of the 16S gene allowed us to obtain an exhaustive taxonomic classification of bacterial populations throughout the processes considered. The main phyla detected throughout the digestion, centrifugation and decanting processes corresponded with microorganisms previously identified in anaerobic digestion of sludge. However, the relative abundance for the phyla throughout the process was very different depending on the treatment phase.

In the digestate (S2) from anaerobic digestion, *Firmicutes* was the predominant phylum, constituted mainly by bacteria of the genus *Clostridia* followed by the genus *Bacilli*. Their presence was crucial for a correct development of the anaerobic process. In related to class level, only important variation was observed within *Proteobacteria* phylum where *α-proteobacteria* was the most abundant after the process.

The relative abundance of the phyla in the clarified (S4) from centrifugation was very similar to that found in the digestate (S2) and only a light decreasing of *Actinobacteria* phylum was detected. This may be an advantage due to the recirculation

of S4 sample to the initial phase of anaerobic digestion. On the contrary, *Firmicutes* showed a sharp decrease in the solid phase (S3), while the phylum *Proteobacteria*. This fact indicates the different predisposition of these microorganisms to be removed by centrifugation. *Proteobacteria* classes were again the most affected being *γ-proteobacteria* the dominant one.

Finally, after the decanting process, in the supernatant (S5), it was observed an increase in the relative abundance of phyla that were minority in the clarified from the centrifugation step (S4), especially the phyla *Proteobacteria*, *Spirochaetes* and *Tenericutes*. On the other hand, *Firmicutes* phylum suffered a sharp descent again in its relative abundance. This was attributed to the presence of oxygen in the decanter which is an open tank. At the class level, an increase of *γ-proteobacteria*, which includes denitrifying microorganisms, was observed. The presence of nitrifying and denitrifying bacteria, i.e. *Nitrosomonas* and *Pseudomonas*, may be beneficial for the subsequent biological treatment. The differences observed on the relative abundances of bacterial phyla and classes are important to understand the influence of operation ways over the efficiencies of the following biological processes.

ACKNOWLEDGMENTS

The work upon which this paper is based on was co-financed by COGERSA, SAU (Projects FUO-EM-138-15, FUO-139-16), IDEPA (Project IDE/2015/000245), Spanish MINECO (Project CTM2015-63864-R) and FEDER funds from European Union. Technical assistance from the Scientific-Technical Services of the University of Oviedo is gratefully acknowledged.

REFERENCES

1. **Cardinali-Rezende, J., Debarry, R.B., Colturato, L.F., Carneiro, E.V., Chartone-Souza, E., and Nascimento, A.M. (2009).** Molecular identification and dynamics of microbial communities in reactor treating organic household waste. Appl. Microbiol. Biotechnol, 84 (4), 777-789.
2. **Venkiteshwaran, K., Bocher, B., Maki, J., and Zitomer, D. (2015).** Relating Anaerobic Digestion Microbial Community and Process Function. Water Research, 8, 37–44. https://doi.org/10.4137/MBI.S33593.
3. **Liu, A.C., Chou, C.Y., Chen, L.L. and Kuo, C.H. (2015).** Bacterial community dynamics in a swine wastewater anaerobic reactor revealed by 16S rDNA sequence analysis. Journal of Biotechnology, 194, 124-131.
4. **Fykse, E.M., Aarskaug, T., Marslien, E.H. and Dybwad, M. (2016).** Microbial community structure in a full-scale anaerobic treatment plant during start-up and first year of operation revealed by high-throughput 16S rRNA gene amplicon sequencing. Bioresource Technology, 222, 380-387.
5. **Zeng, Y., De Guardia, A. and Dabert P. (2016).** Improving composting as a post-treatment of anaerobic digestate. Bioresource Technology, 201, 293–303.
6. **Sancha, M., Laca, A., Laca, A., González, J.M., Mayo, B. and Diaz, M. (2014).** Approaches for Microbiological Characterization of a Landill Leachate Treatment. Journal of Residuals Science & Technology, 11 (2) 1544-8053/14/02 039-06.
7. **Vincent, A.T., Derome, N., Boyle, B., Culley, A.I. and Charette, S.J. (2017).** Next-generation sequencing (NGS) in the microbiological world: How to make the most of your money. Journal of Microbiological Methods 138, 60-71.
8. **Chen, Z., Wang, Y., Li, K., and Zhou, H. (2014).** Effects of increasing organic loading rate on performance and microbial community shift of an up- fl ow

anaerobic sludge blanket reactor treating diluted pharmaceutical wastewater. Journal of Bioscience and Bioengineering, 118 (3), 284–288. https://doi.org/10.1016/j.jbiosc.2014.02.027

9. **Zhou, S., Nikolausz, M., Zhang, J., Riya, S., Terada, A., and Hosomi, M. (2016).** Variation of the microbial community in thermophilic anaerobic digestion of pig manure mixed with different ratios of rice straw. Journal of Bioscience and Bioengineering, 122 (3), 334–340. https://doi.org/10.1016/j.jbiosc.2016.02.012
10. **Nelson, M., Morrison, M. and Yu, Z. (2011).** A meta-analysis of the microbial diversity observed in anaerobic digesters. Bioresource Technology, 102, 3730–3739.
11. **Sundberg, C., Al-Soud, W.A., Larsson, M., Alm, E., Yekta, S.S., Svensson, B.H., Sorensen, S.J., and Karlsson, A. (2013).** 454 pyrosequencing analyses of bacterial and archaeal richness in 21 full-scale biogas digesters. FEMS Microbiol. Eco., 85 (3), 612-626.
12. **Zamanzadeh, M., Hagen, L.H., Svensson, K. and Linjordet, R. (2016).** Anaerobic digestion of food waste - Effect of recirculation and temperature on performance and microbiology. Water Research, 96, 246-254.
13. **Shu, D., He, Y., Yue, H. and Wang, Q. (2015).** Microbial structures and community functions of anaerobic sludge in six full-scale wastewater treatment plants as revealed by 454 high-throughput pyrosequencing. Bioresource Technology, 186, 163–172.
14. **Hu, M., Wang, X., Wen, X. and Xia, Y. (2012).** Microbial community structures in different wastewater treatment plants as revealed by 454-pyrosequencing analysis. Bioresource Technology, 117, 72–79.
15. **Zhang, T., Shao, M. and Ye, L. (2012).** 454 Pyrosequencing reveals bacterial diversity of activated sludge from 14 sewage treatment plants. The ISME Journal, 6, 1137-1147.
16. **Abendroth, C., Vilanova, C., Günther, T., Luschnig, O. and Porcar, M. (2015).** Eubacteria and archaea communities in seven mesophile anaerobic digester plants in Germany. Biotechnology of Biofuels, 8 (87). DOI 10.1186/s13068-015-0271-6
17. **Yang, Z., Xu, R., Zheng, Y., Chen, T., Zhao, L. and Li, M. (2016).** Characterization of extracellular polymeric substances and microbial diversity in

anaerobic co-digestion reactor treated sewage sludge with fat, oil, grease. Bioresource Technology, 212, 164–173.

18. **Narihiro, T., Nobu, M. K., Kim, N.-K., Kamagata, Y. and Liu, W.T. (2015).** The nexus of syntrophy-associated microbiota in anaerobic digestion revealed by long-term enrichment and community survey. Environmental Microbiology, 17, 1707–1720. doi: 10.1111/1462-2920.12616
19. **Gulhane, M., Pandit, P., Khardenavis, A., Singh, D., and Purohit, H. (2017).** Study of microbial community plasticity for anaerobic digestion of vegetable waste in Anaerobic Baffled Reactor. Renewable Energy, 101(February), 59–66. https://doi.org/10.1016/j.renene.2016.08.021
20. **Riviere, D., Desvignes, V., Pelletier, E., Chaussonnerie, S., Guermazi, S., Weissenbach, J., Li, T., Camacho, P. and Sghir, A. (2009).** Towards the definition of a core of microorganisms involved in anaerobic digestion of sludge. ISME J, 3(6), 700-714.
21. **Regueiro, L., Veiga, P., Figueroa, M., Alonso-Gutierrez, J., Stams, A.J.M., Lema, J.M. and Carballa, M. (2012).** Relationship between microbial activity and microbial community structure in six full-scale anaerobic digesters. Microbiological Research, 167(10), 581-589.
22. **Maspolim, Y., Zhou, Y., Guo, C., Xiao, K. and Ng, W.J. (2015).** Determination of the archaeal and bacterial communities in two-phase and single-stage anaerobic systems by 454 pyrosequencing. Journal of Environmental Sciences, 36, 121-129.
23. **Dao, H., Kuroda, K., Nakahara., N., Danshita, T., hatamoto, M. and Yamaguchi, T. (2016).** 16S rRNA gene-based comprehensive analysis of microbial community compositions in a full-scale leachate treatment system. Journal of Bioscience and Bioengineering, 122 (6), 708-715.
24. **Sun, L., Pope, P. B., Eijsink, V. G. H. and Schnürer, A. (2015).** Characterization of microbial community structure during continuous anaerobic digestion of straw and cow manure. Microbial Biotechnology, 8: 815–827. doi:10.1111/1751-7915.12298
25. **Pervin, H. M., Batstone, D. J., and Bond, P. L. (2013).** Previously unclassified bacteria dominate during thermophilic and mesophilic anaerobic pre-treatment of primary sludge. Systematic and Applied Microbiology, 36(4), 281–290. https://doi.org/10.1016/j.syapm.2013.03.003

26. **Ziganshin, A.M., Liebetrau, J., Pröter, J. and Kleinsteuber, S. (2013).** Microbial community structure and dynamics during anaerobic digestion of various agricultural waste materials. Applied Microbiology and Biotechnology, 97(11), 5161-5174.
27. **McDonald, J. E., Houghton, J. N. I., Rooks, D. J., Allison, H. E. and McCarthy, A. J. (2012).** The microbial ecology of anaerobic cellulose degradation in municipal waste landfill sites: evidence of a role for fibrobacters. Environmental Microbiology, 14: 1077–1087. doi: 10.1111/j.1462-2920.2011.02688.x
28. **Michalke, K., Wickenheiser, E. B., Mehring, M., and Hirner, A. V. (2000).** Production of Volatile Derivatives of Metal(loid)s by Microflora Involved in Anaerobic Digestion of Sewage Sludge. Applied and Environmental Microbiology. 66(7), 2791–2796.
29. **Guo, J., Peng, Y., Ni, B.-J., Han, X., Fan, L., and Yuan, Z. (2015).** Dissecting microbial community structure and methane-producing pathways of a full-scale anaerobic reactor digesting activated sludge from wastewater treatment by metagenomic sequencing. Microbial Cell Factories, 14(1), 33. https://doi.org/10.1186/s12934-015-0218-4
30. **Jaenicke, S., Ander, C., Bekel, T., Bisdorf, R., Dröge, M., Gartemann, K. H., and Goesmann, A. (2011).** Comparative and joint analysis of two metagenomic datasets from a biogas fermenter obtained by 454-pyrosequencing. PLoS ONE, 6(1). https://doi.org/10.1371/journal.pone.0014519
31. **Li, A., Chu, Y., Wang, X., Ren, L., Yu, J., Liu, X., and Li, S. (2013).** A pyrosequencing-based metagenomic study of methane-producing microbial community in solid-state biogas reactor. Biotechnology for Biofuels, 6(1), 3. https://doi.org/10.1186/1754-6834-6-3
32. **Di Maria, F., Barratta, M., Bianconi, F., Placidi, P. and Passeri, D. (2017).** Solid anaerobic digestion batch with liquid digestate recirculation and wet anaerobic digestion of organic waste: Comparison of system performances and identification of microbial guilds. Waste Management, 59, 172–180.
33. **Yi, J., Dong, B., Jin, J. and Dai, X. (2014).** Effect of Increasing Total Solids Contents on Anaerobic Digestion of Food Waste under Mesophilic Conditions: Performance and Microbial Characteristics Analysis. PLoS ONE, 9, (7) e102548.doi:10.1371/journal.pone.0102548

34. **Wang, Z., Zhang, X-X., Lu, X., Liu, B., Li, Y., Long, C. and Li, A. (2014).** Abundance and diversity of bacterial nitrifiers and denitrifiers and their functional genes in tannery wastewater treatment plants revealed by high throughput sequencing. PLOS ONE, DOI:10.1371/journal.pone.0113603.
35. **Gao, D., Zhang, T., Tang, C., Wu, W., Wong, C., Lee, Y., Yeh, D. and Criddle, C. (2010).** Membrane fouling in an anaerobic membrane bioreactor: Differences in relative abundance of bacterial species in the membrane foculant layer and in suspension. Journal of Membrane Science, 364, 331–338.
36. **De Gannes, V., Eudoxie, G., and Hickey, W. J. (2013).** Prokaryotic successions and diversity in composts as revealed by 454-pyrosequencing. Bioresource Technology, 133, 573–580. https://doi.org/10.1016/j.biortech.2013.01.138
37. **Partanen, P., Hultman, J., Paulin, L., Auvinen, P., and Romantschuk, M. (2010).** Bacterial diversity at different stages of the composting process. BMC Microbiology, 10(1), 94. https://doi.org/10.1186/1471-2180-10-94
38. **Guo, J., Ni, B. J., Han, X., Chen, X., Bond, P., Peng, Y., and Yuan, Z. (2017).** Unraveling microbial structure and diversity of activated sludge in a full-scale simultaneous nitrogen and phosphorus removal plant using metagenomic sequencing. Enzyme and Microbial Technology, 102(March), 16–25. https://doi.org/10.1016/j.enzmictec.2017.03.009

4.1.2 Physico-chemical pre-treatments of anaerobic digestion liquor for aerobic treatment

Ana Isabel Díaz[1], Paula Oulego[1], J. Manuel González[2], Adriana Laca[1*], and Mario Díaz[1]

[1]Department of Chemical and Environmental Engineering. University of Oviedo. C/Julián Clavería, s/n, E-33006, Oviedo, Asturias, Spain.

[2] R&D, COGERSA SAU. C/ La Zoreda, s/n, E-33697, Gijón, Asturias, Spain.

[*]E-mail (*corresponding author*): lacaadriana@uniovi.es;

Phone: +34 985 10 29 74; Fax: +34 985 10 34 34

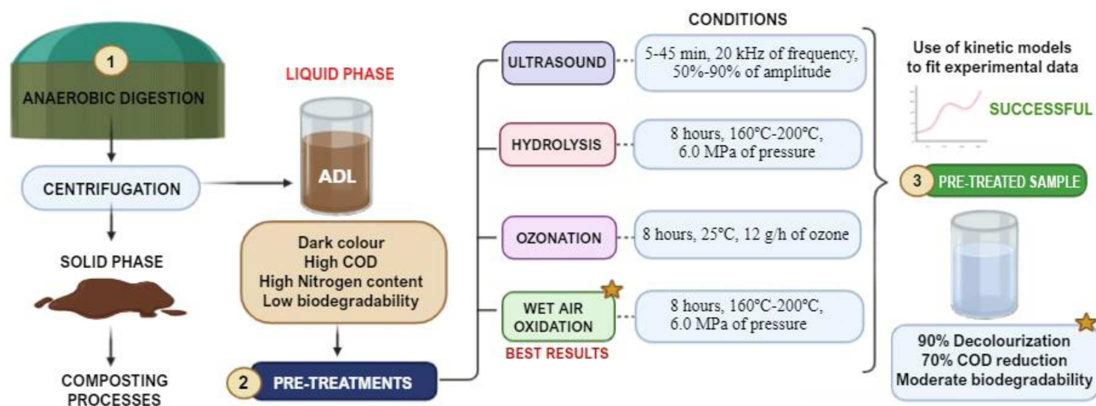


Figura 4. 6. *Resumen gráfico del trabajo 2*

*Artículo publicado en la revista "**Journal of Environmental Management**"

Cita: Ana Isabel Díaz, Paula Oulego, J. Manuel González, Adriana Laca, Mario Díaz, *Physico-chemical pre-treatments of anaerobic digestion liquor for aerobic treatment*, Journal of Environmental Management, Volume 274, **2020**, 111189, ISSN 0301-4797,

https://doi.org/10.1016/j.jenvman.2020.111189

ABSTRACT

Centrifugation of anaerobically digested sewage sludge gives rise to a solid phase, which could be employed as a fertilizer, and a liquid fraction (ADL), which should be treated before being spilled out. This is not an easy task because this liquor is characterized for presenting high COD (~16000 mg O_2/L), high ammonium content (~4000 mg/L) and low biodegradability (BOD_5/COD ~0.2). With the objective to pre-treat this aqueous waste before its treatment by means of more traditional aerobic processes, different physico-chemical methods (ultrasound, ozonation, hydrolysis and wet air oxidation) were assessed in this work. Ultrasound and thermal hydrolysis gave solubilizations around 47% and 68% respectively. The best results in terms of total COD removal were obtained when wet air oxidation (8 h, 160 ºC-200 ºC and 6.0 MPa) and ozonation (8 h, 25 ºC, 12 g/h O_3) techniques were employed achieving COD degradations of 71% and 38%, respectively. The pre-treatment of ADL with the four assayed techniques improved considerably the biodegradability (BOD_5/COD) of the effluent, with values around 0.3-0.4, depending on the treatment. The experimental data were successfully fitted by kinetic models and the kinetic constants for the solubilization and degradation steps were obtained. Application of the proposed models can be of interest for the optimization and selection of the most suitable techniques and operational conditions, in each particular case.

Keywords: anaerobic digestion liquor; wastewater pre-treatment; biodegradability; advanced oxidation processes; hydrolysis; ultrasound

1. INTRODUCTION

Anaerobic digestion (AD) is a widely employed method that transforms organic wastes, such as sludge and organic fraction of municipal solid wastes, into biogas and digestate. The biogas is a product of high added value that can be used as biofuel in a sustainable and eco-friendly way (Xu et al., 2019). The anaerobic digestate, with a high nitrogen content, could be used as organic fertilizer to improve the quality of agricultural soil. However, some characteristics of the digestate, such as odour, viscosity and high content in volatile fatty acids can be harmful to the environment, and its application is not allowed without a previous pre-treatment (Zeng et al., 2016).

One common option is to separate the digestate into liquid and solid phases by filtration or centrifugation processes. After this solid–liquid separation, the solid phase, easy to transport and store, is suitable for composting processes due to the large amount of nutrients and organic components. By contrast, the liquid phase, known as anaerobic digestion liquor (ADL), is more difficult to be processed. This fraction contains 70% - 80% of the total ammonium and 35% - 40% of the total phosphorus from the initial digestate (Peng and Pivato, 2019). Its high organic load and heavy metal content may cause soil contamination and the eutrophication of nearby water. Therefore, it is necessary to treat this effluent before being spilled out in order to reduce the chemical oxygen demand (COD), nitrogen content and high colour, so that rules on wastewater discharged can be accomplished.

When the AD process takes place in a waste management centre, which includes waste disposal, a possible destination for the ADL is the leachate treatment plants, where a biological degradation is usually carried out through denitrification-nitrification processes (Díaz et al., 2019). However, it is not an easy task due to the high nitrogen content and low biodegradability of the organic matter contained in the liquor and, when it is mixed with other leachates, it can result in an effluent with COD and nitrogen levels higher than the design parameters of the treatment plant. Therefore, the addition of an external carbon source, such as methanol, it is frequently necessary to achieve an effective denitrification when ADL and leachates are treated together. For this reason, it is necessary to investigate suitable pre-treatments that allows to obtain a final effluent with physical and chemical characteristics similar to those present in other leachates, so

that ADL can be easily treated by biological treatments without modifying the usual operational parameters.

A novel technique used for the treatment of wastes which contain recalcitrant organic matter, is the irradiation with ultrasounds. This treatment generates pressure waves, gas and vapor bubbles. The implosion of this bubbles create regions with high temperatures and pressures (up to 5000 K and 100 MPa), which promote the physical disintegration of organic matter and/or the extraction of different compounds that could improve the oxidative degradation of organic pollutants (Li et al., 2018; Passos et al., 2014; Somers et al., 2018). In previous studies, the use of low frequencies (20 - 40 kHz) to pre-treat sludge gave the best results. The main advantages of this technique are the short time of treatment and the not necessity of chemical reagents (Tyagi et al., 2014).

Advanced oxidation processes, such as ozonation or wet air oxidation (WAO), are attractive technologies for the treatment of heavily polluted aqueous wastes. These techniques allow the oxidation of organic pollutants due to the generation of powerful chemical oxidants, majority hydroxyl radicals (Miklos et al., 2018). One of their main advantages, compared to conventional technologies, is their capacity to degrade a wide range of recalcitrant components without generating secondary residues (Dewil, 2017). Besides, this technology can also be used in presence of catalyst, such as activated carbon, in order to use less severe operating conditions and/or improve its effectiveness (Abid et al., 2016).

Ozonation is a method based on the high oxidant power of the ozone, which reacts with different organic pollutants such as humic substances (Carrère et al., 2010). Its use, as single technique or in combination with hydrogen peroxide, has been reported by other authors, who indicated its suitability to remove colour from industrial wastewaters, as well as to improve the solubilization and degradability of organic solid wastes (Bakhshi et al., 2018; Cesaro et al., 2019). The use of WAO has been widely studied at different conditions of temperature and pressure for the treatment of sewage sludge and other effluents that contain high concentrations of organic matter. This technique allows to improve the biodegradability and decolourization of the treated effluents (Sivagami et al., 2018; Urrea et al., 2017).

The employment of hydrolysis has been studied for anaerobic digestion processes in order to pre-treat the wastes to increase biogas production, or to post-treat the digestate solid fractions. Nevertheless, its use for the treatment of the ADL has not been almost studied till now. This technique results in decomposition of microbial cells, lipids, proteins and carbohydrates into lighter molecules, releasing it content into liquid fraction, and allowing better fermentation (Hii et al., 2014; Suárez-Iglesias et al., 2017; Svensson et al., 2018).

Most of the published works are focused on the use of these techniques as pre-treatment for sewage sludge or ADL, with the purpose of breaking down organic matter and increasing the biogas generation in anaerobic digestion processes (Gunes et al., 2019; Zhen et al., 2017; Zou et al., 2016). However, part of the generated ADL is not recirculated and needs to be stored for further treatment (Díaz et al., 2018).

In view of these considerations, the main aim of this work was to analyse the effect of different pre-treatment techniques (ultrasound, hydrolysis, ozonation and WAO) in the ADL degradation, in order to solubilize the particulate matter, decrease the chemical oxygen demand (COD) and/or improving the biodegradability. An additional objective of this work was to obtain a kinetic model that helps to select the optimal operating conditions for each particular treatment.

These pre-treatments would allow to adjust the characteristics of the liquor to the conditions of the treatment plant, so that ADL can be easily treated by conventional biological treatments. Additionally, an increase in the soluble COD could be beneficial for the recirculation of ADL to the anaerobic digester, favouring the microbiological hydrolysis step. Studies about the treatment of the liquid obtained after anaerobic digestate centrifugation (ADL) are scarce, and, as far as we know, there is not any published work that compares the four techniques here considered for the treatment of this effluent.

2. MATERIAL AND METHODS

2.1 Effluent description

The ADL used in this study corresponds with the liquid fraction obtained after centrifugation and decantation of the digestate coming from an anaerobic treatment

plant. This digestate was obtained by anaerobic digestion of sewage sludge. The treatment plant is located in the waste treatment centre, COGERSA (Asturias, Spain). For more details of the process, see Diaz et al., (2018).

A detailed description of the waste effluent is shown in Table 1. Slight differences were observed between the batch samples of ADL used for the different experiments, for this reason a range of values for each parameter is shown in Table 4.2.

Tabla 4. 2. *Characteristics of the anaerobic digestion liquor (ADL)*

Parameter	Value
pH	7.96 – 8.26
tCOD (mg O_2/L)	15179 - 16802
sCOD (mg O_2/L)	7205 - 8098
sTOC (mg/L)	3218 - 3788
BOD_5 (mg O_2/L)	2135 - 2960
Colour Number (CN)	0.899 – 1.275
AOSC	0.580 – 0.904
NH_4^+ (mg/L)	3812 - 4166

2.2 Apparatus and procedures

For ultrasound experiments, 100 mL of ADL was treated using a Sonopuls HD 2070 system (Bandelin, Germany), equipped with an VS 70 probe, at a frequency of 20 kHz. Suitable results to enhanced solubilization of COD, TOC and TN have been reported using a sonication frequency of 20 kHz (Garoma and Pappaterra, 2018). During the ultrasound process, temperature was maintained constant at 40 ºC using a water bath. Sonication amplitudes of 50% and 90% were tested. A sonication amplitude of 100% is equivalent to 80 µm. Samples were taken withdraw between 5 - 45 min, each 5 min of sonication.

The ozonation process was carried out introducing 300 mL of ADL in a cylindrical stirred reactor with a capacity of 1 L. For these experiments an ozone flow of 12 g O_3/h was bubbled through the reactor. Shabani et al. (2015) reported significant

removals of COD, colour, and aromatic compounds after the use of this ozone flow during the treatment of landfill leachates. Ozone was generated from industrial grade oxygen using an ozone generator (ZonoSystem). The experiments were carried out at 25 ºC and samples were periodically taken for 8 h. Additionally, an assay with ADL after solid elimination was carried out at the same conditions. These solids were removed by centrifugation at 20 ºC and 13000 g for 15 min.

Hydrolysis experiments were carried out in a 1 L capacity semi-batch reactor (Parr T316SS) equipped with two six-bladed magnetically driven turbine agitators. The reactor was preceded by a 2-L stainless steel water reservoir. The loaded volume in each vessel was 700 mL in order to ensure safe operating conditions. The addition of 20 µL of antifoam 289 (Sigma-Aldrich) was required to avoid the formation of foam during the experiment. The reactor was pressurized and heated up to the desired working conditions (160 ºC, 180 ºC and 200 ºC) and the stirrer speed was adjusted to 350 rpm for all the experiments. The operating pressure was provided by bottled compressed nitrogen that supplied a nitrogen flow of 1400 mL/min, controlled by an electronic mass flow controller (Brooks). The nitrogen was bubbled through the water reservoir in order to become saturated with water before being sparged into the reaction vessel. A valve and a coil fitted to the top of the vessel allowed the withdrawal of samples during the reaction. The pressure was kept constant at 6.0 MPa by means of a back-pressure controller placed at the end of the gas line. Samples were periodically taken for 8 h. Similar conditions of temperature and pressure were used by Urrea et al., (2015) to treat active sludge of a sewage treatment plant. Their results showed this technique as a positive alternative for improving the management of this waste, in the conditions tested.

Finally, for the (WAO) experiments, the equipment and experimental procedure were the same as that used in the hydrolysis experiments, with the only difference that an oxidizing gas (oxygen) was used instead of nitrogen. Similar conditions have been used successfully to treat effluents from the biological treatment of landfill leachates by Oulego et al. (2015). After treatment, the samples were stored at 4 ºC for 24 h prior to be analysed.

2.3 Analytical methods

The concentration of total chemical oxygen demand (tCOD) was spectrophotometrically measured (at 600 nm) by dichromate method according to Standard Methods (APHA, 1998) using a DR2500 spectrophotometer (Hach Company). This method was also employed for the analysis of soluble COD (sCOD) with the only difference that in this case, the sample was previously centrifuged at 20 ºC and 13000 g for 15 min (Kubota 6500 High Speed Refrigerated Centrifuge) to remove the solid particles.

Total organic carbon (TOC) analysis was performed using a TOC analyzer (Shimadzu TOC-VCSH, Japan) and biochemical oxygen demand (BOD_5) was determined using a manometric respirometry measurement system (Lovibond® Water Testing BD 600). The value of pH was measured by means of a pH- meter (Jenway 3510) and the absorbances (Abs) were measured at 436, 525 and 620 nm using a UV/vis spectrophotometer (Thermo Scientific, Heλios γ).

The change in the colour of the ADL by the treatments was determined by means of the colour number (CN), which is defined according to Equation (1) (Tizaoui et al., 2007).

$$CN=\frac{SAC_{436}^2+SAC_{525}^2+SAC_{620}^2}{SAC_{436}+SAC_{525}+SAC_{620}} \qquad \text{Eq (1)}$$

Spectral absorbance coefficients (SAC) are defined as the ratio of the values of the respective absorbance (Abs) over the cell thickness (x). All analytical measurements were done at least in triplicate.

2.4 Parameter calculations

Non-soluble COD (nsCOD) was calculated as the difference between total COD (tCOD) and soluble COD (sCOD) for each reaction time (see Equation 2).

$$nsCOD = tCOD - sCOD \qquad \text{Eq (2)}$$

The percentage of solubilization achieved with each treatment was determined according to Equation (3), where $nsCOD_0$ is the initial value of non-soluble COD, and nsCOD is the value of non-soluble COD at the considered time.

$$\text{Solubilization } (\%) = \frac{nsCOD_0 - nsCOD}{nsCOD_0} \qquad \text{Eq (3)}$$

The average oxidation stage of carbon atoms (AOSC) was also studied. This parameter allowed to evaluate the oxidation degree for the reacting mixture after different treatments. This parameter was obtained as follows (Vogel et al., 2000):

$$AOSC = 4 - 1{,}5 * \left(\frac{COD}{TOC}\right) \qquad \text{Eq (4)}$$

The AOSC values must always lie between the range 4 and –4. Low AOSC values indicate that the oxidation is not complete, whereas high AOSC values indicate a high oxidation state of the organic compounds (Garg and Mishra, 2010).

Finally, biodegradability was calculated as the ratio of BOD_5 over tCOD (or sCOD if solids were removed).

2.5 Kinetic models

Models proposed in this study are based on the kinetics models described by Oulego et al., (2016) for advanced oxidation treatments. In all cases, the kinetic constants were determined by fitting the model to experimental data using Micromath Scientist 3.0.

2.5.1 Ultrasound

The kinetic model proposed to fit the experimental data of ultrasound treatment is shown in Equations (5) and (6), as next indicated:

$$\frac{dnsCOD}{dt} = -k_{U1}(nsCOD - R_1) \qquad \text{Eq (5)}$$

$$\frac{dsCOD}{dt} = k_{U1}(nsCOD - R_1) - k_{U2}CODs \qquad \text{Eq (6)}$$

It is considered that nsCOD was turned into sCOD and this sCOD was subsequently degraded to CO_2. In this model, k_{U1} is the kinetic constant for the

solubilization of nsCOD into sCOD and k_{U2} is the kinetic constant for the degradation of sCOD. R_1 is the value of nsCOD at final times.

2.5.2 Ozonation

For the experiments carried out with solids, the kinetic model for solubilization and degradation followed Equations (7) and (8). In this model, k_{O1} is the kinetic constant for the solubilization of nsCOD into sCOD, k_{O2} is the kinetic constant for the degradation of sCOD and R_1 and R_2 are the values of nsCOD and sCOD, respectively, at final time. In the case of sample without solids, Equation (9) was used:

$$\frac{\mathrm{dnsCOD}}{\mathrm{dt}} = -k_{O1} * (\mathrm{nsCOD} - R_1) \qquad \text{Eq (7)}$$

$$\frac{\mathrm{dsCOD}}{\mathrm{dt}} = k_{O1} * (\mathrm{nsCOD} - R_1) - k_{O2} * (\mathrm{sCOD} - R_2) \qquad \text{Eq (8)}$$

$$\frac{\mathrm{dsCOD}}{\mathrm{dt}} = -k_{O2} * (\mathrm{sCOD} - R_2) \qquad \text{Eq (9)}$$

2.5.3 Hydrolysis

A kinetic model according to Equations (10) and (11) was used to fit the experimental data as shown in Fig. 3a.

$$\frac{\mathrm{dnsCOD}}{\mathrm{dt}} = -k_{H1} * (\mathrm{nsCOD} - R_1) \qquad \text{Eq (10)}$$

$$\frac{\mathrm{dsCOD}}{\mathrm{dt}} = k_{H1} * (\mathrm{nsCOD} - R_1) \qquad \text{Eq (11)}$$

In this model, k_{H1} is the apparent constant for the solubilization of nsCOD into sCOD. The k_{H1} is an apparent constant because the operating conditions were not reached before 60-90 min of treatment, depending on each experiment. So, the first experimental data correspond with lower temperature and pressure values.

2.5.4 Wet Air Oxidation (WAO)

Equations (12) and (13) show the kinetics of the process, considering that nsCOD was transformed into sCOD and this sCOD was subsequently degraded into CO_2.

$$\frac{dnsCOD}{dt} = -k_{W1} * (nsCOD - R_1) \qquad Eq\ (12)$$

$$\frac{dsCOD}{dt} = k_{W1} * (nsCOD - R_1) - k_{W2} * (CODs - R_2) \qquad Eq\ (13)$$

In this model, k_{W1} is the apparent constant for the solubilization of nsCOD and k_{W2} is the apparent constant for the degradation of sCOD. Again, k_{W1} and k_{W2} are apparent constants because the operating conditions were not reached before 60-90 min of treatment.

3. RESULTS AND DISCUSSION

3.1 Ultrasound treatment

The changes of tCOD, sCOD and nsCOD concentrations with time for ultrasound treatment are shown in Fig. 4.7.A Important changes in sCOD and nsCOD were observed, even though tCOD degradation was very low. The most significant increase in sCOD was obtained after 45 min of sonication in both cases, reaching almost an increase of 40% for 90% amplitude. Li et al. (2018), reported an increase of 4.6–23.06 times of sCOD in waste activate sludge after 20–100 min of ultrasound at 20 kHz, concluding that times greater than 80 min was not effective. However, the trend in this study might infer that sonication times higher that 45 min would allow higher increases in sCOD concentrations.

The sCOD/tCOD ratio showed an increase from 0.48 to 0.62 and 0.71 for 50% and 90% amplitudes, respectively. Oz and Uzun, (2015), who carried out an anaerobic digestion of olive mill wastewater pre-treated with ultrasound (20 kHz, 10 min), reported an increase in sCOD/tCOD ratio from 0.59 to 0.79, and consequently, an increase of 20% in biogas production.

With the aim of obtaining a liquor that could be treated by an activated sludge system, it is important to emphasize that a significant increase in the biodegradability of

the final effluent was obtained for 90% amplitude (see Fig. 1b). The BOD_5/tCOD ratio increased progressively throughout the sonication time resulting in a moderately biodegradable leachate after 45 min of sonication (BOD_5/tCOD ~ 0.28).

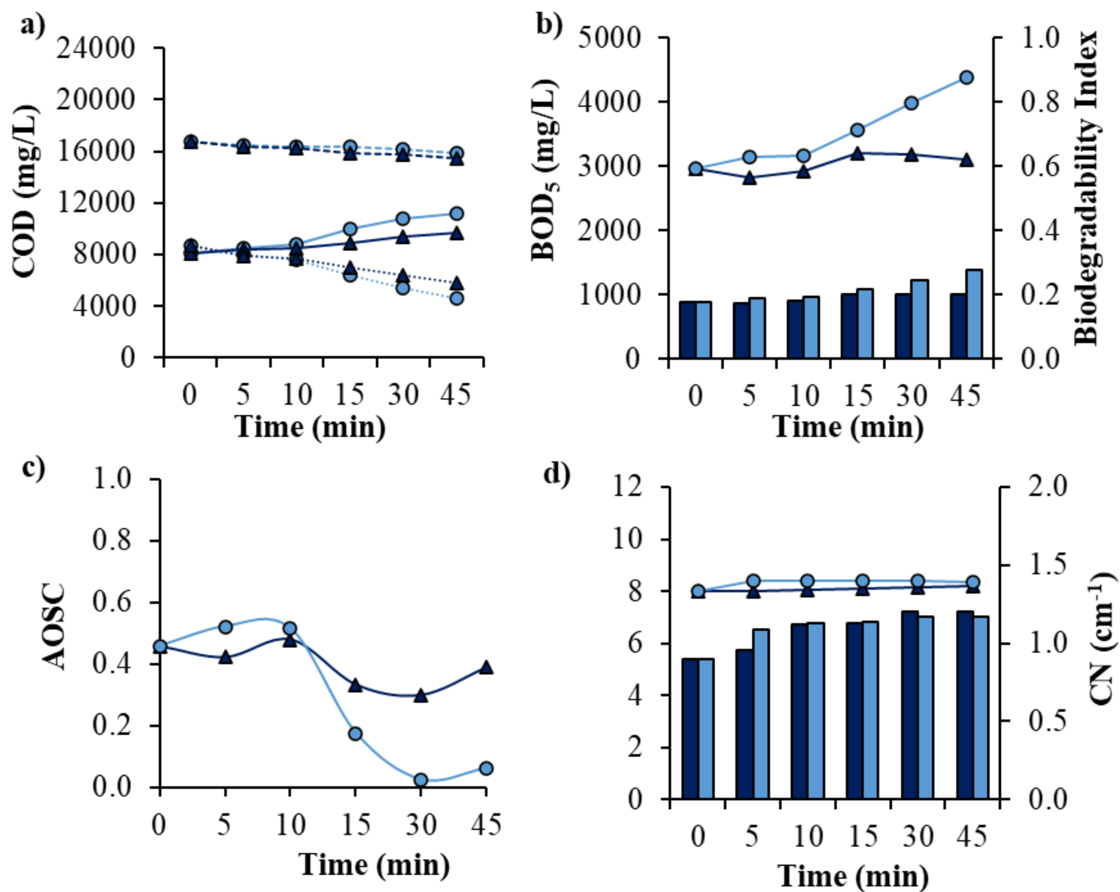


Figura 4. 7. *a) Changes of tCOD (dashed lines), sCOD (solid lines) and nsCOD (dotted lines) during ultrasound treatment at 50% of amplitude (dark colour and triangle markers) and 90% of amplitude (light colour and circle markers).* ***b)*** *Changes of BOD_5 (lines and markers) and biodegradability (columns).* ***c)*** *Change of AOSC and* ***d)*** *Changes of pH (lines and markers) and colour number (columns). The standard deviations (SD) of the experimental data were in all cases lower than 3% of the mean value*

The AOSC value was calculated for each time and amplitude of treatment giving the values shown in Fig. 4.7.C. The use of 90% of amplitude decreased the AOSC value significantly from 15 min of treatment, while for 50% of amplitude AOSC remained almost constant. This technique promotes the physical organic matter disintegration encouraging the solubilization of different compounds, so, the important decrease of

AOSC with 90% of amplitude could be a consequence of the solubilizations achieved at the same time.

The changes in colour and pH are shown in Fig. 4.7.D. The colour number (CN) was increased around 30% in all samples for both amplitudes studied. The value of pH remained almost constant between 8.0 and 8.4.

3.2 Ozonation treatment

When the ADL with solids was treated, a fast solubilization occurred during the first hour of treatment, dropping the nsCOD more than 50%. At the same time, an initial increase occurred in sCOD values and after 45 min decrease again, reaching final values very similar to initial one. While nsCOD was solubilized, sCOD was degraded, so that the tCOD decreased by 35% in just 300 min. For the experiment with centrifuged ADL, a 31% of sCOD removal was achieved at final time (see Fig. 4.8.A).

The results of the biodegradability index are shown in Fig. 4.8.B. An improvement of the biodegradability of treated samples from ADL with and without solids was obtained. In both cases, a moderately biodegradable effluent after 8 h of ozonation (BOD_5/COD~0.3 and BOD_5/COD~0.37, respectively).

The changes of AOSC values showed, in the case of ADL with solids, a fast drop in during the first minutes. This fact indicated that the dissolving of compounds from the solid particles that were rapidly degraded, giving place to the subsequent increase in AOSC values. For the final samples, both in presence and absence of solids, an increase in the AOSC values were observed, reaching values over 1.0 (Fig. 4.8.C).

As observed in Fig. 2d, the ozonation treatment allowed an important removal of colour, decreasing by 73% and 82% during the first 60 min of reaction in presence and absence of solids, respectively. For centrifuge ADL, colour number continues decreasing achieving a removal of 96% after 8 h.

Finally, pH values slightly increased during the first hour of reaction in both cases. Afterwards, when solids were present, the pH values began to decrease with a final pH of 7.5. However, in the absence of solids, pH variations were much lower. Several authors reported that higher values of pH increase the degradation efficiency of

ozonation treatments, due to the faster formation of hydroxyl radicals at higher pH (Sivagami et al., 2018).

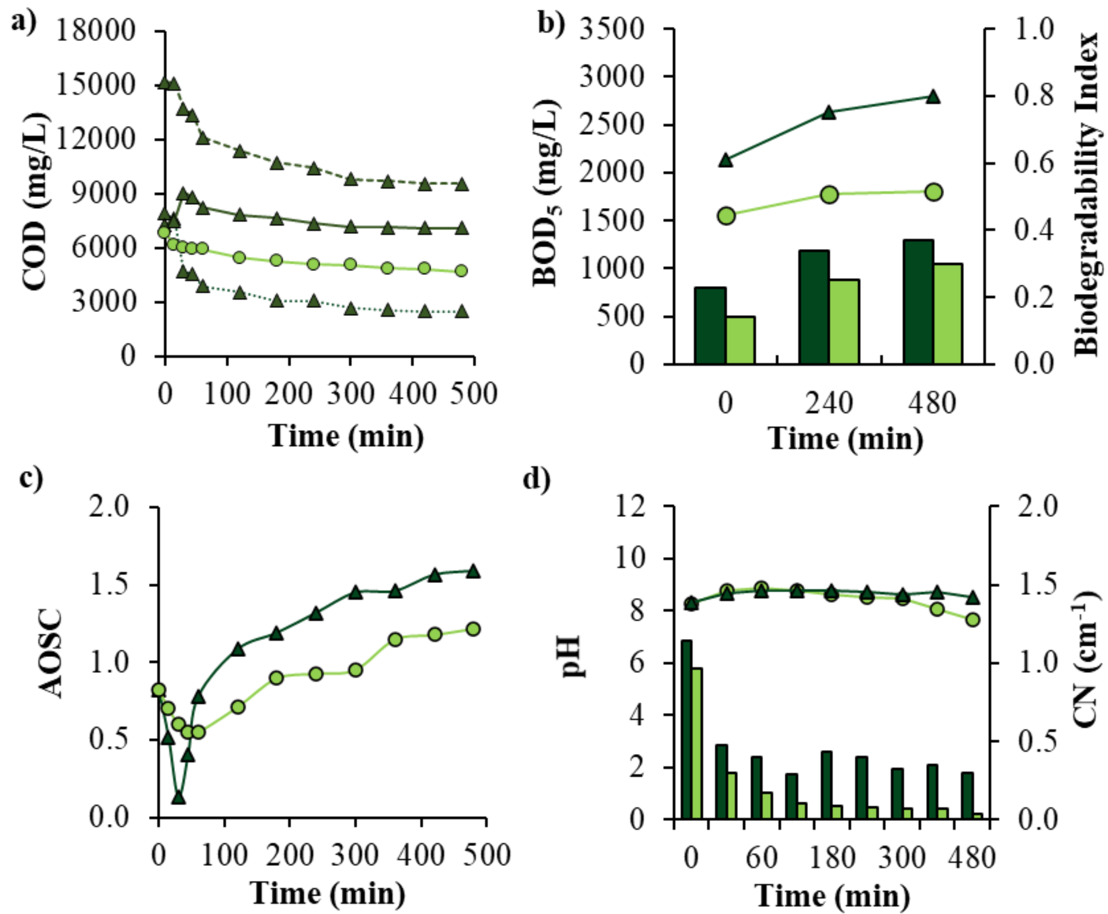


Figura 4. 8. *a) Changes of tCOD (dashed lines), sCOD (solid lines) and nsCOD (dotted lines) during ozonation treatment. The experiment A corresponds to the sample with solids (dark colour and triangle markers) and the experiment B corresponds to the sample without solids (light colour and circle markers). b) Changes of BOD_5 (lines and markers) and biodegradability (columns). c) Changes of AOSC. d) Changes of pH (markers) and CN (columns). The standard deviation (SD) of the experimental data were in all cases less than 4% of mean value.*

3.3 Hydrolysis treatment

As can be shown in Fig. 4.9.A, hydrolysis treatment did not degrade the tCOD of the ADL, which remained practically constant for the three tested temperatures. However, as occurred during ultrasound treatment, sCOD increased as a consequence of the solubilization of nsCOD, which decreased. The use 200 ºC and 6.0 MPa gave an increase of 54% in the sCOD after 90 min of treatment, which corresponds with a solubilisation of nsCOD around 70%.

A moderately biodegradable effluent was reached after 4 h of reaction (BOD_5/COD ~ 0.30), independently of the operating temperature (see Fig. 4.9.B). This improvement in the biodegradability of pre-treated effluent makes easier its treatment in a biological process. In addition, part of the pre-treated effluent could be recirculated to the anaerobic reactor in order to improve biogas production as a consequence of a better use of the organic matter.

Previous studies have reported that sewage sludge treated by hydrolysis is more assimilable by anaerobic microorganisms, allowing a faster and higher methane production (Donoso-Bravo et al., 2011). Yang et al. (2019) analysed the effect of thermal hydrolysis in a digested sludge coming from a domestic WWTP. The digested sludge was hydrolysed, dewatered and recirculated to the digester for re-digestion with raw sludge. They concluded that the hydrolysis treatment increased the concentration of sCOD in the sludge by disintegrating the recalcitrant organic matter and resulting in further improvement of methane production.

As can be seen in Fig. 4.9.C, there is a relationship between the treatment temperature and the final AOSC value. When the higher temperature is used (200 ºC) a drop in AOSC values was observed during the first minutes with negative values after 100 min. These low values indicate a higher breakage of the organic matter as a consequence of the higher temperature employed. The Fig. 4.9.D shows the changes in colour number (CN) and pH. The value of pH progressively increased up to values around 10. Concerning to colour number, it increased gradually, obtaining a treated effluent with greater coloration compared with initial sample.

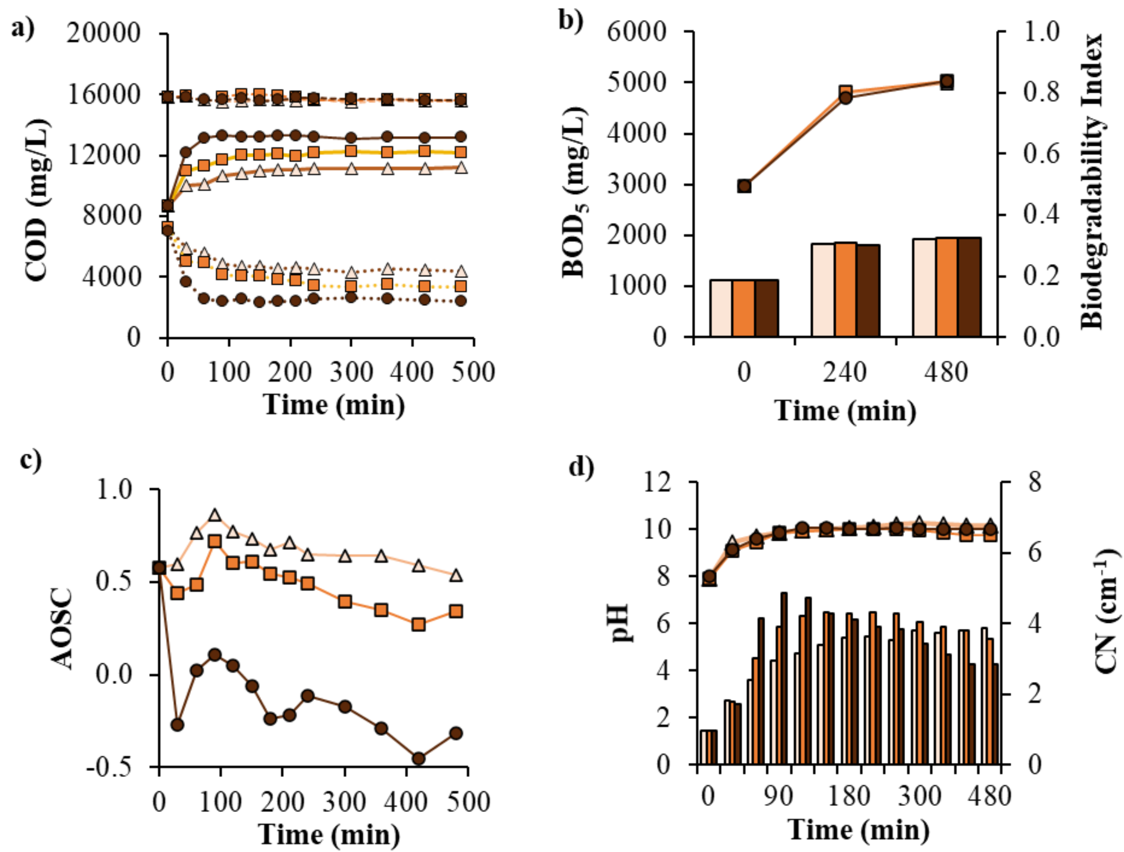


Figura 4. 9. *a) Changes of tCOD (dashed lines), sCOD (solid lines) and nsCOD (dotted lines) during hydrolysis treatment at 160 ºC (light colour and triangle markers), at 180 ºC (intermediate colour and square markers) and at 200 ºC (dark colour and circle markers).* ***b)*** *Changes of BOD_5 (markers) and biodegradability (columns).* ***c)*** *Changes of AOSC* ***d)*** *Changes of pH (markers) and CN (columns). The standard deviation (SD) of the experimental data were in all cases lower than 4% of the mean value.*

3.4 WAO treatment

When the ADL was treated by WAO, both solubility and degradation of pollutants were obtained. Fig. 4.10.A shows the changes of tCOD, sCOD and nsCOD during the experiments at different temperatures.

A fast drop in nsCOD occurred and after 200 min of treatment almost all the nsCOD was solubilised in all cases. As expected, the treatment at highest temperature (200 ºC) achieve the highest removals of tCOD, i.e, 65% and 71%, after 4 h and 8 h of treatment, respectively. For the treatments carried out at 180 ºC and 160 ºC removals

achieved were 57% and 34%, respectively. The tCOD degradation degree showed an increase directly proportional to the temperature used.

Baroutian et al. (2015), studied the treatment of an anaerobically digested municipal sludge and reported similar results during a treatment at 240 ºC and 2.0 MPa. They observed an initial drop of tCOD, that decrease at a slower rate afterwards, reaching removal efficacies above 60%. Chung et al. (2009), who investigated the treatment of sludge by wet oxidation, reported reductions of tCOD around 40% after 90 min of treatment at 200 ºC and pressures of 4.0-5.0 MPa.

Oulego et al. (2016), studied the effect of wet oxidation on a mixture of old and young leachate after nitrification-denitrification process. They reported a tCOD reduction around 51% after 100 min of treatment at 180 ºC and 6.0 MPa. With respect to sCOD, an increase during the initial hours of the treatment was observed for all temperatures studied, due to the solubilization of the solids present in the initial sample.

Results of biodegradability are shown in Fig. 4.10.B. The use of 160 ºC allowed an improvement in the biodegradability of the sample obtaining a moderately biodegradable liquor after 8 h of reaction (BOD_5 /COD ~ 0.38). It is important to highlight that the use of the highest temperature (200 ºC) resulted in a liquor with the high biodegradability index, only after 4 h of oxidation (BOD_5 / COD ~ 0.43).

The AOSC values showed an important variability over time (see Fig. 4.10.C). In all cases an initial decrease directly proportional to the temperature was obtained during the first 30 min of treatment due to the solubilization of nsCOD. After this time, the value was significantly increased. The values obtained for the final samples indicated that the treatment at 160 ºC and 180 ºC gave final samples with AOSC similar to the initial sample. The treatment at 200 ºC caused a decrease of around 30% in the AOSC value, like in the previous techniques. It was maybe consequence for the partially oxidation of the easily oxidizable organics compounds which were degraded into gaseous products. This transformation results in the enrichment of the ADL with other short chain carboxylic acid products, which is consequently reflected in lower AOSC values.

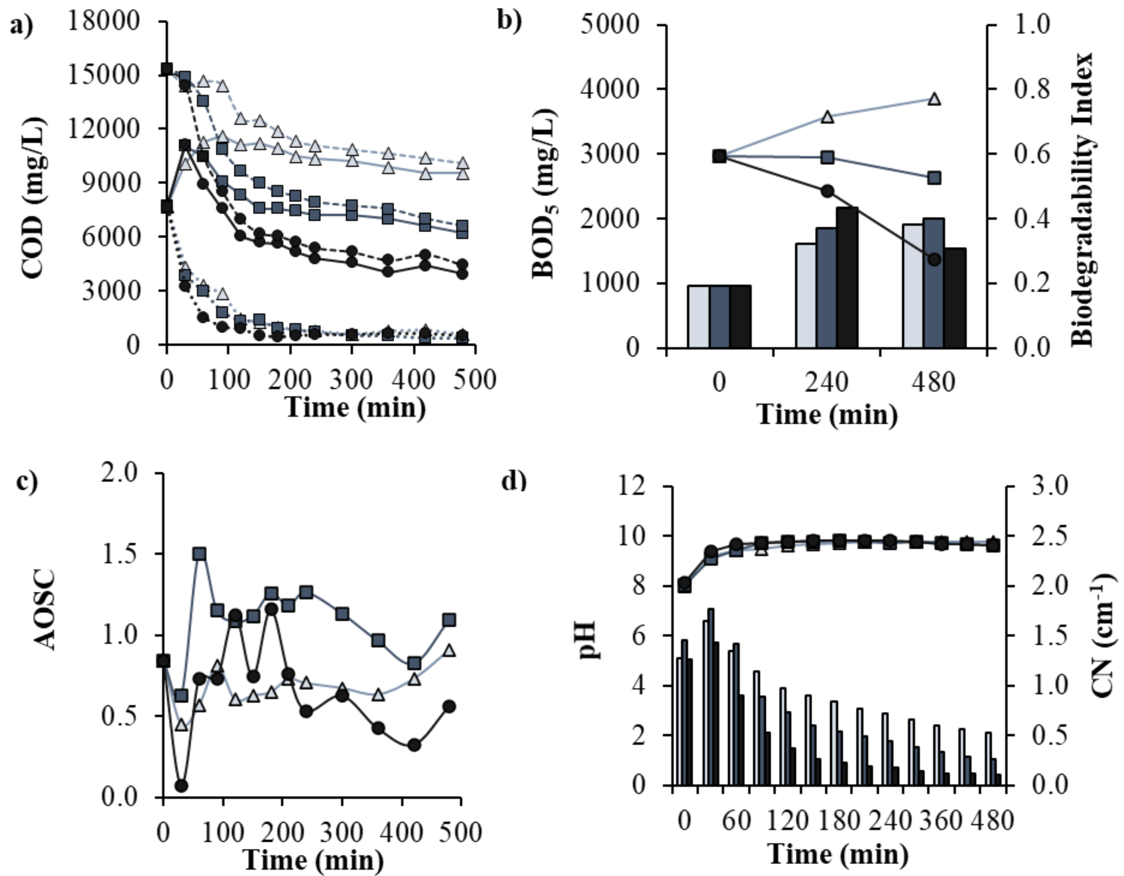


Figura 4. 10. *a) Changes of tCOD (dashed lines), sCOD (solid lines) and nsCOD (dotted lines) during wet air oxidation treatment at 160 ºC amplitude (light colour and triangle markers), at 180 ºC (intermediate colour and square markers) and at 200 ºC (dark colour and circle markers).* ***b)*** *Changes of BOD_5 (markers) and biodegradability (columns).* ***c)*** *Changes of AOSC.* ***d)*** *Changes of pH (markers) and CN (columns). The standard deviation (SD) of the experimental data were in all cases lower than 10% of mean value.*

The colour number was increased during the first 30 min, coinciding with the increase in sCOD and COT at this time. After 1 hour of reaction, the colour number, shown in Fig. 4.10.D, began to decrease until achieving a practically complete colour removal (91%) after 8 h of reaction. Finally, the data obtained after WAO treatment shown the increase in pH from values of 8 to almost 10. The increase in pH can be due to the degradation of the acids present in the sample. This fact could be considered as disadvantage for the subsequent treatment, however, the increase in the sample could be damped due to the mix of the ADL treated with young and mature leachates, which are added in greater proportion and present lower values of pH.

3.5 Comparison of the techniques in terms of the solubilization and degradation efficacies

The percentages of solubilization and degradation were calculated for each treatment at final time (Fig. 4.11.A and Fig. 4.11.B, respectively) and important differences were observed between them.

The percentages of nsCOD solubilization obtained with WAO were significantly higher than those obtained for the rest of the assayed treatments, reaching values around 90% for the three temperatures tested. When hydrolysis was used for the treatment, the solubilization degree showed an increase with temperature, reaching values of 39%, 52% and 68% for 160°C, 180°C and 200°C, respectively. It has been reported that the use of temperatures higher than 170°C in hydrolysis treatments gives high solubilizations of nsCOD, mainly due to the solubilization of the proteins present in samples (Donoso-Bravo et al., 2011). When strong operating conditions were used (200 ºC and 6.0 MPa), solubilizations around 65% were achieved after 4 h of reaction. This value was similar to that achieved with ozonation after 8 h.

The percentages of nsCOD solubilization obtained with WAO were significantly higher than those obtained for the rest of the assayed treatments, reaching values around 90% for the three temperatures tested. When hydrolysis was used for the treatment, the solubilization degree showed an increase with temperature, reaching values of 39%, 52% and 68% for 160°C, 180°C and 200°C, respectively. It has been reported that the use of temperatures higher than 170°C in hydrolysis treatments gives high solubilizations of nsCOD, mainly due to the solubilization of the proteins present in samples (Donoso-Bravo et al., 2011). When strong operating conditions were used (200 ºC and 6.0 MPa), solubilizations around 65% were achieved after 4 h of reaction. This value was similar to that achieved with ozonation after 8 h.

The use of ultrasounds with the highest amplitude (90%) gave a solubilization of 47% just after 45 min, whereas the values obtained for amplitudes of 50% at the same time was 33%. Oz and Yarimtepe, (2014), who studied the effect of sonication on leachates, reported solubilizations of 63% after 45 min at 20 kHz.

In relation to tCOD degradation, the highest percentage was achieved with WAO at 200 ºC, obtaining a final tCOD degradation of 71%, as shown in Fig. 4.11.B.

The use of a lower temperature (160 ºC) gave a tCOD degradation around 35%, similar to that obtained after the ozonation treatment (37%).

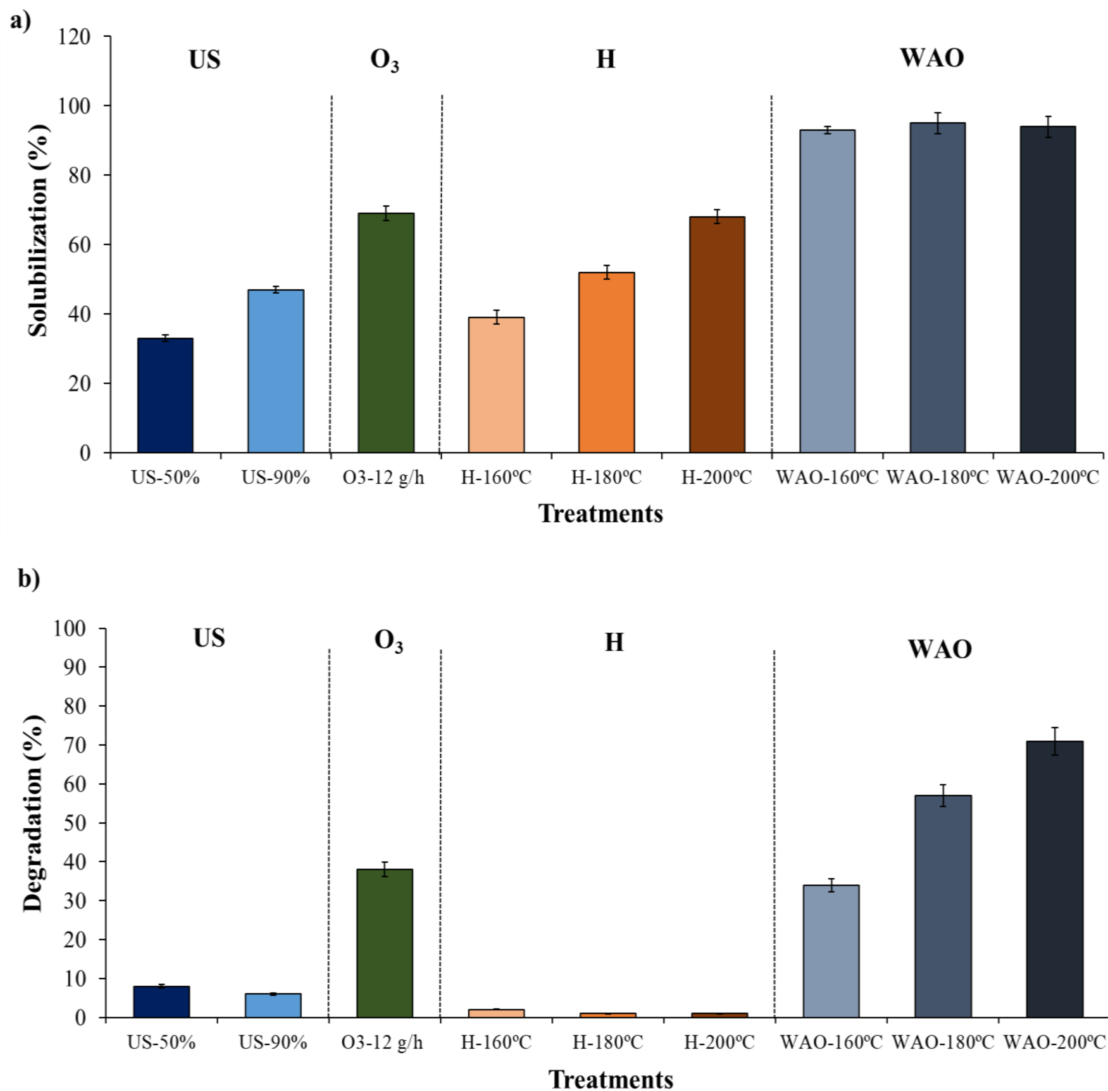


Figura 4. 11. *Results of solubilization degree **(a)** and tCOD degradation **(b)** for the different treatments. The segments in the upper part of the bars indicate the standard deviations.*

The use of any of the assayed treatments increased the biodegradability index and enabled to obtain an effluent moderately biodegradable, which is beneficial for a subsequent biological treatment such as nitrification-denitrification and activated sludge. In this sense, ozonation (8 h, 25 ºC, 12 g/h O_3) and WAO (8 h, 160-200 ºC and 6.0 MPa) were the most appropriate techniques, achieving a BOD_5/COD ratio around

0.4. In particular, after only 4 h of reaction, WAO gave a treated effluent with similar biodegradability and tCOD values to the mixture of young and mature leachates that are treated in a biological plant sited in the waste treatment centre that supply the ADL samples.

The tCOD degradations obtained using hydrolysis and ultrasound methods was low, i.e. lower than 8% in both cases. However, significant solubilizations were obtained, increasing the biodegradability index without decreasing the content in organic matter. The effluents so obtained could be interesting for their recirculation to the anaerobic digester. Microbiological hydrolysis of sewage sludge is the rate limiting step of anaerobic digestion, that could be enhance with the additional input of easily-biodegradable organic matter from solid solubilization. Hence, these techniques could also be useful to improve the biogas production and decrease the time to reach the maximum biogas volume (Rasapoor et al., 2019).

3.6 Kinetic model

The values of the kinetic constants and the apparent kinetic constants obtained by fitting the models (previously described in Material and Methods section) to the experimental data are shown in Table 4.3. The comparison between model and experimental data for each technique can be seen in Supplementary Material (Figures 5.2.S1-5.2.S4).

For ultrasound technique, the solubilization constants were higher than the degradation constants for both amplitudes. This fact corresponds with the low tCOD degradation observed in relation with the increase in sCOD. The use of the highest amplitude showed an increase in the value of the solubilization constant, although a decrease in the value of the degradation constant was observed.

Considering hydrolysis, it was observed an increase in the values of the solubilization constants with temperature. Xue et al., (2015), studied the effect of the thermal hydrolysis on organic matter solubilization in sludge. They reported that the use of this technique increased the sCOD concentration in the sludge. A direct increase of the solubilization with temperature was also reported.

Tabla 4. 3. *Kinetic constants calculated from experimental data*

Treatment	Conditions		k (min^{-1})		r^2
US	50% of amplitude	Eq (5)	k_{1U}	0.010	0.998
		Eq (6)	k_{2U}	0.004	0.997
	90% of amplitude	Eq (5)	k_{1U}	0.014	0.998
		Eq (6)	k_{2U}	0.002	0.997
O_3	With solids	Eq (7)	k_{1O}	0.016	0.998
		Eq (8)	k_{2O}	0.027	0.987
	Without solids	Eq (9)	K_{2O}	0.006	0.998
H	160 ºC. 6.0 MPa	Eq (10,11)	k_{1H}	0.014	0.997
	180 ºC. 6.0 MPa	Eq (10,11)	k_{1H}	0.018	0.998
	200 ºC. 6.0 MPa	Eq (10,11)	k_{1H}	0.045	0.995
WAO	160 ºC. 6.0 MPa	Eq (12)	k_{1W}	0.015	0.998
		Eq (13)	k_{2W}	0.013	0.991
	180 ºC. 6.0 MPa	Eq (12)	k_{1W}	0.019	0.995
		Eq (13)	k_{2W}	0.014	0.982
	200 ºC. 6.0 MPa	Eq (12)	k_{1W}	0.036	0.994
		Eq (13)	k_{2W}	0.014	0.995

For ozonation the degradation constant for centrifuged ADL was lower than that obtained for ADL with solids, indicating that the organic compounds that came from solids solubilization were more easily oxidizable than the compounds that were initially dissolved in the ADL. In relation with this, Bakhshi et al. (2018), who carried out a research on the use of ozonation as pre-treatment for sludge, reported that anaerobic digestion of ozonated municipal sludge showed better performance than conventional anaerobic digestion in terms of biosolids reduction and increasing biogas yield.

Regarding WAO method, the solubilization constants increased with temperature, whereas degradation constants remained almost constant. This is translated in the higher degradation of tCOD previously commented.

4. CONCLUSIONS

This work focuses on the pre-treatment of the liquid obtained after anaerobic digestion of sludge (ADL). This is an issue scarcely studied before, despite of the fact of being a real environmental problem due to the high content in non-biodegradable organic matter of ADL and the increasing number of anaerobic digestion plants worldwide.

The four pre-treatment methods here evaluated (ultrasounds, ozonisation, hydrolysis and WAO) were effective for the solubilization of nsCOD, reaching values above 90% for the best cases. Additionally, the application of these techniques allowed to improve considerably the biodegradability of ADL samples.

Besides, when ozonation (8 h, 25 ºC, 12 g/h O3) and WAO (8 h, 200 ºC and 6.0 MPa) were used, significant decolourization and tCOD removal were achieved (37% and 71% respectively) obtaining physico-chemical characteristics in ADL similar to those found in other municipal landfill leachates. These results could be interesting to subsequently processed ADL in an aerobic system, together with other leachates. In this sense, the treated ADL could be added to the process, without significant changes in the usual operating conditions. Other potential advantage would be the recirculation of part of the pre-treated ADL to the anaerobic reactor in order to improve the biogas production. These both possibilities have not been yet tested, so further investigation would be of interest.

The kinetic models proposed for each of the four techniques have successfully fitted the experimental data in the conditions tested. These models may be useful in order to estimate the best operating conditions for the treatment of ADL, depending on each particular composition and on the objectives of the treatment, i.e. improving its biodegradability or removing its COD content.

ACKNOWLEDGMENTS

This work was co-financed by COGERSA, S.A.U. (Project FUO-EM-138-15), IDEPA (Project IDE/2015/000245), Spanish MINECO, (Projects CTM2015-63864-R and MINECO-17-BES-2016-077963), Foundation for the Promotion of Applied Scientific Research and Technology in Asturias (Project FC-GRUPIN-IDI/2018/000127) and FEDER funds from European Union.

REFERENCES

[1] APHA, AWWA, WEF, 1999. Standard Methods for Examination of Water and Wastewater (twentieth ed.), American Public Health Association, Washington DC.

[2] Abid, M.F., Alwan, G.M., Abdul-Ridha, L.A., 2016. Study on Catalytic Wet Air Oxidation Process for Phenol Degradation in Synthetic Wastewater Using Trickle Bed Reactor. Arab. J. Sci. Eng. 41, 2659–2670. https://doi.org/10.1007/s13369-016-2171-x

[3] Bakhshi, Z., Jauffur, S., Frigon, D., 2018. Assessing energy benefits of operating anaerobic digesters at low temperature with solids pre-ozonation. Renew. Energy 115, 1303–1311. https://doi.org/10.1016/J.RENENE.2017.08.080

[4] Baroutian, S., Smit, A.-M.M., Andrews, J., Young, B., Gapes, D., 2015. Hydrothermal degradation of organic matter in municipal sludge using non-catalytic wet oxidation. Chem. Eng. J. 260, 846–854. https://doi.org/10.1016/J.CEJ.2014.09.063

[5] Carrère, H., Dumas, C., Battimelli, A., Batstone, D.J., Delgenès, J.P., Steyer, J.P., Ferrer, I., 2010. Pretreatment methods to improve sludge anaerobic degradability: A review. J. Hazard. Mater. 183, 1–15. https://doi.org/10.1016/J.JHAZMAT.2010.06.129

[6] Cesaro, A., Belgiorno, V., Siciliano, A., Guida, M., 2019. The sustainable recovery of the organic fraction of municipal solid waste by integrated ozonation and anaerobic digestion. Resour. Conserv. Recycl. 141, 390–397. https://doi.org/10.1016/J.RESCONREC.2018.10.034

[7] Chung, J., Lee, M., Ahn, J., Bae, W., Lee, Y.-W., Shim, H., 2009. Effects of operational conditions on sludge degradation and organic acids formation in low-critical wet air oxidation. J. Hazard. Mater. 162, 10–16.

https://doi.org/10.1016/J.JHAZMAT.2008.05.038

[8] Collado, S., Oulego, P., Suárez-Iglesias, O., Díaz, M., 2018. Biodegradation of dissolved humic substances by fungi. Appl. Microbiol. Biotechnol. 102, 3497–3511. https://doi.org/10.1007/s00253-018-8851-6

[9] Dewil, R., 2017. New perspectives for Advanced Oxidation Processes. J. Environ. Manage. 195, 93–99. https://doi.org/10.1016/J.JENVMAN.2017.04.010

[10] Díaz, A.I., Oulego, P., Collado, S., Laca, A., González, J.M., Díaz, M., 2018. Impact of anaerobic digestion and centrifugation/decanting processes in bacterial communities fractions. J. Biosci. Bioeng. 126. https://doi.org/10.1016/j.jbiosc.2018.05.024

[11] Díaz, A.I., Oulego, P., Laca, A., González, J.M., Díaz, M., 2019. Metagenomic Analysis of Bacterial Communities from a Nitrification–Denitrification Treatment of Landfill Leachates. Clean - Soil, Air, Water 47, 1–8. https://doi.org/10.1002/clen.201900156

[12] Donoso-Bravo, A., Pérez-Elvira, S., Aymerich, E., Fdz-Polanco, F., 2011. Assessment of the influence of thermal pre-treatment time on the macromolecular composition and anaerobic biodegradability of sewage sludge. Bioresour. Technol. 102, 660–666. https://doi.org/10.1016/J.BIORTECH.2010.08.035

[13] Garg, A., Mishra, A., 2010. Wet oxidation-an option for enhancing biodegradability of leachate derived from municipal solid waste (msw) landfill. Ind. Eng. Chem. Res. 49, 5575–5582. https://doi.org/10.1021/ie100003q

[14] Garoma, T., Pappaterra, D., 2018. An investigation of ultrasound effect on digestate solubilization and methane yield. Waste Manag. 71, 728–733. https://doi.org/10.1016/j.wasman.2017.03.021

[15] Gunes, B., Stokes, J., Davis, P., Connolly, C., Lawler, J., 2019. Pre-treatments to enhance biogas yield and quality from anaerobic digestion of whiskey distillery and brewery wastes: A review. Renew. Sustain. Energy Rev. 113, 109281. https://doi.org/10.1016/J.RSER.2019.109281

[16] Hii, K., Baroutian, S., Parthasarathy, R., Gapes, D.J., Eshtiaghi, N., 2014. A review of wet air oxidation and Thermal Hydrolysis technologies in sludge treatment, Bioresource Technology. Elsevier Ltd. https://doi.org/10.1016/j.biortech.2013.12.066

[17] Li, X., Guo, S., Peng, Y., He, Y., Wang, S., Li, L., Zhao, M., 2018. Anaerobic digestion using ultrasound as pretreatment approach: Changes in waste activated sludge, anaerobic digestion performances and digestive microbial populations. Biochem. Eng. J. 139, 139–145. https://doi.org/10.1016/J.BEJ.2017.11.009

[18] Miklos, D.B., Remy, C., Jekel, M., Linden, K.G., Drewes, J.E., Hübner, U., 2018. Evaluation of advanced oxidation processes for water and wastewater treatment – A critical review. Water Res. 139, 118–131. https://doi.org/10.1016/J.WATRES.2018.03.042

[19] Oulego, P., Collado, S., Laca, A., Díaz, M., 2016. Impact of leachate composition on the advanced oxidation treatment. Water Res. 88, 389–402. https://doi.org/10.1016/j.watres.2015.09.048

[20] Oulego, P., Collado, S., Laca, A., Díaz, M., 2015. Tertiary treatment of biologically pre-treated landfill leachates by non-catalytic wet oxidation. Chem. Eng. J. 273, 647–655. https://doi.org/10.1016/j.cej.2015.03.089

[21] Oz, N.A., Uzun, A.C., 2015. Ultrasound pretreatment for enhanced biogas production from olive mill wastewater. Ultrason. Sonochem. 22, 565–572. https://doi.org/10.1016/j.ultsonch.2014.04.018

[22] Oz, N.A., Yarimtepe, C.C., 2014. Ultrasound assisted biogas production from landfill leachate. Waste Manag. 34, 1165–1170. https://doi.org/10.1016/j.wasman.2014.03.003

[23] Passos, F., Astals, S., Ferrer, I., 2014. Anaerobic digestion of microalgal biomass after ultrasound pretreatment. Waste Manag. 34, 2098–2103.

[24] Peng, W., Pivato, A., 2019. Sustainable Management of Digestate from the Organic Fraction of Municipal Solid Waste and Food Waste Under the Concepts of Back to Earth Alternatives and Circular Economy. Waste and Biomass Valorization. https://doi.org/10.1007/s12649-017-0071-2

[25] Rasapoor, M., Adl, M., Baroutian, S., Iranshahi, Z., Pazouki, M., 2019. Energy performance evaluation of ultrasonic pretreatment of organic solid waste in a pilot-scale digester. Ultrason. Sonochem. 51, 517–525. https://doi.org/10.1016/j.ultsonch.2018.04.021

[26] Shabani, M., Fatehifar, E., Khoshfetrat, A.B., 2015. Contamination Removal of Landfill Leachate by using Ozone Treatment Contamination Removal of Landfill Leachate by using Ozone Treatment.

[27] Sivagami, K., Sakthivel, K.P., Nambi, I.M., 2018. Advanced oxidation processes

for the treatment of tannery wastewater. J. Environ. Chem. Eng. 6, 3656–3663. https://doi.org/10.1016/j.jece.2017.06.004

[28] Somers, M.H., Azman, S., Sigurnjak, I., Ghyselbrecht, K., Meers, E., Meesschaert, B., Appels, L., 2018. Effect of digestate disintegration on anaerobic digestion of organic waste. Bioresour. Technol. 268, 568–576. https://doi.org/10.1016/J.BIORTECH.2018.08.036

[29] Suárez-Iglesias, O., Urrea, J.L., Oulego, P., Collado, S., Díaz, M., 2017. Valuable compounds from sewage sludge by thermal hydrolysis and wet oxidation. A review. Sci. Total Environ. 584–585, 921–934. https://doi.org/10.1016/j.scitotenv.2017.01.140

[30] Svensson, K., Kjørlaug, O., Higgins, M.J., Linjordet, R., Horn, S.J., 2018. Post-anaerobic digestion thermal hydrolysis of sewage sludge and food waste: Effect on methane yields, dewaterability and solids reduction. Water Res. 132, 158–166.

[31] Tizaoui, C., Bouselmi, L., Mansouri, L., Ghrabi, A., 2007. Landfill leachate treatment with ozone and ozone/hydrogen peroxide systems. J. Hazard. Mater. 140, 316–324. https://doi.org/10.1016/J.JHAZMAT.2006.09.023

[32] Tyagi, V.K., Lo, S.-L., Appels, L., Dewil, R., 2014. Ultrasonic Treatment of Waste Sludge: A Review on Mechanisms and Applications. Crit. Rev. Environ. Sci. Technol. 44, 1220–1288. https://doi.org/10.1080/10643389.2013.763587

[33] Urrea, J.L., Collado, S., Laca, A., Díaz, M., 2015. Rheological behaviour of activated sludge treated by thermal hydrolysis. J. Water Process Eng. 5, 153–159. https://doi.org/10.1016/j.jwpe.2014.06.009

[34] Urrea, J.L., Collado, S., Oulego, P., Díaz, M., 2017. Wet oxidation of the structural sludge fractions. J. Clean. Prod. 168, 1163–1170. https://doi.org/10.1016/j.jclepro.2017.09.033

[35] Vogel, F., Harf, J., Hug, A., von Rohr, P.R., De, F.Â., Vogel, R., Harf, J., Hug, A., Rudolf, P., Rohr, V., 2000. The mean oxidation number of carbon (MOC) - A useful concept for describing oxidation processes, Water Research. Pergamon. https://doi.org/10.1016/S0043-1354(00)00029-4

[36] Xu, Y., Lu, Y., Zheng, L., Wang, Z., Dai, X., 2019. Perspective on enhancing the anaerobic digestion of waste activated sludge. J. Hazard. Mater. 121847. https://doi.org/10.1016/J.JHAZMAT.2019.121847

[37] Xue, Y., Liu, H., Chen, S., Dichtl, N., Dai, X., Li, N., 2015. Effects of thermal

hydrolysis on organic matter solubilization and anaerobic digestion of high solid sludge. Chem. Eng. J. 264, 174–180. https://doi.org/10.1016/j.cej.2014.11.005

[38] Yang, D., Hu, C., Dai, L., Liu, Z., Dong, B., Dai, X., 2019. Post-thermal hydrolysis and centrate recirculation for enhancing anaerobic digestion of sewage sludge. Waste Manag. 92, 39–48. https://doi.org/10.1016/j.wasman.2019.04.044

[39] Zeng, Y., De Guardia, A., Dabert, P., 2016. Improving composting as a post-treatment of anaerobic digestate. Bioresour. Technol. 201, 293–303. https://doi.org/10.1016/j.biortech.2015.11.013

[40] Zhen, G., Lu, X., Kato, H., Zhao, Y., Li, Y.-Y.Y., 2017. Overview of pretreatment strategies for enhancing sewage sludge disintegration and subsequent anaerobic digestion: Current advances, full-scale application and future perspectives, Renewable and Sustainable Energy Reviews.

[41] Zou, S., Wang, X., Chen, Y., Wan, H., Feng, Y., 2016. Enhancement of biogas production in anaerobic co-digestion by ultrasonic pretreatment. Energy Convers. Manag. 112, 226–235. https://doi.org/10.1016/j.enconman.2015.12.087

4.1.3. Fungal treatment of an effluent from sewage sludge digestion to remove recalcitrant organic matter

Ana Isabel Díaz[1], Adriana Laca[1*], and Mario Díaz[1]

[1]Department of Chemical and Environmental Engineering. University of Oviedo. C/Julián Clavería, s/n, E-33006, Oviedo, Asturias, Spain.

[*]E-mail (*corresponding author*): lacaadriana@uniovi.es;

Phone: +34 985 10 29 74; Fax: +34 985 10 34 34

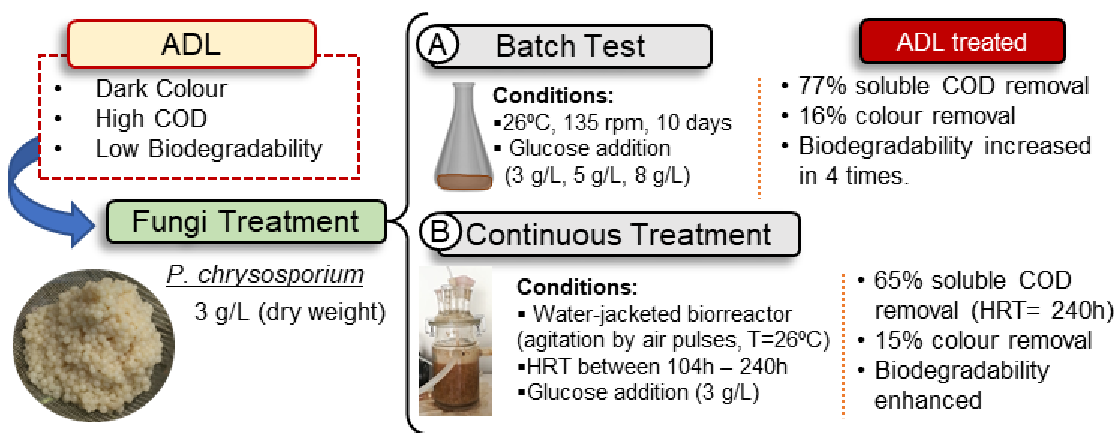


Figura 4. 12. *Resumen gráfico del trabajo 3*

*Artículo publicado en la revista "**Biochemical Engineering Journal**"

Cita: Ana Isabel Díaz, Adriana Laca, and Mario Díaz, *Fungal Treatment of an effluent from sewage sludge digestion to remove recalcitrant organic matter*, Biochemical Engineering Journal, Volume 172, **2021**, ISSN 1369-703X,

https://doi.org/10.1016/j.bej.2021.108056

ABSTRACT

In this study, *Phanerochaete chrysosporium* was tested for first time to treat the liquid effluent from a sewage sludge digestion. This anaerobic digestion liquor (ADL) has a COD concentration of around 7500 mg/L, a very low biodegradability (BOD_5/COD=0.02) and deep colour, being impossible their effective treatment by conventional biological methods. Assays inoculating the fungi were carried out at 26° C during 10 days in a batch reactor, following the evolution of colour, COD and BOD_5. The effect of adding different concentrations of glucose was evaluated, resulting to be a key factor for increasing the efficacy of the treatment. Based on batch results, a continuous fungal water-jacketed bioreactor was successfully started up. COD removals above 65% were achieved both in batch and continuous operations. These results show the possibilities of this sustainable and economic approach to remove refractory COD in the low-biodegradable effluents derived from anaerobic digestion of sludge or other complex wastes.

Keywords: fungi; biotreatment; COD; degradation; digestate

Abbreviations: **ADL**, anaerobic digestion liquor; **COD**, chemical oxygen demand; **BOD_5**, 5-day biological oxygen demand; **CN**, colour number; **WRF**, white-rot fungi; **LiP**, lignin peroxidase; **MnP**, manganese peroxidase; **Lac**, laccase; **SAC**, spectral absorbance coefficients; **HRT**, hydraulic retention time.

1. INTRODUCTION

Anaerobic digestion is an extensively method used to treat sewage sludge. After the biomethanization step, the digestate is normally subjected to a centrifugation process, obtaining a solid and a liquid phase as final products. The agronomical valorisation of the solid fraction has been widely studied, and normally consists of mixing it with vegetable remains in order to obtain a marketable biofertilizer by composting [1,2].

The management of the liquid phase, commonly named anaerobic digestion liquor (ADL), is more difficult. This fraction is characterized for containing high concentrations of organic matter and nitrogen and dark colour. Although an important fraction of ADL is usually recirculated to the digester during the anaerobic digestion process, which increases methane production, around 45% of the ADL generated is not recirculated and needs to be stored and treated [3,4]. Its discharge without treatment may cause eutrophication of nearby water and chemical, biological, or physical soil contamination [5,6]. As this effluent comes from a previous biological treatment, its organic matter content, measure as chemical oxygen demand (COD), is mainly recalcitrant material that has not been degraded during the anaerobic digestion [4,7] and cannot be removed during conventional biological treatments.

Different physico-chemical techniques, such as ozonation and wet air oxidation, have been studied to treat the ADL with good results in terms of colour and COD removal [8]. Other methods have focused on concentring nitrogen and phosphorus to use ADL as fertilize, i.e. evaporation, stripping and membrane technologies. Nevertheless, these physico-chemical treatments usually imply higher operational cost, in terms of energy requirements, than biological treatment methods [9–11].

In solid waste management facilities, the treatment of ADL together with landfill leachates by nitrification-denitrification processes is a common practice. During this treatment, diverse microbial communities are used to remove nitrogen, biodegradable organic matter and hazardous substances [12–14]. However, the degradation of humic substances and other recalcitrant organics contained in the ADL cannot be efficiently carried out by bacteria [15,16]. The addition of an external carbon source, as methanol, is frequently necessary to achieve a suitable denitrification and, at the same time, most

part of the recalcitrant COD from ADL remains in the effluent after the biological treatment. So, to avoid the final discharge of these contaminants, it is necessary to complement the conventional biological treatment with other that allows the effective removal of recalcitrant organic matter [17].

The use of the white-rot fungi (WRF) has been considered a hopeful alternative to treat complex effluents with low biodegradability in an economical, sustainable and eco-friendly way [18,19]. Fungi have greater resistance to inhibitory compounds than bacterial species and they can survive in adverse conditions such as low pH, low temperature and low nitrogen content [20,21]. WRF can synthesise extracellular oxidative enzymes such as lignin peroxidase (LiP), manganese peroxidase (MnP) and laccase (Lac), which take part in the degradation of recalcitrant compounds. Due to the extracellular enzyme system, it is not observed substrate diffusion limitations, generally encountered in bacteria [22].

Several works about the use of WRF for the treatment of landfill leachates, dairy wastewaters and effluents from textile and pharmaceutical industry have been published with good results [23,24]. Specifically, Yang et al. [25] obtained decolourization efficiencies around 70-80% after the treatment of textile manufacturing effluent by a fungi consortium reactor, Djelal and Amrane [26] showed that the addition of fungi consortium improved COD degradation until removal values about 75% on a synthetic dairy wastewater, Dalecka et al. [27] achieved an almost complete removal of the diclofenac present in synthetic municipal wastewaters by using the fungus *Trametes versicolor,* and Kalĉíková et al. [28] reported COD removals around 60% when mature landfill leachates were treated by *Dichomitus squalens*. In particular, the treatment with *Phanerochaete chrysosporium* has been considered as one promising method in bioremediation, due to its ability to degrade many chemical pollutants, both in soil and in liquid culture, achieving high colour, metals, polycyclic aromatic hydrocarbons (PAHs) and total organic carbon (TOC) removal efficiencies [29,30]. Based on these previous studies, the use of this fungus to treat liquid effluents coming from anaerobic digestion seems to be a possible alternative that deserves being investigated.

Regarding to the application of fungi in anaerobic digestion processes, the literature has been mainly focused on its use to pre-treat sewage sludge and solid digestate in order to increase biogas production or to improve the subsequent

composting process [31,32]. Besides, most of the researchers have used synthetic effluents or sterilized samples to investigate the fungi potential and it is well-known that the presence of bacteria may cause a competition for the substrate, reducing fungi growth, the expression of fungal enzymes and consequently the fungal degradative capacity [33].

As far as we know, the use of *P.chrysosporium* to treat ADL has not been previously investigated. Considering the behaviour of this fungus with other complex effluents and the necessity of new alternatives to treat low-biodegradable effluents from anaerobic digestion treatments, the aim of this research was to evaluate in batch and continuous operation the capacity of this fungus to remove recalcitrant organic matter, colour and/or enhance the biodegradability of a non-sterile ADL.

2. MATERIAL AND METHODS

2.1. Sample description

The anaerobic digestion liquor (ADL) corresponds with the liquid phase of the digestate obtained after a digestion of sewage sludge. The sample was collected from an anaerobic digestion treatment plant sited in Asturias. A detail description of the process can be seen in Díaz et al. [4]. For the fungal treatment, the sample was centrifuged for 20 min at 10000g and filtered by 0.45 μm filter (Millipore) in order to remove the solids presents in ADL.

2.2. Fungus and pellets obtention

A basidiomycete white-rot fungus, *Phanerochaete chrysosporium* Burdsall 1974 from Spanish Type Culture Collection (CECT) was used in this study. The recovery of freeze-dried strain was carried out in a laminar air flow cabinet to prevent the contamination. Firstly, the freeze-dried strain CECT 2798 was resuspended, avoiding air bubbles, on 0.3 mL of malt extract broth (VWR Chemicals BDH), previously sterilized at 121ºC for 20 min. Then, 100 μL of the suspension were used to inoculate an agar Petri plate with 25 mL of 1.5% malt extract agar (MEA), which was incubated at 26ºC for 7 days. Before using the fungus for pellets obtention, three subcultures were carried out. To preserve the fungus on Petri plates with MEA, subcultures were routinely made every month.

To obtain the fungus pellets, the methodology described by Blánquez et al. [34], undergoing some modifications, was used as next explained. Five cylinders of 1 cm of diameter from the growing zone of Petri plates were inoculated in a 500 mL Erlenmeyer flask containing 150 mL of malt extract broth, previously sterilized at 115ºC for 10 minutes. The flask was incubated at 26ºC with a constant orbital shaking at 135 rpm for 6 days to obtain the mycelial mass.

The mycelial mass obtained was separated from the liquid medium with a 1 mm mesh sieve, resuspended in 0.8% NaCl (w/v) in a ratio of 1:3 (w/v) and blended at 11000 rpm for 5 minutes with a homogenizer (Heidolph Silent Crusher). This suspension was used to obtain the pellets by inoculating 600 µL in a 1 L Erlenmeyer flask with 250 mL of malt extract broth, previously sterilized at 115ºC for 10 minutes and with a pH between 4.5 and 5. It was incubated at 26ºC with a constant orbital shaking at 135 rpm for 6 days. The pellets obtained was removed with a sieve and conserved in 0.8% NaCl (w/v) solution at 4ºC until use.

2.3. Fungus discontinuous treatment

A total of seven batch experiments were carried out to evaluate the potential of fungus to degrade ADL. Firstly, according to Hu et al. [35] the sample was adjusted to pH=6.0 with 1 M NaOH or 1 M HCl. Then, the ADL was mixed with phosphate buffer (0.1 M, pH=6) in a ratio of 3:1 (v/v) in order to maintain pH constant during the assays. The characterization of the diluted ADL is shown in Table 4.4.

To carry out the biological treatment, the sample was inoculated with 3 g/L (dry weight) of *P. chrysosporium*, corresponding with the test **S1.** With the aim of evaluating the effect of fungus addition in relation with the endogenous microflora, an experiment without fungus addition was assayed and used as control 1 (**C1**).

To evaluate the effect of glucose as co-substrate, the sample was inoculated with 3 g/L (dry weight) of fungus and different glucose concentrations (3 g/L, 5 g/L and 8 g/L), corresponding with the assays **S2**, **S3**, and **S4**, respectively. A test with the intermediate glucose concentration (5 g/L) and without fungus inoculum was used as control 2 (**C2**). Additionally, to know the effect of endogenous microflora in the treatment, an experiment using sterilized effluent (121ºC for 20 min) with the addition of 5 g/L of glucose and inoculated with fungus was also carried out (**C3**).

***Tabla 4. 4.** Characteristics of anaerobic digestion liquor (after dilution with buffer)*

Parameter	Value
pH	6,05 ± 0,02
sCOD (mg O_2/L)	5589 ± 52
BOD_5 (mg O_2/L)	109 ± 1,4
Biodegradability (IB)	0,02 ± 0,005
Nitrogen (mg/L)	2327 ± 146
Phosphorus (mg/L)	71 ± 57
Colour Number (CN)	0,984 ± 0,01

All the batch experiments were carried out in a 1 L Erlenmeyer flasks with 200 mL of non-sterile sample incubated at 26ºC and with a constant orbital shaking at 135 rpm for 10 days. All the experiments were carried out in duplicate. Periodically, samples were taken for the analysis of soluble COD, colour, pH and BOD_5.

2.4. Fungus continuous treatment

The start-up of a continuous fungal bioreactor to treat the ADL was carried out and different operating conditions were evaluated. The continuous experiments were carried out in a 1.5 L capacity water-jacketed bioreactor loaded with 1.25 L of ADL inoculated with 3 g/L (dry matter) of *P. chrysosporium.* In view of batch results, 3 g/L of glucose were added to the ADL before being fed into the reactor. In this case, the pH was kept between 5.6 and 6.5 by adding NaOH 0.5M or HCl 0.5M using a pH-burette (CRISON, PH-BURETTE 24 1S). Temperature was set up at 26ºC and fungus pellets was maintained fluidized by air pulses generated by an electrovalve. The electrovalve was controlled by a cyclic timer (60 seconds open, 30 seconds close) with an air flow of 36 L/h. Fine-bubble diffuser stones was used to assure homogeneity and avoid biomass mechanical stress. A 1 mm mesh sieve was placed at the outlet of the reactor to avoid the loss of fungus pellets. At the end of each experiment, the reactor was emptied and refilled with new ADL inoculated with the fungus. Three different hydraulic retention time (HRT) were tested (104, 208 and 240 hours). The analysis of soluble COD, colour, pH and BOD_5 was carried out in outcoming samples taken at different times.

2.5. Analytical methods

Samples was taken periodically each 24 hours, centrifuged at 10000g during 10 min, filtered by 0.45 µm filter (Millipore) and stored at -20ºC until being analysed. All analytical measurements were done at least in triplicate.

The concentration of soluble COD was spectrophotometrically measured (at 600 nm) by dichromate method according to Standard Methods [36], using a DR2500 spectrophotometer (Hach Company).

Biochemical oxygen demand (BOD_5) was determined using a manometric respirometry measurement system (Lovibond® Water Testing BD 600) and biodegradability was calculated as the ratio of BOD_5 over soluble COD.

The change in the colour of the ADL was determined by means of the colour number (CN), which is defined according to Equation (1) [37].

$$CN=\frac{SAC_{436}^2 + SAC_{525}^2 + SAC_{620}^2}{SAC_{436} + SAC_{525} + SAC_{620}} \qquad \text{Eq (1)}$$

Spectral absorbance coefficients (SAC) are defined as the ratio of the values of the respective absorbance (Abs) over the cell thickness (x). The absorbances (Abs) were measured at 436, 525 and 620 nm using a UV/vis spectrophotometer (Thermo Scientific, Heλios γ). Finally, the value of pH was measured by means of a pH-meter (Jenway 3510).

2.6. Calculation section

For batch tests (S1, S2, S3 and S4), a first order kinetic model was used to fit the data of soluble COD removal by fungus, so the Equation (2) was used. The parameter R is the soluble COD at final times and *k* is the kinetic constant.

$$\frac{dCOD}{dt} = -k * (COD - R) \qquad \text{Eq (2)}$$

Regarding continuous treatment, assuming a completely mixed continuous flow system, the kinetic constants (k_{104}, k_{208}, and k_{240}) for the soluble COD removals in steady state were calculated according to Equation (3). In this equation, the parameter

HRT is the hydraulic retention time, and COD_i and COD_o are the incoming and outcoming soluble COD concentrations for each case.

$$k = \frac{\mathrm{COD_i} - \mathrm{COD}_o}{\mathrm{HRT} * \mathrm{COD}_o} \quad \text{Eq (3)}$$

3. RESULTS AND DISCUSSION

3.1. Fungal biotreatment of ADL

Several batch experiments were carried out to know the efficacy of using *P. chrysosporium* for the biotreatment of non-sterilized ADL. Experiments without fungi inoculation, C1 without glucose addition and C2 with 5 g/L of glucose, were used as control tests. The experiments S1 (without glucose addition), S2 (with 3 g/L of glucose), S3 (with 5 g/L of glucose) and S4 (with 8 g/L of glucose) were carried out inoculating 3 g/L (dry matter) of fungus. Additionally, an experiment with sterilized ADL inoculated with fungus and with 5 g/L of glucose addition (C3) was carried out to know the role played by the endogenous microflora. The evolution of soluble COD concentrations for all the experiments is shown in Fig. 4.13

Results showed that the inoculation of *P. chrysosporium* significantly improved the removal of soluble COD. The sample treated with the fungus without adding glucose (S1) showed soluble COD removals around 20% at final time, whereas a value of 11% was obtained for the non-inoculated sample without glucose used as control (C1). In both, S1 and C1, pH increased slightly until values around 6.4 and COD decreased progressively throughout the treatment. Similar low removal values have been reported when fungi were used to treat poorly biodegradable effluents without adding external nutrient sources [38].

Higher degradation values have been reported when crude fungal enzymes were directly used. For example, Wan Razarinah et al. [39], who investigated the treatment of landfill leachates by immobilized *Trametes menziesii*, reported a COD removal of 32% after 7 days. Hu et al. [29], reported TOC removal efficiencies around 75% after 72 hours of treating landfill leachates by immobilized *P. chrysosporium.* In addition, a degradation of around 42% was reported when non-sterile textile effluents were treated by fungus immobilized and Tween80 (0.05 w/v) was added [40]. In the present work,

the degradation observed in C1 and part of the degradation in S1 is due to the activity of the endogenous microorganisms of the ADL. This effluent is characterised by containing a high microbial load, mainly bacteria belonging to *Firmicutes* and *Proteobacteria* phyla [4]. Microorganisms within these phyla are known to have antagonistic effects on fungal growth and they are used in the control of fungal diseases in plants [41].

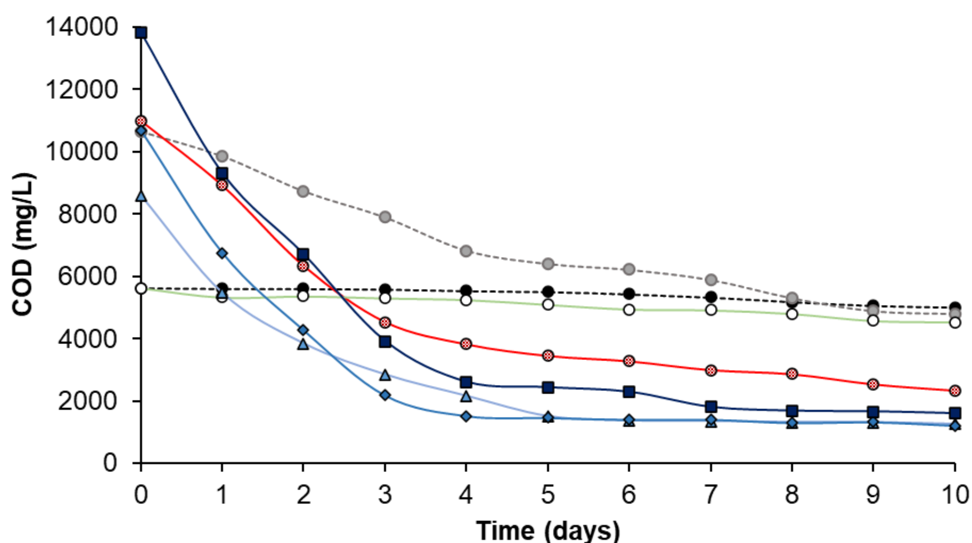


Figura 4. 13. *Changes in COD concentration during batch test for the different samples. The dashed lines shown the non-inoculated tests* ***C1*** *(●) and* ***C2*** *(●), used as controls. The solid lines shown the inoculated tests* ***C3*** *(●),* ***S1*** *(○),* ***S2*** *(▲),* ***S3*** *(◆) and* ***S4*** *(■). A concentration of 3 g/L, 5 g/L and 8 g/L of glucose was added to S2, S3 and S4, respectively. The tests C2 and C3 were supplied with 5 g/L of glucose. In addition, the experiment C3 was carried out with sterile sample while C1, C2, S1, S2, S3 and S4 were carried out with non-sterile sample. The standard deviation (SD) of the experimental data were in all cases less than 9% of mean value.*

Previous studies have reported that the concentrations of nutrients, i.e. carbon, nitrogen and phosphorus, in the effluent play a key role for the production of fungal ligninolytic enzymes [42–44]. Specifically, for the treatment of industrial wastewaters by *P. chrysosporium*, the literature reported higher decolourization and COD degradation efficiencies when glucose was added [45]. Saetang and Babel [46], tested the effect of 0–3 g/L of glucose, corn starch or cassava on leachate degradation by immobilized white-rot fungi. These authors reported an optimum glucose concentration of 3 g/L obtaining a COD removal of 57% after 10 days of treatment with *Trametes*

versicolor. Nevertheless, a too high sugar concentration can result in a decline in LiP o MnP activity [47].

Therefore, in order to improve COD degradation, in the next experiments, glucose was added as external carbon source in the concentration of 3 g/L, 5 g/L and 8 g/L, which correspond with S2, S3 and S4, respectively. As a consequence of glucose addition, the initial soluble COD values increased to 8596 mg/L for S2, 10665 mg/L for S3 and 13846 mg/L for S4. As can be seen in Fig.4.13, the addition of 3 g/L and 5 g/L (S2 and S3 respectively) gave the best results, removing 73% of soluble COD after 5 days of treatment (the percentage was calculated with respect to the initial COD in the sample without glucose addition). In both cases, the COD reduction was progressive until the 4th or 5th day. The addition of a fermentable carbon source promoted the releasing of ligninolytic enzymes that enhanced the biodegradation of soluble COD. After the 5th day, COD remained almost constant, achieving COD removals around 78% after 10 days. Concerning S4 test, where 8 g/L of glucose was added, the degradation continued until the 7th day and then stabilized in a value slightly higher than that obtained with lower glucose concentrations (see Fig.4.13). In this case, only a 72% degradation was achieved after 10 days. In our case, the results show that, under the conditions tested, a dosage of 3 g/L is enough, being even disadvantageous using a glucose concentration higher than 5 g/L. In all cases, the pH showed a decrease during the first four days reaching values of 6.06, 5.76 and 5.72, respectively. This fact is surely related with glucose consumption during the initial phase of treatment. After this time, pH increased and remained almost constant with values around 6.5.

With respect to the availability of nitrogen and phosphorus, for aerobic biological treatments, a COD/N/P ratio around 100/5/1 is usually maintained to ensure the nutritional requirements for biomass growth [48,49]. The concentration of soluble COD, nitrogen and phosphorus in the ADL effluent after dilution with the buffer was 5589 mg/L, 2327 mg/L and 71 mg/L, respectively. So, the COD/N/P ratio of initial ADL was around 100/42/1, indicating that the effluent contained an excess of nitrogen and phosphorous enough. When glucose was added, the COD/N/P ratios were of 100/27/0.8 for 3 g/L (S2), 100/22/0.7 for 5 g/L (S3) and 100/17/0.5 for 8 g/L (S4). In these cases, nitrogen is still in excess, but phosphorus might be a little scarce, especially for the addition of 8 g/L of glucose. In addition, an excessive phosphorus and nitrogen

load has been related with a reduction of the fungal enzyme activity, especially for MnP [35,50].

Nevertheless, in this work, the high removals of soluble COD obtained in S2 and S3 indicated that fungal activity was not affected for the high concentration of nitrogen or the low concentration of phosphorous. This is not the case of S4, where the low availability of phosphorous might explain the lower efficacies obtained in this experiment.

When the results obtained in S3 are compared with those obtained in the test C2, carried out without fungus inoculation and with 5 g/L of glucose, it can be observed that without fungus the efficacy in COD degradation was significantly lower, achieving a removal only of 15% after 10 days of treatment. This fact revealed the role of the fungus to degrade the recalcitrant organic matter present in ADL. The fungal enzyme activities are related with COD removals and decolourization efficiencies with a greater influence of MnP in the decolourization processes and LiP enzyme in COD biodegradation [40].

In addition, to evaluate the impact of the endogenous microflora of the ADL in the organic matter degradation when the fungus is inoculated, the experiment C3, with sterile medium inoculated with fungus and supplied with 5 g/L of glucose, was carried out. In this case, the COD removal achieved at the end of the experiment was 59%. This value is quite lower than that achieved in S3, which indicates an important contribution of the endogenous microorganism in the COD degradation. This fact, together with the almost non-existent degradation in the control non-sterilized and supplemented with glucose without fungi inoculation (C2), revealed that endogenous microbiota only is able to degrade the organic matter when the fungi release its enzymes. Therefore, there is a synergistic effect between the fungus *Phanerochaete chrysosporium* and the microbiota present in the ADL. In this sense, Dhouib et al. [51] reported values around 65% of COD removal during the treatment of olive mill wastewater using white-rot fungi and activated sludge, whereas only 34% of COD removal was obtained after treatment with active sludge. The kinetic model proposed for COD removal in batch tests successfully fitted the experimental data in all the conditions tested. The values of *k* constants are shown in Table 4.5

Tabla 4. 5. *Kinetic constants calculated from experimental data obtained after batch tests*

Treatment	Glucose addition	k (d^{-1})	r^2
S1	-	0.170	0.97
S2	3 g/L	0.577	0.99
S3	5 g/L	0.622	0.97
S4	8 g/L	0.598	0.98

Regarding the effect of fungal treatment in the BOD_5, significant differences between the different experiments were not observed after 10 days of treatment. As can be seen in Fig. 4.14, for C1 and S1 (without glucose), BOD_5 remained almost constant during the 10 days. Control C2 began with a BOD_5 of 2865 mg/L due to the glucose addition and then, it showed, as expected, a progressive decrease in BOD_5 due to glucose consumption by the endogenous microbiota of the ADL. Likewise, tests S2, S3 and S4 showed a faster reduction in BOD_5 thanks to the fungus activity. In these experiments, the enzymes released by the fungus allowed the transformation of non-biodegradable organic matter into biodegradable one that was consumed simultaneously to its generation, as indicated by the fact that the BOD_5 values were low during all the treatment. Even though the final BOD_5 concentration was not influenced by the treatment, as in tests S2, S3 and S4, the COD decreased, the biodegradability (BOD_5/COD) was improved from an initial value of 0.02 to final values of 0.8, 0.9 and 0.7, respectively. The addition of 3-5 g/L of glucose gave the best results also in relation with biodegradability.

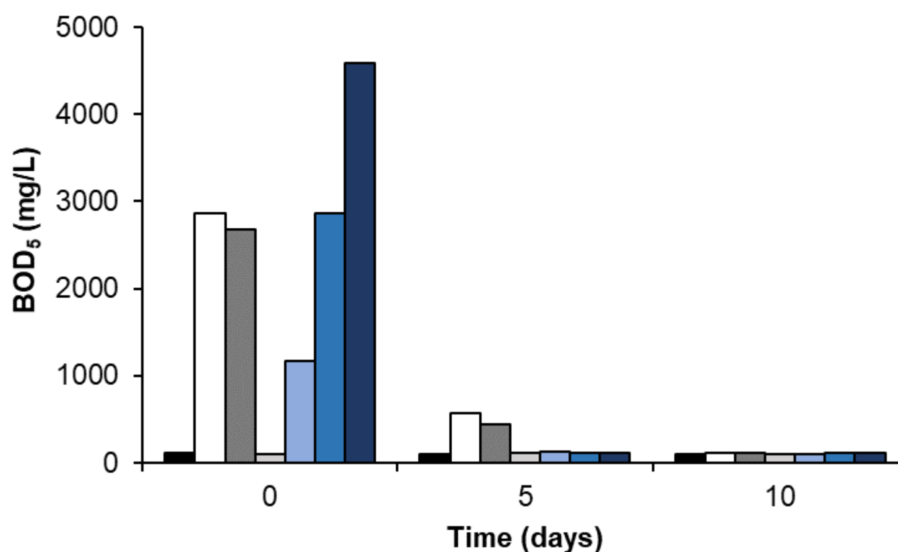


Figura 4. 14. *Data of BOD_5 values during batch test at initial time, 5 days, and 10 days for the non-inoculated tests* ***C1*** *(■) and* ***C2*** *(□), and inoculated tests* ***C3*** *(■),* ***S1*** *(■),* ***S2*** *(■),* ***S3*** *(■) and* ***S4*** *(■). A concentration of 3 g/L, 5 g/L and 8 g/L of glucose was added to S2, S3 and S4, respectively. The tests C2 and C3 were supplied with 5 g/L of glucose. In addition, the experiment C3 was carried out with sterile sample while C1, C2, S1, S2, S3 and S4 were carried out with non-sterile sample. The standard deviation (SD) of the experimental data were in all cases less than 6% of mean value.*

The changes in colour index (CN) for the treated samples are shown in Fig. 4.15. For the non-inoculated samples (C1 and C2), the colour index remained stable throughout the treatment, unlike samples inoculated with *P. chrysosporium*, that presented great variability. For sample S1, inoculated with the fungus but without glucose addition, no colour removals were obtained at the final times, compared to the initial sample. However, it should be noted that there was an initial decolourization that achieved a 22% colour removal on the third day of treatment. After that, the colour increased progressively until the end of the treatment, indicating that, despite of the fact that COD almost did not change, some transformations occurred during the process. The best results were obtained again with the addition of glucose, according to Pakshirajan and Kheria [45], who reported that the addition of 5 g/L of glucose led to an optimal decolourisation of 80%. So, for tests S2, S3 and S4, a rapid decrease of the colour number occurred during the initial phase of treatment, reaching on the fourth day of treatment the maximum values of removal, i.e. 29%, 22% and 23%, respectively. These results matched with the fast decrease observed in soluble COD during these first hours of treatment.

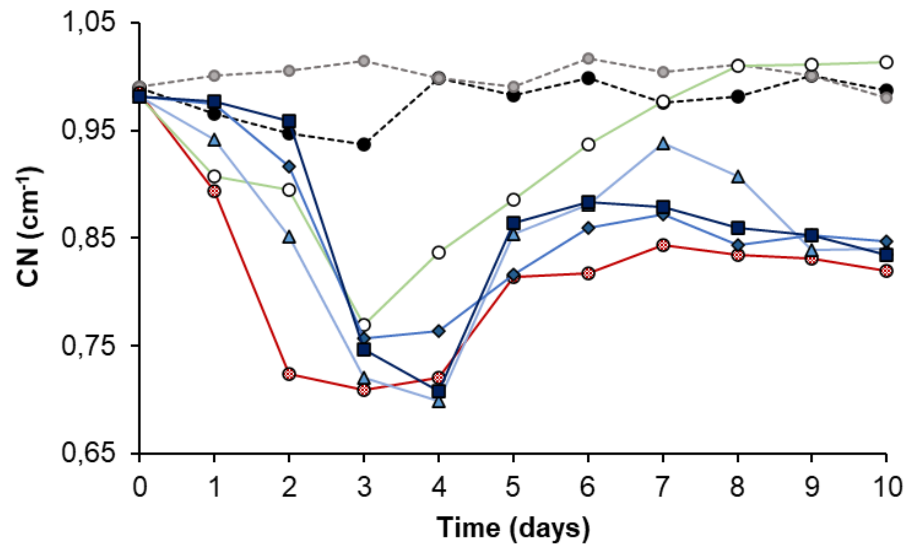


Figura 4. 15. *Changes in colour index (CN) during batch test for the different samples. The dashed lines shown the non-inoculated tests* ***C1*** *(●) and* ***C2*** *(●), used as controls. The solid lines shown the inoculated tests* ***C3*** *(●),* ***S1*** *( ),* ***S2*** *(▲),* ***S3*** *(◆) and* ***S4*** *(■). A concentration of 3 g/L, 5 g/L and 8 g/L of glucose was added to S2, S3 and S4, respectively. The tests C2 and C3 were supplied with 5 g/L of glucose. In addition, the experiment C3 was carried out with sterile sample while C1, C2, S1, S2, S3 and S4 were carried out with non-sterile sample. The standard deviation (SD) of the experimental data were in all cases less than 4% of mean value.*

According to the literature, this fact could be due to a higher fungal enzyme activity during the first days of treatment favoured by the presence of glucose. For times longer than 5 days, the colour increased again. This increase in colour may be due to the generation of coloured compounds during COD removal. Junnarkar et al. [52], reported a dark brown colour generation throughout the fermentation process of wheat straw by *P. chrysosporium*. This colour was related with the polyphenols compounds that were released from lignocellulosic material due to the degradative action of the fungal ligninolytic enzymes. With respect to C3 (with sterilized medium), the absence of endogenous microorganisms led to higher colour removals during the first 48 hours, although the final values were almost the same as those obtained for S2, S3 and S4 (around 15%).

Yang et al. [53] reported that MnP activities with 5 g/L of glucose were about 2–8 times those obtained with 10 g/L. This agrees with the fact that the colour removal was higher with an addition of 3 g/L of glucose than for higher initial concentrations of this sugar. Besides, Ellouze et al. [50], who studied the treatment of landfill leachates with *Trametes trogii* and *P. chrysosporium*, reported that the addition of 2 g/L of

ammonia showed a reduction of MnP and LiP activity around 60% and 50%, respectively. As above commented, the wastewater treated in this work contained a high concentration of nitrogenous, which could affect the production MnP, related with decolourization processes, and explain that the efficiencies for colour removal here achieved were not as high as those reported for another industrial wastewater.

3.2. Start-up of a continuous bioreactor.

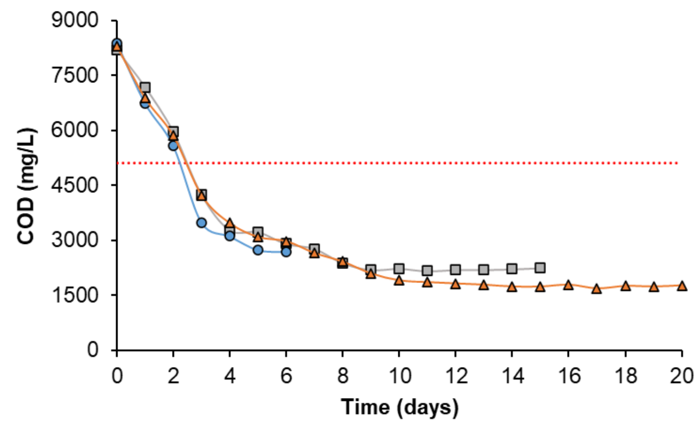
The operational conditions were selected based on the results obtained from batch tests. Because similar values of COD, colour and biodegradability were obtained for S2 and S3 tests, the addition of 3 g/L of glucose was selected to carry out the continuous treatment.

Considering that a minimum time of around 100 h were necessary in batch experiments to achieve the maximum COD removal, different hydraulic retention times (HRT) between 104 and 240 hours were evaluated. The reactor was emptied and refilled with fresh fungus and ADL sample before each experiment with different HRT. The evolution of soluble COD, BOD_5 concentrations and colour index over time for each HRT is shown in Fig.4.16.

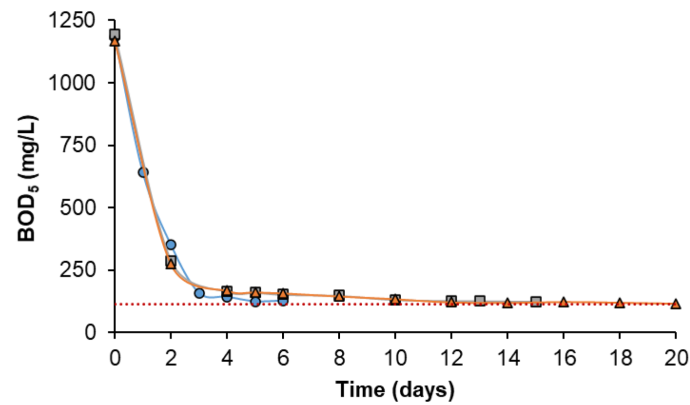
Regarding COD and BOD_5 concentrations (Fig. 4.16.A and 4.16.B), the continuous system reached the steady state in a time around the hydraulic retention time for each case. The reactor was considered to be operating at steady state when the variability of the soluble COD values obtained for the outgoing samples was less than 5%. The reactor operated stable after reaching the steady state at least for 10 days and morphological modifications were not observed in fungus pellets at the end of each experiment.

As can be seen in Fig 4.16.A, for soluble COD degradation, greater removals were observed when the highest HRT where used. The values obtained for the *k* constants were 0.463 d^{-1}, 0.304 d^{-1} and 0.362 d^{-1} for HRT values of 104 h, 208 h and 240 h, respectively. These values were in all cases lower than that obtained in batch experiments (0.577 d^{-1}).

a)



b)



c)

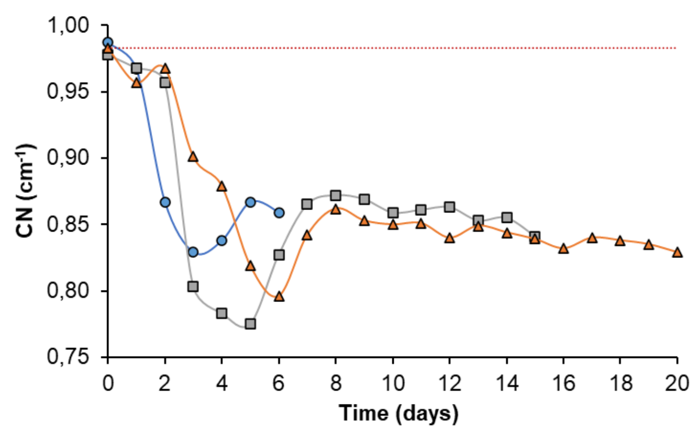


***Figura 4. 16**. Changes in **a)** COD, **b)** BOD_5 and **c)** colour index (CN) during the continuous treatments with different HRT values: 104 h (●), 208 h (■) and 240 h (▲). The red dashed line shown the value of each parameter for the ADL treated.*

In relation with colour index (CN), slight variations were observed when the system worked in steady state and colour removals around 15% were obtained for all cases (see Fig 4.16.C).

The percentage of soluble COD and colour removals, as well as the biodegradability index (COD/BOD_5) in steady state for each HRT are shown in Fig.4.17.

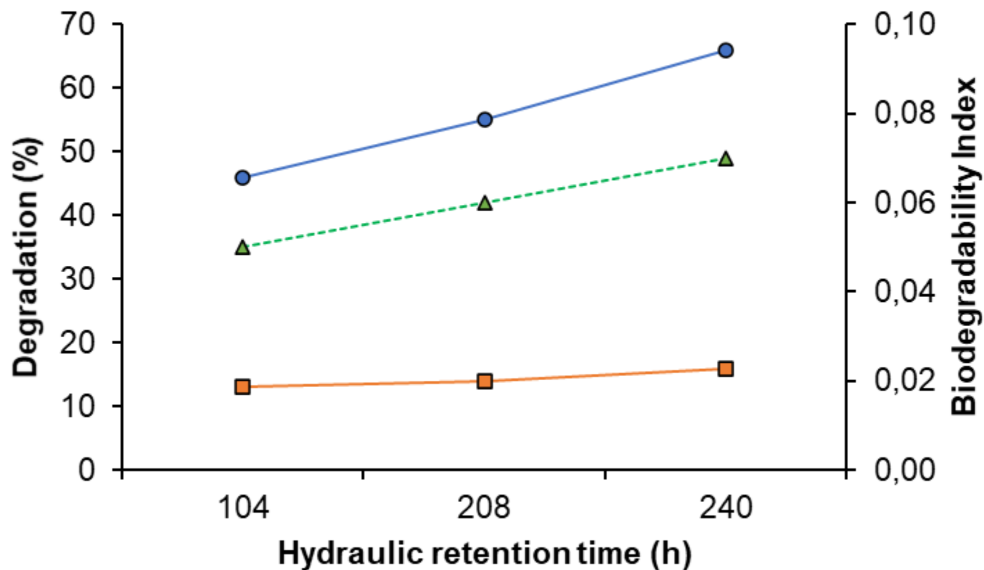


Figura 4. 17. Percentages of COD (●) and colour removal (■) during continuous treatment (steady state) for each HRT. The biodegradability (▲) is shown in the right axis.

The use of the shortest hydraulic retention time (104 hours) allowed to obtain a soluble COD removal of 45%, reaching final concentrations of 2889 mg/L. When HRT was increased, an improvement in the COD degradation was observed and the best results were achieved using a retention time of 240 hours. In this case, soluble COD removals greater than 65% were achieved, whereas removal percentage did not exceed of 55% for the experiment where 208 hours of HRT were used. However, even for the highest HTR, the COD removal obtained in continuous was lower than that obtained in batch treatment (S2), where a 77% of soluble COD removal was achieved (also indicated by the kinetic constants above commented). This fact could be due to the agitation system used during the treatment. For batch tests, the agitation was orbital while in the continuous system it was provided by gas bubbling. Espinosa-Ortiz et al.

[54], reported that the use of this system can generate dark areas in the reactor, with insufficient mixing, and an undefined fluid flow pattern inside the reactor. These disadvantages could explain that the efficiencies achieved in the continuous treatments were lower than those achieved in the batch experiments.

Other authors have been reported similar results. For example, Lu et al. [55] who studied the biodegradation of phenolic compounds from coking wastewater by white-rot fungus *P. chrysosporium,* reported removal values of 68% using free fungus. In addition, when the fungus was fixed onto wood chips, the efficiency was improved, achieving removal values around 81%. They attributed this difference to the fact that the fungus fixed on a surface increases its ability to synthesize enzymes.

Regarding the BOD_5 values, no significant differences were observed in the final effluent for the different HRT employed. However, the biodegradability of the treated effluent was enhanced due to the soluble COD removals, giving a treated effluent with a biodegradability index (BOD_5/COD) between 0.05 and 0.07, depending on the HRT employed, compared with a value of 0.02 for the untreated sample. Therefore, the highest biodegradability was obtained for the highest HRT assayed.

4. CONCLUSIONS

In this work, *Phanerochaete chrysosporium* was for first time employed for the treatment of non-sterile ADL. This effluent from the anaerobic treatment of sewage sludge contained a high concentration of low-biodegradable organic matter. The addition of glucose was key to enhance the removal of COD by the fungus. So, batch experiments without glucose achieved a COD removal of just 20%, whereas with 3 g/L of glucose the COD removal after 10 days of treatment was 77%. Additionally, colour removals of around 30% were achieved during the first days of treatment and, after 10 days, the biodegradability was four times greater than the initial one. Higher glucose concentrations did not improve the efficacy of the treatment. Using a glucose concentration of 3 g/L and HRT between 104 and 240 h, a continuous water-jacketed bioreactor was started-up for the fungi treatment and it operated stably for at least 10 days. A COD removal of 65% was achieved when the highest HRT was used, with an increase in biodegradability similar to that achieved in batch experiments. These results

showed the potential of this fungus to treat liquid effluents from anaerobic treatments although the operational conditions should be optimized in each particular case.

ACKNOWLEDGEMENTS

This work was co-financed by Spanish MINECO (Project CTM2015-63864-R) and Foundation for the Promotion of Applied Scientific Research and Technology in Asturias (Project FC-GRUPIN-IDI/2018/000127). Ana Isabel Díaz acknowledges an FPI grant from Spanish MICINN (BES-2016-077963). The authors particularly thank to COGERSA, S.A.U. for their support in the sample collection.

AUTHORSHIP STATEMENTS

Ana Isabel Díaz: Conceptualization, Methodology, Validation, Investigation, Formal analysis, Data curation, Writing - original draft, Visualization.

Adriana Laca: Conceptualization, Methodology, Validation, Formal analysis, Writing - review & editing, Supervision.

Mario Díaz: Conceptualization, Methodology, Funding acquisition, Project administration, Supervision, Writing - review & editing.

REFERENCES

[1] S. Wainaina, M.K. Awasthi, S. Sarsaiya, H. Chen, E. Singh, A. Kumar, B. Ravindran, S.K. Awasthi, T. Liu, Y. Duan, S. Kumar, Z. Zhang, M.J. Taherzadeh, Resource recovery and circular economy from organic solid waste using aerobic and anaerobic digestion technologies, Bioresour. Technol. 301 (2020) 122778. https://doi.org/10.1016/J.BIORTECH.2020.122778.

[2] Y. Zeng, A. De Guardia, P. Dabert, Improving composting as a post-treatment of anaerobic digestate, Bioresour. Technol. 201 (2016) 293–303. https://doi.org/10.1016/j.biortech.2015.11.013.

[3] Y.Q. Li, C.M. Liu, A.C. Wachemo, X.J. Li, Effects of liquid fraction of digestate recirculation on system performance and microbial community

structure during serial anaerobic digestion of completely stirred tank reactors for corn stover, Energy. 160 (2018) 309–317. https://doi.org/10.1016/j.energy.2018.06.082.

[4] A.I. Díaz, P. Oulego, S. Collado, A. Laca, J.M. González, M. Díaz, Impact of anaerobic digestion and centrifugation/decanting processes in bacterial communities fractions, J. Biosci. Bioeng. 126 (2018) 742–749. https://doi.org/10.1016/J.JBIOSC.2018.05.024.

[5] A. Xia, J.D. Murphy, Microalgal Cultivation in Treating Liquid Digestate from Biogas Systems, Trends Biotechnol. 34 (2016) 264–275. https://doi.org/10.1016/j.tibtech.2015.12.010.

[6] R. Nkoa, Agricultural benefits and environmental risks of soil fertilization with anaerobic digestates: A review, Agron. Sustain. Dev. 34 (2014) 473–492. https://doi.org/10.1007/s13593-013-0196-z.

[7] F. Tambone, V. Orzi, M. Zilio, F. Adani, Measuring the organic amendment properties of the liquid fraction of digestate, Waste Manag. 88 (2019) 21–27. https://doi.org/10.1016/j.wasman.2019.03.024.

[8] A.I. Díaz, P. Oulego, J.M. González, A. Laca, M. Díaz, Physico-chemical pre-treatments of anaerobic digestion liquor for aerobic treatment, J. Environ. Manage. 274 (2020) 111189. https://doi.org/https://doi.org/10.1016/j.jenvman.2020.111189.

[9] W. Tao, K.P. Fattah, M.P. Huchzermeier, Struvite recovery from anaerobically digested dairy manure: A review of application potential and hindrances, J. Environ. Manage. 169 (2016) 46–57. https://doi.org/10.1016/j.jenvman.2015.12.006.

[10] E. Tampio, S. Marttinen, J. Rintala, Liquid fertilizer products from anaerobic digestion of food waste: Mass, nutrient and energy balance of four digestate liquid treatment systems, J. Clean. Prod. 125 (2016) 22–32. https://doi.org/10.1016/j.jclepro.2016.03.127.

[11] H. Gong, Z. Yan, K.Q. Liang, Z.Y. Jin, K.J. Wang, Concentrating process of

liquid digestate by disk tube-reverse osmosis system, Desalination. 326 (2013) 30–36. https://doi.org/10.1016/j.desal.2013.07.010.

[12] S. Collado, P. Oulego, O. Suárez-Iglesias, M. Díaz, Leachates and natural organic matter. A review of their biotreatment using fungi, Waste Manag. 96 (2019) 108–120. https://doi.org/10.1016/J.WASMAN.2019.07.018.

[13] Z. Zhao, J. Luo, B. Jin, J. Zhang, B. Li, B. Ma, X. An, S. Zhang, B. Shan, Analysis of Bacterial Communities in Partial Nitritation and Conventional Nitrification Systems for Nitrogen Removal, Sci. Rep. 8 (2018). https://doi.org/10.1038/s41598-018-30532-4.

[14] A.I. Díaz, P. Oulego, A. Laca, J.M. González, M. Díaz, Metagenomic Analysis of Bacterial Communities from a Nitrification–Denitrification Treatment of Landfill Leachates, Clean - Soil, Air, Water. 47 (2019) 1–8. https://doi.org/10.1002/clen.201900156.

[15] J. Margot, L. Rossi, D.A. Barry, C. Holliger, A review of the fate of micropollutants in wastewater treatment plants, Wiley Interdiscip. Rev. Water. 2 (2015) 457–487. https://doi.org/10.1002/wat2.1090.

[16] A. Akhiar, A. Battimelli, M. Torrijos, H. Carrere, Comprehensive characterization of the liquid fraction of digestates from full-scale anaerobic co-digestion, Waste Manag. 59 (2017) 118–128. https://doi.org/10.1016/J.WASMAN.2016.11.005.

[17] L. Sheng, Z. Lei, M. Dzakpasu, Y.Y. Li, Q. Li, R. Chen, Application of the anammox-based process for nitrogen removal from anaerobic digestion effluent: A review of treatment performance, biochemical reactions, and impact factors, J. Water Process Eng. 38 (2020) 101595. https://doi.org/10.1016/j.jwpe.2020.101595.

[18] S. Collado, P. Oulego, O. Suárez-Iglesias, M. Díaz, Biodegradation of dissolved humic substances by fungi, Appl. Microbiol. Biotechnol. 102 (2018) 3497–3511. https://doi.org/10.1007/s00253-018-8851-6.

[19] J.A. Mir-Tutusaus, R. Baccar, G. Caminal, M. Sarrà, Can white-rot fungi be a

real wastewater treatment alternative for organic micropollutants removal? A review, Water Res. 138 (2018) 137–151. https://doi.org/10.1016/j.watres.2018.02.056.

[20] T.T. More, S. Yan, R.D. Tyagi, R.Y. Surampalli, Potential use of filamentous fungi for wastewater sludge treatment, Bioresour. Technol. 101 (2010) 7691–7700. https://doi.org/10.1016/j.biortech.2010.05.033.

[21] S. Sankaran, S.K. Khanal, N. Jasti, B. Jin, A.L. Pometto, J.H. Van Leeuwen, Use of filamentous fungi for wastewater treatment and production of high value fungal byproducts: A review, Crit. Rev. Environ. Sci. Technol. 40 (2010) 400–449. https://doi.org/10.1080/10643380802278943.

[22] A.F. Ariste, R.A. Batista-García, V.K. Vaidyanathan, N. Raman, V.K. Vaithyanathan, J.L. Folch-Mallol, S.A. Jackson, A.D.W. Dobson, H. Cabana, Mycoremediation of phenols and polycyclic aromatic hydrocarbons from a biorefinery wastewater and concomitant production of lignin modifying enzymes, J. Clean. Prod. 253 (2020) 119810. https://doi.org/10.1016/j.jclepro.2019.119810.

[23] M.B. Asif, F.I. Hai, L. Singh, W.E. Price, L.D. Nghiem, Degradation of Pharmaceuticals and Personal Care Products by White-Rot Fungi—a Critical Review, Curr. Pollut. Reports. 3 (2017) 88–103. https://doi.org/10.1007/s40726-017-0049-5.

[24] T. Kadri, T. Rouissi, S. Kaur Brar, M. Cledon, S. Sarma, M. Verma, Biodegradation of polycyclic aromatic hydrocarbons (PAHs) by fungal enzymes: A review, J. Environ. Sci. (China). 51 (2017) 52–74. https://doi.org/10.1016/j.jes.2016.08.023.

[25] Q. Yang, C. Li, H. Li, Y. Li, N. Yu, Degradation of synthetic reactive azo dyes and treatment of textile wastewater by a fungi consortium reactor, Biochem. Eng. J. 43 (2009) 225–230. https://doi.org/10.1016/j.bej.2008.10.002.

[26] H. Djelal, A. Amrane, Biodegradation by bioaugmentation of dairy wastewater by fungal consortium on a bioreactor lab-scale and on a pilot-scale, J. Environ. Sci. (China). 25 (2013) 1906–1912. https://doi.org/10.1016/S1001-

0742(12)60239-3.

[27] B. Dalecka, T. Juhna, G.K. Rajarao, Constructive use of filamentous fungi to remove pharmaceutical substances from wastewater, J. Water Process Eng. 33 (2020) 100992. https://doi.org/10.1016/j.jwpe.2019.100992.

[28] G. Kalčíková, J. Babič, A. Pavko, A.Ž. Gotvajn, Fungal and enzymatic treatment of mature municipal landfill leachate, Waste Manag. 34 (2014) 798–803. https://doi.org/10.1016/j.wasman.2013.12.017.

[29] L. Hu, Y. Liu, G. Zeng, G. Chen, J. Wan, Y. Zeng, L. Wang, H. Wu, P. Xu, C. Zhang, M. Cheng, T. Hu, Organic matters removal from landfill leachate by immobilized Phanerochaete chrysosporium loaded with graphitic carbon nitride under visible light irradiation, Chemosphere. 184 (2017) 1071–1079. https://doi.org/10.1016/j.chemosphere.2017.06.065.

[30] A. Hassan, A. Pariatamby, I.C. Ossai, F.S. Hamid, Bioaugmentation assisted mycoremediation of heavy metal and/metalloid landfill contaminated soil using consortia of filamentous fungi, Biochem. Eng. J. 157 (2020) 107550. https://doi.org/10.1016/j.bej.2020.107550.

[31] U. Brémond, A. Bertrandias, D. Loisel, J. Jimenez, J.P. Steyer, N. Bernet, H. Carrere, Assessment of fungal and thermo-alkaline post-treatments of solid digestate in a recirculation scheme to increase flexibility in feedstocks supply management of biogas plants, Renew. Energy. 149 (2020) 641–651. https://doi.org/10.1016/j.renene.2019.12.062.

[32] S. Voběrková, M.D. Vaverková, A. Burešová, D. Adamcová, M. Vršanská, J. Kynický, M. Brtnický, V. Adam, Effect of inoculation with white-rot fungi and fungal consortium on the composting efficiency of municipal solid waste, Waste Manag. 61 (2017) 157–164. https://doi.org/10.1016/j.wasman.2016.12.039.

[33] W. Tian, Q. Sun, D. Xu, Z. Zhang, D. Chen, C. Li, Q. Shen, B. Shen, Succession of bacterial communities during composting process as detected by 16S rRNA clone libraries analysis, Int. Biodeterior. Biodegrad. 78 (2013) 58–66. https://doi.org/10.1016/j.ibiod.2012.12.008.

[34] P. Blánquez, N. Casas, X. Font, X. Gabarrell, M. Sarrà, G. Caminal, T. Vicent, Mechanism of textile metal dye biotransformation by Trametes versicolor, Water Res. 38 (2004) 2166–2172. https://doi.org/10.1016/j.watres.2004.01.019.

[35] L. Hu, G. Zeng, G. Chen, H. Dong, Y. Liu, J. Wan, A. Chen, Z. Guo, M. Yan, H. Wu, Z. Yu, Treatment of landfill leachate using immobilized Phanerochaete chrysosporium loaded with nitrogen-doped TiO2 nanoparticles, J. Hazard. Mater. 301 (2016) 106–118. https://doi.org/10.1016/J.JHAZMAT.2015.08.060.

[36] APHA; AWWA; WEF, APHA Standard Methods for Examination of Water and Wastewater (twentieth ed.), Am. Public Heal. Assoc. Washingt. DC. (2005).

[37] C. Tizaoui, L. Bouselmi, L. Mansouri, A. Ghrabi, Landfill leachate treatment with ozone and ozone/hydrogen peroxide systems, J. Hazard. Mater. 140 (2007) 316–324. https://doi.org/10.1016/J.JHAZMAT.2006.09.023.

[38] J. Saetang, S. Babel, Effect of glucose on enzyme activity and color removal by Trametes versicolor for high strength landfill leachate, Water Sci. Technol. 62 (2010) 2519–2526. https://doi.org/10.2166/wst.2010.552.

[39] W.A.R.W. Razarinah, M.N. Zalina, N. Abdullah, Treatment of landfill leachate by immobilized Ganoderma australe and crude enzyme, ScienceAsia. 40 (2014) 335–339. https://doi.org/10.2306/scienceasia1513-1874.2014.40.335.

[40] M. Sedighi, A. Karimi, F. Vahabzadeh, Involvement of ligninolytic enzymes of Phanerochaete chrysosporium in treating the textile effluent containing Astrazon Red FBL in a packed-bed bioreactor, J. Hazard. Mater. 169 (2009) 88–93. https://doi.org/10.1016/j.jhazmat.2009.03.070.

[41] I. Petatán-Sagahón, M.A. Anducho-Reyes, H.V. Silva-Rojas, A. Arana-Cuenca, A. Tellez-Jurado, I.O. Cárdenas-Álvarez, Y. Mercado-Flores, Isolation of bacteria with antifungal activity against the phytopathogenic fungi Stenocarpella maydis and Stenocarpella macrospora, Int. J. Mol. Sci. 12 (2011) 5522–5537. https://doi.org/10.3390/ijms12095522.

[42] A. Bardi, Q. Yuan, G. Siracusa, I. Chicca, M. Islam, F. Spennati, V. Tigini, S. Di Gregorio, D.B. Levin, G. Petroni, G. Munz, Effect of cellulose as co-substrate on old landfill leachate treatment using white-rot fungi, Bioresour. Technol. 241 (2017) 1067–1076. https://doi.org/10.1016/J.BIORTECH.2017.06.046.

[43] J. Mallerman, R. Itria, P. Babay, M. Saparrat, L. Levin, Biodegradation of nonylphenol polyethoxylates by litter-basidiomycetous fungi, J. Environ. Chem. Eng. 7 (2019) 103316. https://doi.org/10.1016/j.jece.2019.103316.

[44] W.D.H. Schneider, R.C. Fontana, S. Mendonça, F.G. de Siqueira, A.J.P. Dillon, M. Camassola, High level production of laccases and peroxidases from the newly isolated white-rot basidiomycete Marasmiellus palmivorus VE111 in a stirred-tank bioreactor in response to different carbon and nitrogen sources, Process Biochem. 69 (2018) 1–11. https://doi.org/10.1016/j.procbio.2018.03.005.

[45] K. Pakshirajan, S. Kheria, Continuous treatment of coloured industry wastewater using immobilized Phanerochaete chrysosporium in a rotating biological contactor reactor, J. Environ. Manage. 101 (2012) 118–123. https://doi.org/10.1016/j.jenvman.2012.02.008.

[46] J. Saetang, S. Babel, Effect of leachate loading rate and incubation period on the treatment efficiency by T. versicolor immobilized on foam cubes, Int. J. Environ. Sci. Technol. 6 (2009) 457–466. https://doi.org/10.1007/BF03326085.

[47] S. Senthilkumar, M. Perumalsamy, H. Janardhana Prabhu, Decolourization potential of white-rot fungus Phanerochaete chrysosporium on synthetic dye bath effluent containing Amido black 10B, J. Saudi Chem. Soc. 18 (2014) 845–853. https://doi.org/10.1016/j.jscs.2011.10.010.

[48] B.Y. Ammary, Nutrients requirements in biological industrial wastewater treatment, African J. Biotechnol. 3 (2004) 236–238. https://doi.org/10.5897/ajb2004.000-2042.

[49] S.Z.. Ahammad, J.. Gomes, T.R. Sreekrishnan, Wastewater treatment forproduction of H2S-free biogas, J. Chem. Technol. Biotechnol. 83 (2008)

1163–1169. https://doi.org/10.1002/jctb.

[50] M. Ellouze, F. Aloui, S. Sayadi, Effect of high ammonia concentrations on fungal treatment of Tunisian landfill leachates, Desalination. 246 (2009) 468–477. https://doi.org/10.1016/J.DESAL.2008.03.068.

[51] A. Dhouib, M. Ellouz, F. Aloui, S. Sayadi, Effect of bioaugmentation of activated sludge with white-rot fungi on olive mill wastewater detoxification, (n.d.). https://doi.org/10.1111/j.1472-765X.2006.01858.x.

[52] N. Junnarkar, N. Pandhi, N. Raiyani, N. Bhatt, R. Raiyani, Production of LiP by Phanerochaete chrysosporium MTCC 787 Through Solid State Fermentation of Wheat Straw and Assessing its Activity Against Reactive Black B, Int. J. Adv. Res. J. 4 (2016) 812–819. http://www.journalijar.com/uploads/275_IJAR-8575.pdf.

[53] Q. Yang, L. Tao, M. Yang, H. Zhang, Effects of glucose on the decolorization of Reactive Black 5 by yeast isolates, J. Environ. Sci. 20 (2008) 105–108. https://doi.org/10.1016/S1001-0742(08)60016-9.

[54] E.J. Espinosa-Ortiz, E.R. Rene, K. Pakshirajan, E.D. Van Hullebusch, P.N.L.L. Lens, E.D. van Hullebusch, P.N.L.L. Lens, Fungal pelleted reactors in wastewater treatment: Applications and perspectives, Chem. Eng. J. 283 (2016) 553–571. https://doi.org/10.1016/j.cej.2015.07.068.

[55] Y. Lu, L. Yan, Y. Wang, S. Zhou, J. Fu, J. Zhang, Biodegradation of phenolic compounds from coking wastewater by immobilized white rot fungus Phanerochaete chrysosporium, J. Hazard. Mater. 165 (2009) 1091–1097. https://doi.org/10.1016/J.JHAZMAT.2008.10.091.

4.2. Tratamiento de lixiviados

En este apartado se recogen todas las investigaciones relacionadas con la caracterización y el tratamiento del permeado de lixiviados. Debido a la elevada concentración de compuestos fenólicos altamente recalcitrantes en lixiviados, a menudo es empleo exclusivo de un tratamiento biológico convencional no es suficiente, y se necesitan tratamientos adicionales para la degradación de los compuestos más refractarios y la adecuación del lixiviado final para su vertido.

Esta sección está compuesta por dos apartados, en el **4.2.1** se ha llevado a cabo la caracterización microbiológica de varios puntos del tratamiento de nitrificación-desnitrificación de lixiviados de vertedero. Estos resultados permiten conocer detalladamente la microbiota predominante en el proceso biológico de tratamiento y la influencia de los procesos operacionales en la distribución de la microbiota, y se recogen en un artículo publicado cuya cita se destaca al inicio del apartado. En el punto **4.2.2**, se ha evaluado el empleo del hongo de pudrición blanca *P. chrysosporium*, para el tratamiento biológico del licor de biometanización, evaluando también la influencia de la adición de fuentes de nitrógeno y carbono durante el proceso de degradación.

4.2.1 Metagenomic analysis of bacterial communities from nitrification-denitrification treatment of landfill leachates by Ion PGM System

Ana Isabel Díaz[1], Paula Oulego[1], Adriana Laca[1*], José Manuel González[2] and Mario Díaz[1]

[1]Department of Chemical and Environmental Engineering, University of Oviedo, Oviedo, Spain

[2] R&D, COGERSA SAU. Gijón, Asturias, Spain E-33697

Correspondence: Dra. Adriana Laca, Department of Chemical and Environmental Engineering, University of Oviedo, Oviedo, Spain

email: lacaadriana@uniovi.es; Phone: +34 985 10 29 74; Fax: +34 985 10 34 34

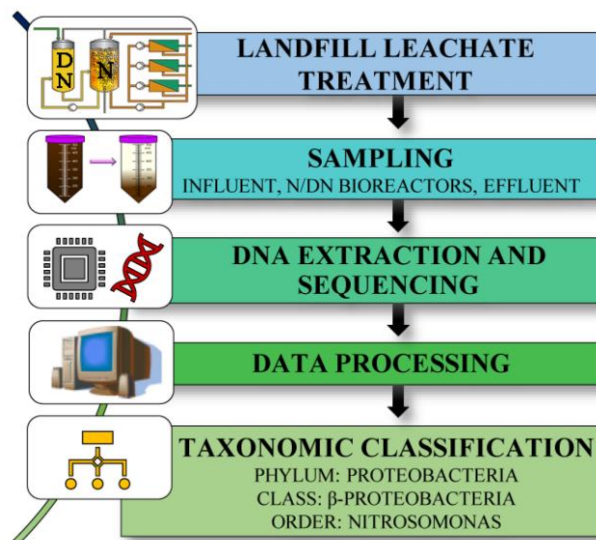


Figura 4. 18. *Resumen gráfico del trabajo 4*

*Artículo publicado en la revista "**Clean – Soil, Air, Water**"

Cita: Díaz, A. I., Oulego, P., Laca, A., González, J. M., Díaz, M., Metagenomic Analysis of Bacterial Communities from a Nitrification–Denitrification Treatment of Landfill Leachates. *Clean - Soil, Air, Water* **2019**, 47, 1900156.

https://doi.org/10.1002/clen.201900156

ABTRACT

The efficiency of the biological removal of carbon and nitrogen from leachates is determined by the activity of microbial populations present in biological reactors. In this work, a complete characterization of bacterial communities revealed by Personal Genome Machine (PGM) sequencing has been carried out from different point of a nitrification-denitrification process operated in an urban landfill sited in the North of Spain. The leachate fed to the treatment was a mixture of young leachate, old leachate and effluent from an anaerobic digestion process, in a volume ratio of 1:0.9:0.12 respectively. The anoxic and oxic reactors were followed by an ultrafiltration step. Samples were taken from different points of the process and PGM sequencing was used to characterize microbial communities. Results revealed the microbial diversity of samples, which included detection of minority populations that are difficult to be explored by other methods. Bacteria belonging to *Bacteroidetes* and *Proteobacteria* were dominant in all the samples analysed. *Proteobacteria* represented more than 50% of the total population in all cases. Samples taken after the biological treatment showed a significant reduction in the relative abundance of *Firmicutes*, *Tenericutes* and *Lentisphaerae* phyla in comparation with the initial leachate. The relative abundance of the classes was also studied and the most abundant in the samples were *β-Proteobacteria* and *Flavobacteria*.

ABBREVIATIONS: **PGM**, personal genome machine, **COD**, chemical oxygen demand, **BOD**, biological oxygen demand, **VFA**, volatile fatty acids.

KEYWORDS: Bacterial community; nitrification-denitrification; PGM sequencing; wastewater treatment; metagenomic.

1. INTRODUCTION

Landfill leachate is the liquid that results from water percolating through waste deposits. The specific composition of leachates depends on the type of wastes, landfill age, climate conditions and hydrogeology of the landfill site [1]. These effluents are usually characterized by high concentrations of organic matter, ammonium as well as potentially toxic metal and chlorinated salts [2]. Young leachates are commonly characterized by high biochemical oxygen demand (BOD) and chemical oxygen demand (COD), as consequence of a rapid anaerobic fermentation that generates volatile fatty acids (VFA) as main products [3]. In mature leachates, the methanogenic phase occurs and the VFA are converted to biogas. Therefore, the organic fraction of the leachate becomes dominated by recalcitrant or bio-refractory compounds [4].

Pollutants present in the leachate can contaminate groundwater, rivers and soils, causing high environmental impact. Therefore, its collection and treatment is one of the main problems in urban waste landfills. Biological processes have been reported as the most effective for the treatment of such wastewater [3, 5]. These processes take advantage of the abilities of microbes to degrade organic matter, remove nutrients and transform toxic compounds into harmless products [6]. During biological treatment, the nitrogen of landfill leachate is removed through nitrification and denitrification processes, which are carried out by ammonium-oxidizing bacteria (AOB), nitrite-oxidizing bacteria (NOB), and denitrifying bacteria [7]. These bacterial communities are highly sensitive to environmental factors, such as pH, salinity, temperature or dissolved oxygen [8, 9]. It is essential to characterize the microbiota present at each stage of the processes in order to understand the biological transformations that determine the efficiency of the treatments. Several molecular techniques, such as terminal restriction fragment length polymorphism (T-RFLP), denaturing gradient gel electrophoresis (DGGE), single-strand conformation polymorphism (SSCP) and Sanger sequencing of clone libraries, have been employed in the last decades to describe microbial communities in wastewater processes [10]. However, the information obtained from these techniques was limited because only a few hypervariable regions are considered. In recent years, the application of more advanced techniques, i.e. the next generation of sequencing (NGS) based on 16S rRNA gene sequencing, has provided a cheaper and higher throughput alternative to sequencing DNA [11]. This technology allows the

generation of millions of short sequencing reads for massive studies of genes, giving higher taxonomic resolution. It offers a great opportunity and new insights to rapidly examine the composition as well as the interaction of the great diversity of microorganisms involved in wastewater treatments [12].

However, despite of the evident interest, as far as we know, Personal Genome Machine (PGM) sequencing has not yet been employed for the study in depth of microbial ecology in nitrification-denitrification processes of landfill leachates. This technique was used in this work to carry out a microbial characterization throughout a real biological treatment of wastewater mainly composed by a mixture of young and old leachates. In particular, the aims of this work were: i) To characterize the bacterial population in the raw leachate and in the nitrification-denitrification reactors and ii) to determine the effect of operational parameters on the distribution of bacterial communities and its repercussions in the effectiveness.

2. MATERIAL AND METHODS

2.1. Plant operation parameters

The samples used in this study were taken from the biological leachate treatment plant sited in COGERSA, the wastes treatment center of Asturias (Spain). This center has a non-hazardous-wastes landfill with a capacity of 16 million m^3, a hazardous-waste landfill with a capacity of 600 m^3 and an anaerobic digestion plant, which can treat 30000 t/years of sludge from urban wastewater treatment plants and the organic fraction of municipal solid waste.

The treated process was fed with a mixture of young leachate, old leachate and an effluent from the anaerobic digestion process, in an approximate volume ratio of 1:0.9:0.12 respectively. Approximately 700 m^3 of leachates/day were treated by the biological treatment, which consisted of one denitrification reactor (anoxic), one mixed reactor which operated as denitrifying or nitrifying depending on the conditions of the plant, and four nitrification reactors (oxic). At the time when the samples were taken, the mixed reactor was operating as a nitrifying reactor.

The nitrification-denitrification process occurred under pressure (2.5 bar) at mesophilic temperatures (37-40°C). The volume of each reactor was 175 m^3 with a total hydraulic retention time of 7 h. During the process the pH maintained between 6.5 and 7, and 3 m^3 of methanol/day were supplied as carbon source. Oxygen was supplied by air compressors through bottom ejectors to the nitrification reactors in order to assure an oxygen concentration of 2.5 ppm. The injection pumps circulated the air-mud mixture, favoring the dissolution of oxygen and the homogenization of the sludge inside the reactors. After the treatment process, a recirculation from the last nitrification tank (OXIC-4) to the initial denitrification tank (ANOXIC-1) was carried out in a ratio of 80.5%. The rest of treated water was separated from the biological sludge by ultrafiltration process formed by five units of ultrafiltration with a total membrane surface of 280 m^2 with a pore size of 0.02 μm. The efficiency of nitrogen removal was higher than 80%. A flow diagram of the treatment plant is shown in Fig. 4.19.

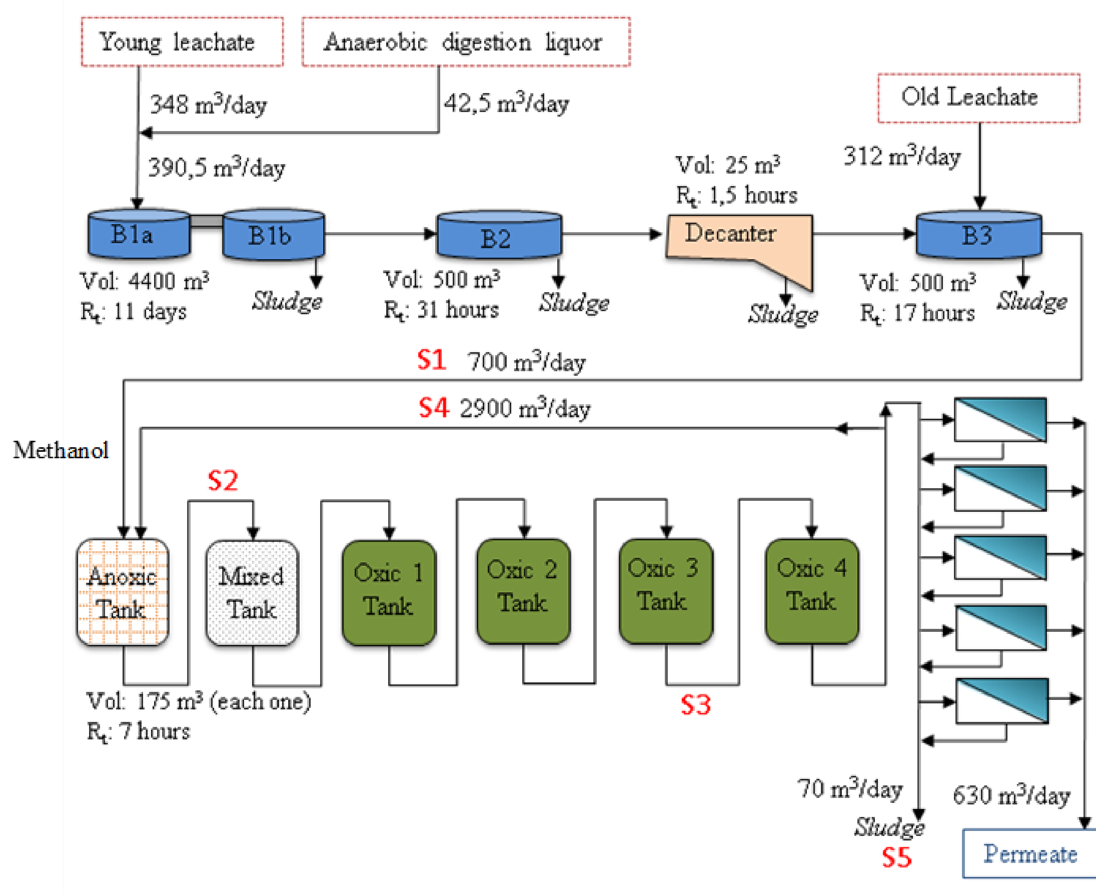


Figura 4. 19. Process flow diagram of biological treatment plant.

2.2. Sampling

Five different samples were collected throughout the biological treatment to be analysed microbiologically. Sample 1 (S1) corresponds to the raw leachate fed to the biological treatment. This sample was taken before mixing with the recirculate permeate from the ultrafiltration process. Sample 2 (S2) was taken from the effluent of the denitrification reactor, sample 3 (S3) corresponds to the effluent of nitrification reactor OXIC-3 and sample 4 (S4) corresponds to the recirculated effluent to the head of the process coming from nitrification reactor OXIC-4. Finally, sample 5 (S5) was taken from the sludge of the ultrafiltration process. Detailed information from each point of sampling is shown in Fig. 4.19 and Table 4.6.

2.3. Sample processing and DNA extraction

Sample processing was preformed according to Diaz et al. [13]. A volume of 160 mL of each sample was centrifuged for 20 min at 13000 × *g*. The supernatant was discarded, and the solid fraction was preserved at -20°C for DNA extraction. With this aim, Power Biofilm DNA Isolation Kit (MoBio Laboratories, Carlsbad, CA, USA), specific for leachate samples, was employed and 0.25 g of the solid fraction were weighted and treated according to the manufacturer's instructions. Due to the excessive color of samples, 200 µL of buffer solution 3 (BF3) were added (amount recommended in the kit protocol for colored samples). The extracted DNA was concentrated using the Concentrator Plus Vacufuge (Eppendorf, Hamburg, Germany) and a BioPhotometer Plus (Eppendorf, Hamburg, Germany) was used to ensure that the amount of DNA was high enough to continue the process.

2.4. DNA amplification and purification

An Ion 16S Metagenomics Kit (Ion Torrent, Life Technologies) was employed for DNA amplification. This kit allows the simultaneous examination of seven of the nine hypervariable regions in the bacterial 16S rRNA gene, using one primer for the V2-4-8 regions and another primer for the V3-6 and V7-9 regions. The DNA samples were amplified by PCR reaction, which was performed in several steps: i) heating at 95 °C for 10 min, ii) 25 cycles of denaturation at 95 °C for 30 s, iii) alignment at 58 °C for 30 s, iv) extension at 72 °C for 30 s, v) elongation at 72 ° C for 7 min and vi) preservation at 4 °C for 20 min. The resulting products were purified using the

Agencourt AMPure XP Kit (Beckman Coulter, Atlanta, GA, USA) and the 16S rRNA amplicons were quantified with a Qubit 2.0 Fluorometer using dsDNA HS Assay Kit (Invitrogen, Carlsbad, CA, USA).

2.5. Library construction and sequence analysis

The DNA obtained in the purification phase was fragmented in order to obtain smaller fragments of up to 150 base pairs (bp) by using an Ion Plus Fragment Library Kit (AB Library Builder). For the library construction, each fragment of the obtained DNA was coupled to a marker and two adapters. Each library corresponds to a different collection of DNA fragments to be sequenced and is unique to each sample. Construction of the library was conducted using the PGM Hi-Q OT2 Kit. Subsequently, the samples were sequenced using the PGM Hi-Q Sequencing Ion Kit and the Ion 318 Chip Kit v2, which has a minimum capacity of four million readings.

The results obtained were analysed by using Life Technologies Ion Reporter Software, that uses both the Premium Curated MicroSEQ ID 16S rRNA reference database and the Curated Greengenes Database. The restriction criteria applied was as follows: i) read length filter: 150 bp, ii) minimum alignment coverage: 90%, iii) read abundance filter: 10, iv) genus cut off: 97%, and v) species cut off: 99%. These criteria were selected according to previous works about microbial identification that used databases employed in this study [13, 14].

2.6. Nucleotide sequence accession numbers

The sequences obtained in this study are available in the National Center for Biotechnology Information (NCBI) under accession numbers SAMN09765719 to SAMN09765723. The SRA database accession number is SRP156554.

Tabla 4. 6. *Characteristics of the samples analysed. The values correspond to the averages (± standard deviations) of four samples taken along 2016.*

	Parameters							
	pH (ud.)	**COD_T (mg/L)**	**COD_S (mg/L)**	**BOD_5 (mg/L)**	**NH_4^+ (mg/L)**	**NO_3^- (mg/L)**	**NO_2^- (mg/L)**	**TS (mg/L)**
S1	8.56 ± 0.10	5155 ± 1159	4988 ± 966	1788 ± 798	2330 ± 171	< 60	2330 ± 171	10565 ± 1416
S2	7.53 ± 0.41	23738 ± 2375	7988 ± 491	2500 ± 616	412 ± 83	< 60	412 ± 83	28250 ± 2371
S3	6.73 ± 0.23	25775 ± 2207	7890 ± 616	2275 ± 618	78 ± 40	352 ± 138	78 ± 40	20009 ± 1825
S4	6.68 ± 0.21	21475 ± 6974	7788 ± 709	1950 ± 656	41 ± 34	368 ± 136	41 ± 34	29168 ± 2593
S5	6.81 ± 0.16	25150 ± 2362	9142 ± 2847	2125 ± 591	80 ± 43	316 ± 154	80 ± 43	29008 ± 2643

3. RESULTS AND DISCUSSION

The PGM sequencing and the amplification of hypervariable regions of 16S rRNA provided a detailed taxonomic bacterial classification throughout the nitrification-denitrification treatment. A total of 21 phyla, 250 families, 128 genera and 77 species were identified in the five samples analysed. The classification of microorganisms up to species level is shown in the Supporting Information Figs. S1-S5. After the analysis with Ion Reporter Software, a total of 1056150 effective sequences were obtained. In general, the hypervariable V3 and V6-7 regions presented a greater number of mapped reads, followed by the V4 and V8 regions. This information highlights the importance of sequencing all hypervariable regions to obtain a more accurate identification of microorganisms.

3.1. Raw leachate (S1)

The raw leachate is a mixture of young leachate, mature leachate and an effluent from an anaerobic digestion process as indicated in Section 2. This sample, as shown in Table 1, was characterized by high concentrations of ammonium (>2000 mg/L) and CODt (>4000 mg/L) with moderate biodegradability (BOD_5/COD ~ 0.3). As shown in Fig. 4.20, in the initial leachate (S1), *Proteobacteria* and *Firmicutes* phyla were the most abundant, achieving 51 and 18% of total relative abundance, respectively. Previous studies highlighted the dominance of these phyla in landfill leachates and wastewater treatments, followed by other groups such as *Bacteroidetes* and *Tenericutes*, also found in this sample, but with relative abundances < 8% [6].

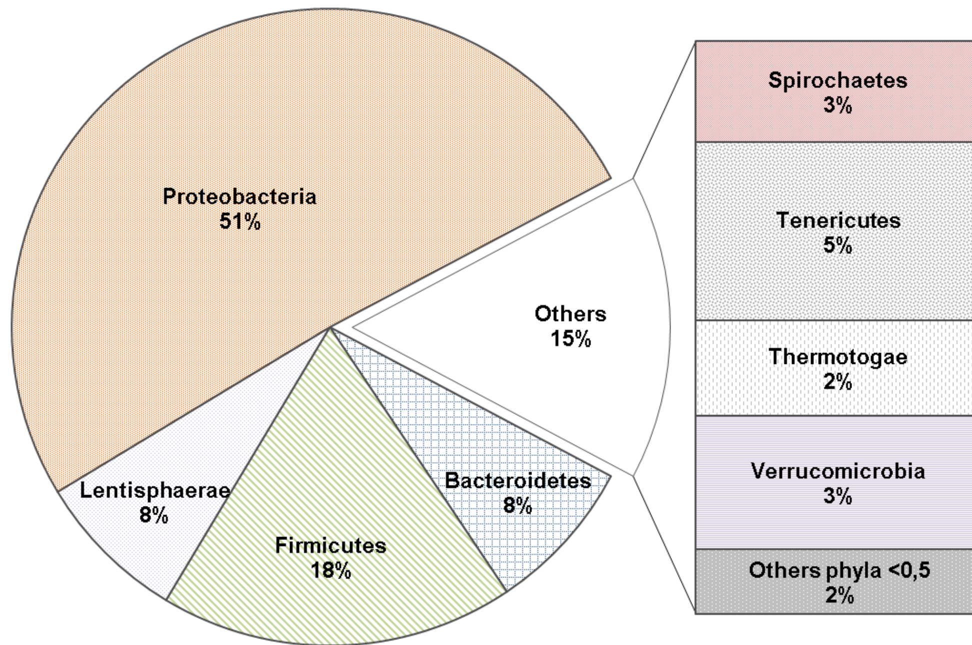
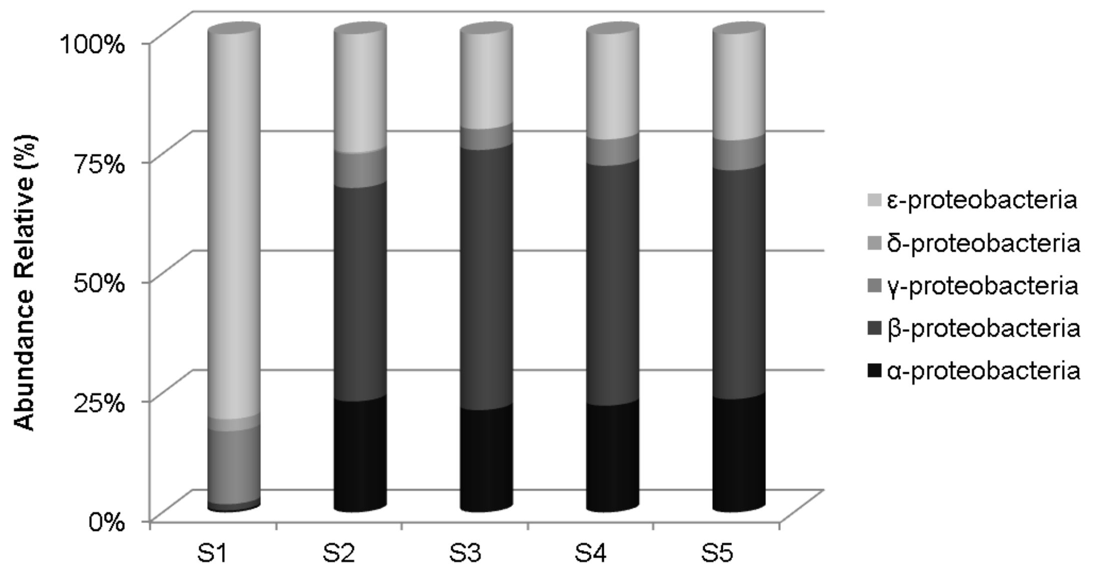


Figura 4. 20. *Relative abundance for the phyla detected in the raw leachate (S1).*

The relative abundance of classes within *Proteobacteria* phylum is shown in Fig. 4.21.A, where *ε-Proteobacteria* which accounted for 80% of the total bacteria, was the most abundant. Within this class, the genus *Arcobacter* was detected in S1. The presence of this genus has been reported as typical in urban wastewater and some microorganisms within it as e.g., *Arcobacter butzleri* has been described as potential pathogens and fecal pollution indicator [16]. Lu et al. [17] reported the efficiency of activated sludge in full-scale water treatment systems for the elimination of this species. In this study, *Arcobacter skirrowii* and *Arcobacter venerupis* were detected in S1, S2 and S3. Nevertheless, its relative abundance was significantly reduced throughout the process, and it has not been detected in S4 and S5. This fact indicates that the nitrification-denitrification process considered here is effective for the elimination of this pathogenic bacterium.

A)



B)

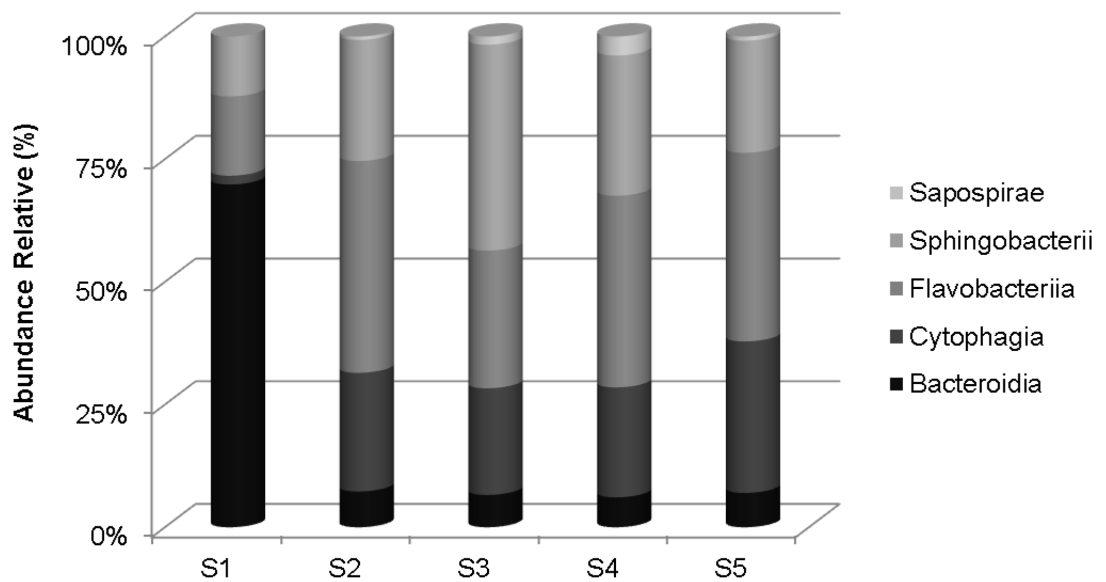


Figura 4. 21. *Relative abundance for the classes detected in the A) Proteobacteria and B) Bacterioidetes phyla.*

The second class in order of relative abundance within *Proteobacteria* phylum was *γ-Proteobacteria*, which accounted for approximately 15%. Genera as *Pseudomonas*, *Teredinibacter*, *Idiomarina* and *Marinospirillum* were the most abundant. Previous studies reported *Pseudomonas* genus as bacteria with capacity to biodegrade organic substances and to reduce the biotoxicity caused by xenobiotic organic chemicals. Besides, it is known that these bacteria use primarily nitrate as an electron acceptor and play an important role in the conversion of nitrite to molecular nitrogen [18]. Du et al. [19] applied bacteria of this genus as a bioaugmented system to treat complex and high concentrated wastewater with high levels of nitrate and nitrite. This class also includes important nitrifiers and denitrifiers microorganisms which have an important role during the biological process. Genera such as *Nitrosomonas*, *Nitrosospira*, *Nitrosococcus*, *Tissierella*, *Pseudomonas*, *Clostridium* and *Paracoccus* were detected in S1, all of them have been associated with fermentative metabolism of macromolecular organic compounds [20].

Köchling et al. [21], who analysed microbial communities in raw leachates of different ages, reported that *Proteobacteria*, mainly *Pseudomonadales* order, were more abundant in rainy seasons, whereas microorganisms belonging to *Firmicutes*, mainly *Clostridiales* order, were predominant in dry seasons and they increased their proportion with the landfill age. *Firmicutes* was related to the secretion of extracellular enzymes as cellulases, lipases and proteases. Thus, their main function in landfills consists in degrading complex polysaccharides, such as starch and cellulose [22]. Here, the landfill is located in a high rainfall zone and the proportion of old leachate was lower than the proportion of young leachate, which is reflected by the lower relative abundance of *Firmicutes* phylum. *Clostridia* class was the most representative within *Firmicutes* phylum, with more than 50% of relative abundance. The following microorganisms belonging to this class were identified: *Cellulosibacter alkalithermophilus*, *Clostridium* sp., *Tissierella creatinini*, *Syntrophomonas byantii*, *Syntrophomonas sapovorans*, and *Proteiniborus ethanoligenes*.

As shown in Fig. 4.20, the *Bacteroidetes* phylum accounted for 8% of total microorganisms in S1. Microorganisms within this phylum have been described as important bacteria for the degradation of high molecular weight organic matter to acetic and propionic acid [20a]. Their presence has been reported in anaerobic digestion processes fed with vegetal biomass, sludge or mixed organic residues [13, 23]. Within

Bacteroidetes phylum, *Bacteroidia* class was the most abundant representing around 70% of the phylum. This class plays an important role in hydrolyzing and fermenting organic materials, producing organic acids, CO_2 and H_2 during the anaerobic digestion process that takes place in landfills [24]. Within this class, species of the order *Bacteroidales*, i.e. *Petrimonas* sp., were detected. *Flavobacteria* and *Sphingobacterium* classes were also detected with relative abundances of 12 and 16%, respectively. These classes have been described as typical populations in leachates [2, 22].

3.2. Denitrification reactor output (S2)

During the denitrification step (anoxic tank), organic matter is consumed by heterotrophic bacteria responsible for the transformation of nitrate into molecular nitrogen. In this reactor, methanol was added as carbon source to increase the biodegradable organic matter available for denitrifying bacteria. For this reason, COD values reported in Table 4.6 for S2 were higher than values reported for S1. As can be estimated from data shown in Table 4.6, nitrate recirculated to the anoxic tank is removed in this step with efficiencies >80%. The relative abundances of majority phyla found in S2 are shown in Fig. 4.22. With respect to S1, the relative abundance of Bacteroidetes increased, accounting 27% of total. This phylum together with Proteobacteria has been described as dominant in denitrification processes [25].

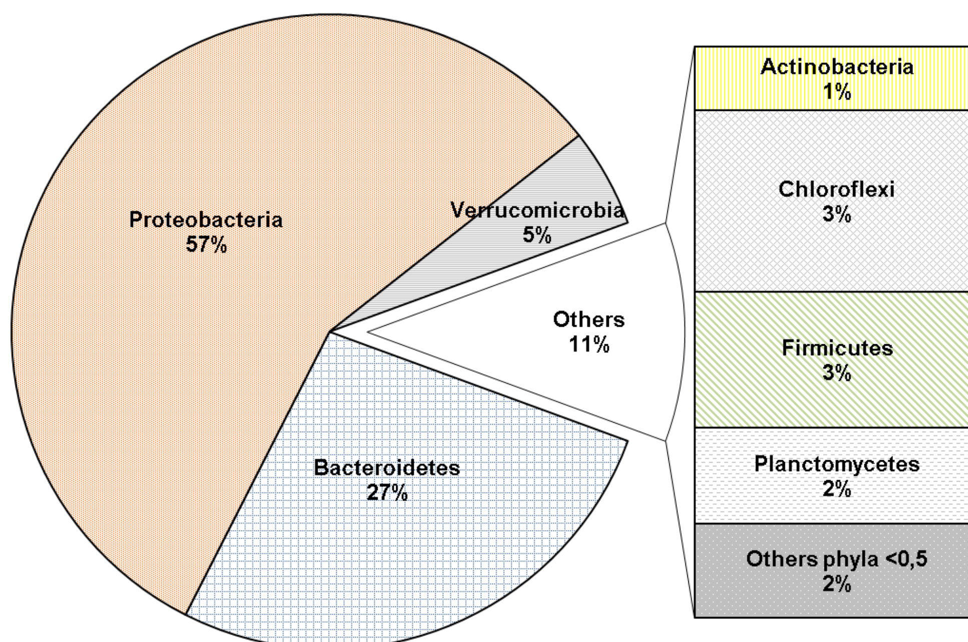


Figura 4. 22. *Relative abundance for denitrification reactor output (S2).*

The relative abundance of the phylum *Proteobacteria* was increased by 6% with respect to the raw leachate. This fact was expected since it was reported that the relative abundance of *Proteobacteria* phylum was higher when the ammonium concentration was reduced [2] and the ammonium concentration in S2 was five times lower than in S1 due to the recirculation (see Table 4.6 and Fig. 4.19). Potential denitrifying genera within this phylum, i.e. *Thauera*, *Comamonas* and *Azoarcus*, were detected.

With respect to the relative abundance of classes within this phylum (Fig 4.21.A), a significant decrease in *ε-Proteobacteria* and an increase in *α-Proteobacteria* and *β-Proteobacteria* in comparison with S1 were observed. These three classes have been reported as being involved in nitrification-denitrification processes. The AOB are phylogenetically restricted to *β-Proteobacteria*, including the genera *Nitrosomonas*, *Nitrosospira*, *Nitrosovibrio* and *Nitrosolobus* and to *γ-proteobacteria*, including the genus *Nitrosococcus* [26]. *Nitrosomonas* and *Nitrosococcus* were detected in S2, whereas *Nitrosospira*, *Nitrosovibrio* and *Nitrosolobus* were not identified in this study. The relative abundance of *Firmicutes* in S2 was very low, whereas in S1 it was one of the most abundant phyla. *Firmicutes* has been described as one of the most abundant in anaerobic processes [27]. Again, the high recirculation from the oxic reactors seems to be the reason for the decrease in the relative abundance of this phylum in S2.

The second dominant phylum, *Bacteroidetes*, has been related with the degradation of particulate organic matter, especially high-molecular-weight compounds [28]. Regarding the relative abundance within *Bacteroidetes* phylum (Fig. 4.21.B), *Cytophagia*, *Flavobacteria* and *Sphingobacterii* classes were dominant. Guo et al. [29] described *Flavobacteria* and *Sphingobacterii* as dominant classes in activate sludge treatments plants. Gabarró et al. [30] who investigated microbial communities in the treatment of mature landfill leachates reported that these classes are key in nitrification processes. Microorganisms belonging to these classes utilize complex organic substrates as cellulose, which might suggest that they can promote the degradation of recalcitrant compounds [31]. With respect to *Bacteroidia* class, the relative abundance decreased until values $<7\%$ in all samples during the treatment process. This fact was expected since most of these microorganisms are known to be obligate anaerobes. The subsequent nitrification step was carried out under aerobic conditions, inhibiting bacteria belonging to *Bacteroidia* class and decreasing its relative abundance in all the samples, except for S1. Hu et al. [32] reported that microorganisms within

Proteobacteria phylum were most abundant in aerobic conditions whereas *Bacteroidetes* phylum, to which *Bacteroidia* class belongs, was most abundant in anaerobic bioreactors. Other phyla as *Verrucomicrobia*, *Actinobacteria*, *Chloroflexi*, *Firmicutes* and *Planctomycetes* were detected with relative abundances <5%.

3.3. Nitrification reactors output (S3 and S4) and ultrafiltration sludge (S5)

Nitrification processes are typically conducted by autotrophic bacteria. Consequently, as shown in Table 1, the concentration of COD and BOD_5 were similar for S2, S3 and S4. However, due to the activity of nitrifying bacteria, more than 80% of the ammonium contained in S2 was removed. Results obtained for the samples S3 and S4 from the nitrification process are shown in Fig. 4.23.A and 4.23.B, respectively.

Therefore, *Proteobacteria* and *Bacteroidetes* phyla were again the most abundant, representing around 90% of total relative abundance in these samples. Most of the microorganisms responsible for carrying out nitrification processes, (AOB and NOB) are found within these phyla [33].

It is striking the higher relative abundance of *Proteobacteria* in sample S3. Heterotrophic nitrifiers from genera belonging to this phylum, such as *Comamonas*, *Thauera*, *Paracoccus* and *Azoarcus* were detected in S3 reaching the classification of the microorganisms up to species level, i.e., *Comamomas denitrificans*, *Thauera amoniaromatica*, *Thauera phenylacetica* and *Paracoccus solventivorans*. These genera have been reported in activated sludge reactors treating ammonium-rich, high-organic tannery and coking wastewater [34].

Guo et al. [29] studied the microbial structure and diversity of activated sludge in a full-scale simultaneous nitrogen and phosphorus removal plant. They described *Proteobacteria*, *Nitrospirae*, *Bacteroidetes*, *Actinobacteria* and *Firmicutes* as dominant phyla. In addition to *Proteobacteria* and *Bacteroidetes* phyla, *Actinobacteria*, *Firmicutes* and *Nitrospirae* were also detected in S3 and S4, but with low relative abundance. Other phyla as *Chloroflexi*, *Verrucomicrobia* and *Planctomycetes* were detected in these samples with relative abundances <5%.

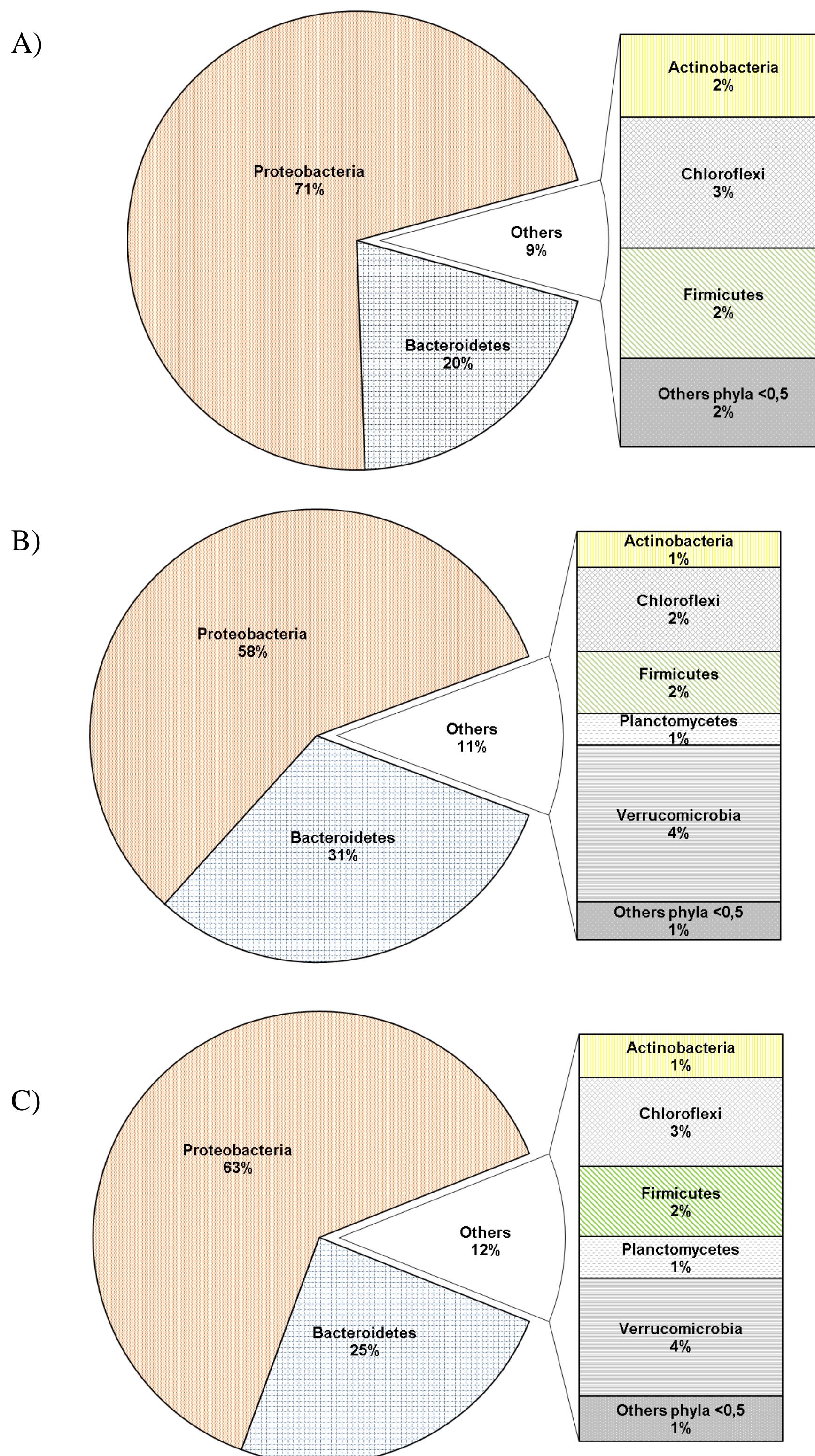


Figura 4. 23. *Relative abundance for the phyla detected in the A,B) nitrification reactors output and C) ultrafiltration sludge samples (S3, S4, and S5, respectively).*

The ultrafiltration sludge (S5) is basically a concentrated mixture of sludge resulting from the last oxic reactor. Thus, as expected, the microbiota found in this sample was similar to S3 and S4 microbiota with again *Bacteroidetes* and *Proteobacteria* as the dominant phyla (Fig. 4.23.C).

The relative abundance of classes within these phyla (Fig. 4.21) remained almost constant in all the samples analysed throughout the process (S2 to S5), and only slight variations could be observed.

CONCLUDING REMARKS

Results here obtained proved that the Ion Torrent methodology, based on PGM sequencing and amplification of all variable regions of 16S rRNA gene, provides an exhaustive taxonomic classification of bacterial populations in complex samples taken from biological treatments, such as the nitrifying-denitrifying process analysed here. The predominant phylum throughout the leachate treatment was *Proteobacteria* with more than 50% of total relative abundance in all analysed samples. This predominance was expected because most of microorganisms involved in nitrification-denitrification processes are included within this phylum, mainly in β-Proteobacteria and *γ-Proteobacteria* classes.

In the initial leachate (S1), the relative abundance of *Firmicutes* was higher than in samples taken at the outlet of biological reactors (S2 to S5). On the contrary, *Bacteroidetes* abundances were higher throughout the biological process, reaching values between 20 and 30%. This phylum together with *Proteobacteria* represented more than 90% in samples S2 to S5.

In relation to class level, *ε-Proteobacteria* was the most abundant in the initial leachate. However, throughout the biological process *α-Proteobacteria* and *β-Proteobacteria* became also dominant classes, according with others works that analysed leachate treatments [25, 26, 33].

The high recirculation contributes to achieve a high degree of mixture, so the phyla with higher values of relative abundance (*Proteobacteria* and *Bacteroidetes*) were found throughout the biological treatment. However, in relation with the relative abundance of phyla, significative differences have been detected between oxic and

anoxic reactors, with higher percentages of *Proteobacteria* in the samples taken from the oxic reactors. In addition, several differences could be detected with respect to minority populations, depending on the specific environments of each reactor.

ACKNOWLEDGMENTS

The work upon which this paper is based on was co-financed by Consortium for the Management of Solid Wastes of Asturias (Projects FUO-EM-138-15, FUO-139-16), Institute for Economic Development of the Principality of Asturias (Project IDE/2015/000245), Spanish Ministry of Economy and Business (Project CTM2015-63864-R) and European Regional Development Funds from European Union. Technical assistance from the Scientific-Technical Services of the University of Oviedo is gratefully acknowledged. The authors have declared no conflict of interest

REFERENCES

[1] R.B. Brennan, E. Clifford, C. Devroedt, L. Morrison, M.G. Healy, *J. Environ. Manage.* **2018**, *188*, 64.

[2] H.T.N. Dao, K. Kuroda, N. Nakahara, T. Danshita, M. Hatamoto, T. Yamaguchi, *J. Biosci. Bioeng.* **2016**, *122*, 708.

[3] Y. Peng, *Arab. J. Chem.* **2017**, *10*, S2567.

[4] a) P. Mandal, B.K. Dubey, A.K. Gupta, *Waste Manag.* **2017**, *69*, 250, b) J. Wiszniowski, D. Robert, J. Surmacz-Gorska, K. Miksch, J.V. Weber, *Environ. Chem. Lett.* **2006**, *4*, 51.

[5] S. Renou, J.G. Givaudan, S. Poulain, F. Dirassouyan, P. Moulin, *J. Hazard. Mater*. **2008**, 150.

[6] I. Ferrera, O. Sánchez, *Biotechnol. Adv.* **2016**, *34*, 790.

[7] a) S. Dong, M. Li, Y. Chen, Y, *Sci. Rep.* **2017**, *7*, 12215; b) P.H. Nielsen, H. Daims, H. Lemmer, *FISH Hadbook for Biological Wastewater Treatment.* **2009**, IWA Publishing: London.

[8] T. Jin, T. Zhang, Q. Yan, *Appl. Microbiol. Biotechnol*. **2010**, *87*, 1167.

[9] Q. Yao, D.C. Peng, *AMB Express.* **2017**, *7*, 25.

[10] a) A.C. Liu, C.Y. Chou, L.L. Chen, C.H. Kuo, *J. Biotechnol.* **2015**, *194*, 124; b) M. Sancha, A. Laca, A. Laca, J.M. González, B. Mayo, M. Diaz, *J. Residuals Sci. Technol.* **2014**, *11* (2) ,1544.

[11] A. Grada, K. Weinbrecht, *J. Invest. Dermatol.* **2013**, *133*, e11.

[12] a) A.T. Vincent, N. Derome, B. Boyle, A.I. Culley, S.J. Charette, *J. Microbiol. Methods.* **2017**, *138*, 60; b) K.A. Gilbride, D.Y. Lee, L.A. Beaudette, *J. Microbiol. Methods.* **2006**, *66*, 1; c) L.A.B. Siniscalchi, L.R. Leite, C.A.L. Chernicharo, J.C. De Araújo, *Environ. Sci. Pollut. Res.* **2017**, *24*, 16751; d) L. Song, S. Yang, H. Lui, J. Hu, *Appl. Microbiol. Biotechnol.* **2017**, *101*, 761.

[13] A.I. Díaz, P. Oulego, S. Collado, A. Laca, J.M. González, M. Díaz, *J. Biosci. Bioeng.* **2018**, *126*, 742.

[14] V. L. Lozano, N. Defarge, L-M. Rocque, R. Mesnage, D. Hennequin, R. Cassier, J.S. de Vendômois, J-M Panoff, G-E. Séralini, C. Amiel, *Toxicol. Rep.* **2018**, *5*, 96.

[15] a) MK Byun, J Chang, HJ Kim, SH Jeong , *PLOS ONE*. **2017**, *12(8),* e0183553. b) V. Pylro, L.F.W. Roesch, D.K. Morais, I.M. Clark, P.R. Hirsch, M.R. Tótola, *J. Microbiol. Methods.* **2014**, *107*, 30.

[16] a) S. McLellan, S. Huse, S. Mueller Spitz, E. Andreishcheva, M. Sogin, *Environ. Microbiol* **2010**, *12*, 378; b) L. Collado, L. Inza, J. Guarro, MJ. Figueras, *Environ. Microbiol.* **2008**, *10 (6)*, 1635

[17] X. Lu, XX. Zhang, Z. Wang, K. Huang, Y. Wang, L. Weigang, T. Yunfei, L. Bo, T. Yunging, *PLOS ONE*, **2014**, *10(5),* e0125549.

[18] a) B. Xie, S. Xiong, S. Liang, C. Hu, X. Zhang, J. Lu, *Bioresour. Technol* **2012**, *103*, 71; b) J.O. Eberly, K.J. Indest, D.E. Hancock, C.M. Jung, F.H. Crocker, *J Ind Microbiol Biotechnol* **2016**, *43*, 795.

[19] C. Du, C.W. Cui, S. Qiu, S.N Shi, A. Li, F. Ma, *Environmental Science and Pollution Research* **2017**, *24*, 11435.

[20] a) T. Narihiro, M.K. Nobu, N.K. Kim, Y. Kamagata, W.T. Liu, *Environ. Microbiol*, **2015** *17*, 1707; b) S.H. Yang, H.K. Ahn, B.S. Kim, S.S. Chang, K.Y. Chung, E.M. Lee, K.S. Ki, E.G. Kwon, *Asian-Australas J Anim Sci* **2017**, *30*, 1660.

[21] T. Köchling, J.L. Sanz, S. Gavazza, L. Florencio, *Appl. Microbiol. Biotechnol* **2015**, *99*, 5657.

[22] K. Yang, Q. Yue, J. Kong, P. Zhao, Y. Gao, K. Fu, B. Gao, *Chem. Eng. J* **2016**, *285*, 319.

[23] a) D. Riviere, V. Desvignes, E. Pelletier, S. Chaussonnerie, S. Guermazi, J. Weissenbach, T. Li, P. Camacho, A. Sghir, *ISME J.* **2009**, *3(6)*, 700. b) L.

Regueiro, P. Veiga, M. Figueroa, J. Alonso-Gutierrez, A.J.M. Stams, J.M Lema, M. Carballa, *Microbiol. Res.* **2012**, *167(10)*, 581.

[24] J.H. Guo, Y.Z. Peng, B.J. Ni, X.Y. Han, L. Fan, Z.G. Yuan, *Microb. Cell. Factories.* **2015**, *14*, 33.

[25] H. Lu, K. Chandran, D. Stensel, *Water Res.* **2014**, *64*, 237

[26] W. Zhu, C. Wang, F. Sun, L. Zhao, W. Dou, Z. Mao, W. Wu, *Mar. Pollut. Bull.* **2018**, *126*, 540.

[27] a) T. Zhang, M.F. Shao, L. Ye, *ISME J*, **2012**, *6*, 1137; b) M. Hu, X. Wang, X. Wen, Y. Xia, *Bioresour. Technol*. **2012**, *117*, 72.

[28] a) J. Wang, B. Gong, W. Huang, Y. Wang, J. Zhou, *Bioresour. Technol.* **2017**, *228*, 31; b) M.L.B. Da Silva, M.E. Cantão, M.P. Mezzari, J. Ma, C.W. Nossa, *Microb. Ecol.* **2015**, *70*, 77.

[29] J. Guo, B.J. Ni, X. Han, X. Chen, P. Bond, Y. Peng, Z. Yuan, *Enzyme Microb. Technol.* **2017**, *102*, 16.

[30] J. Gabarró, E. Hernández-del Amo, F. Gich, M. Ruscalleda, M.D. Balaguer, J. Colprim, *Water Res.* **2013**, *47*, 7066.

[31] D.L. Kirchman, *FEMS Microbiol. Ecol* **2002**, *39*, 91.

[32] M. Hu, X. Wang, X. Wen, Y. Xia, *Bioresour. Technol.* **2012**, *117*, 72.

[33] L. Ye, M.F. Shao, T. Zhang, A.H.Y. Tong, S. Lok, *Water Res* **2011**, *45*, 4390.

[34] a) Z. Wang, X.X. Zhang, X. Lu, B. Liu, Y. Li, C. Long, A. Li, *PLoS One.* **2014**, *9*, 1; b) Q. Ma, Y. Qu, W. Shen, Z. Zhang, J. Wang, Z. Liu, D. Li, H. Li, J. Zhou, *Bioresour. Technol.* **2015**, *179*, 436.

4.2.2 Evaluation of using white-rot fungi as post-treatment of biologically and physical-chemically pre-treated landfill leachate

Ana Isabel Díaz[1], Adriana Laca[1*], and Mario Díaz[1]

[1]Department of Chemical and Environmental Engineering. University of Oviedo. C/Julián Clavería, s/n, E-33006, Oviedo, Asturias, Spain.

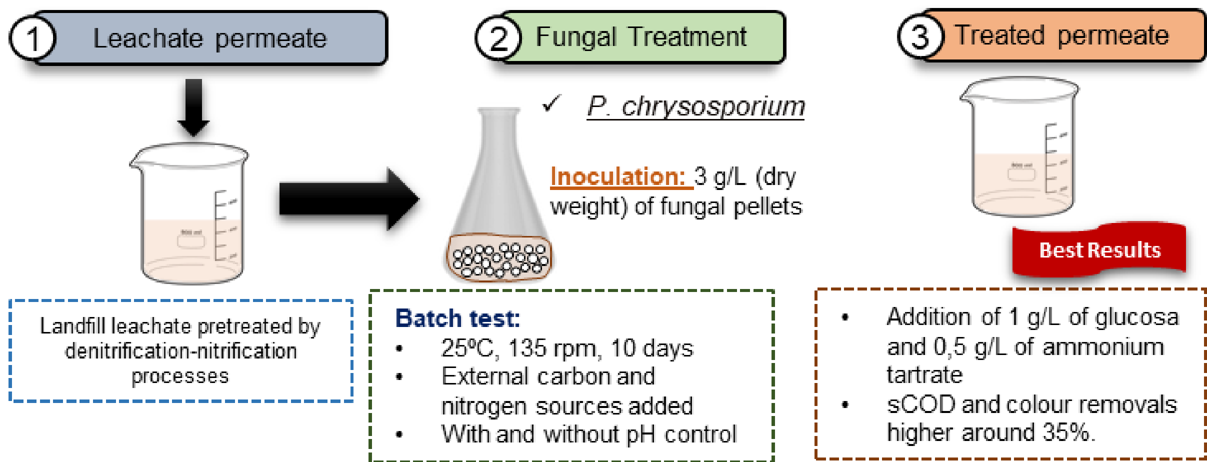


Figura 4. 24. *Resumen gráfico del trabajo 5*

ABSTRACT

Ultrafiltration permeate from a landfill leachate treatment plant is characterized by a high load of refractory compounds that are not degraded by bacteria during the previous nitrification-denitrification process. So, this effluent needs to be treated before being discharged to avoid serious environmental problems. The use of white-rot fungi to treat recalcitrant compounds has been promoted because of their ability to synthesize extracellular enzymes that degrade recalcitrant compounds.

In this work, the use of the white-rot fungus *P. chrysosporium* to treat a biologically and physical-chemically pre-treated landfill leachate was evaluated. Batch tests were carried out at 26ºC and 135 rpm for 15 days. The soluble chemical oxygen demand (sCOD), soluble biological oxygen demand ($sBOD_5$) and colour, as well as the lignin peroxidase (LiP) and manganese peroxidase (MnP) enzymatic activities were analysed. Also, the effect of different operational conditions including pH control, permeate dilution and supplementation with external carbon and nitrogen sources on the efficiency of fungal treatment were investigated. The pH control was shown to be key for fungal treatment. In addition, results showed that glucose and nitrogen sources were required to improve the fungal growth and enzymatic synthesis, considering 1 g/L of glucose and 0.5 of ammonium tartrate the optimal values. Data here obtained open the possibility of using fungi to treat recalcitrant effluents from a landfill leachate treatment plant.

1. INTRODUCTION

Municipal and industrial solid waste disposal in a landfill remains a common practice in most countries. Although this technique is generally the cheapest, the leachates generated may pose various environmental problems, such as groundwater pollution. Landfill leachate contains a large amount of organic and inorganic contaminants such as ammonia, chlorinated salts, and metal ions (eg, chromium, lead, and copper) [1,2]. Several biological and physical-chemical techniques, such as nitrification-denitrification, chemical oxidation, membrane filtration and activated carbon adsorption, as well as the combination between them, have been studied to remove refractory pollutants from landfill leachate [3,4].

Nevertheless, although these methods reduce organic and inorganic loads, their application has some drawbacks such as the high energy and operating cost of chemical oxidation processes, the need to regenerate activated carbon to avoid clogging problems or the membrane fouling in filtration processes [5]. Regarding biological methods, they are less effectives when the effluent treated contains high load of refractory compounds, because bacteria are not capable of degrading these complex compounds [2,6]. Therefore, it is essential to look for alternative methods to reduce these refractory compounds and/or enhancing the biodegradability of the leachates.

The application of white-rot fungi for the treatment and detoxification of landfill leachates has been considered as one of the most promising alternatives in recent years, due to its ability to synthetize powerful extracellular oxidative enzymes, including lignin peroxidase (LiP), manganese peroxidase (MnP) and laccase (Lac), which degrade the complex contaminants present in landfill leachates [7,8]. Furthermore, fungi have greater tolerance to adverse environmental conditions than bacterial species, withstanding high temperature, low pH and high concentration of toxic and recalcitrant compounds [8–10].

The effluent studied in this work has already been subjected to a nitrification-denitrification process followed by an ultrafiltration stage, which separate the treated water (permeate) from the biological sludge. Although the chemical oxygen demand (COD) and ammonia concentration have been considerably reduced after biological and physical pre-treatments, the permeate still contains refractory compounds that must be

treated before discharge. In addition, the biological chemical demand (BOD_5) of the permeate is very low (<30 mg O_2 / L), which indicates that most of the organic matter is resistant to a conventional biological treatment [6]. Therefore, the use of fungi seems to be a good alternative for the degradation of recalcitrant organic matter in the permeate.

Studies found in the literature are mainly focused on the use of fungi as pre-treatment of landfill leachates [7,8,11,12]. Nevertheless, as far as we know, there is not any published work that reported the use of fungi to treat the permeate from a combined physical and biological treatment of landfill leachates.

For these reasons, the aim of this work was to investigate the capacity of the white-rot fungus *Phanerochaete chrysosporium* to treat a landfill permeate to reduce its refractory chemical oxygen demand (COD) and colour. In addition, it has been studied the effect of different operational parameters such as the pH control, the dilution of the permeate and the addition of carbon and nitrogen sources, on the efficacy of fungal treatment and enzymatic synthesis. This information would be useful as an approach to determining the best operational conditions for futures fungal treatments of this effluent.

2. MATERIAL AND METHODS

2.1 Effluent description

The sample used in this work was a permeate coming from the ultrafiltration stage after the nitrification-denitrification treatment of landfill leachates. The treatment plant is in the waste treatment centre, COGERSA (Asturias, Spain). For more details of the process, see Diaz et al. [2]. A detailed description of the effluent is shown in Table 4.7.

2.2. Fungal strain and pellets obtention

The white-rot fungus, *Phanerochaete chrysosporium* Burdsall 1974 was used for the fungal treatment. The freeze-dried strain CECT 2798 was provided by Spanish Type Culture Collection (CECT). The recovery of freeze-dried strain as well as the obtention of the fungus pellets used for the treatment were carried out according to the methodology described by Díaz et al. [13].

Tabla 4. 7. *Characteristics of the initial permeate*

Parameter	Value
pH	6,78 ± 0,11
Conductivity (µS/cm)	11723 ± 453
sCOD (mg O_2/L)	1265 ± 6
$sBOD_5$ (mg O_2/L)	28 ± 3
Biodegradability (BI)	0,02 ± 0,003
Ammonium (mg/L)	21 ± 3
Nitrate (mg/L)	469 ± 79
Nitrite (mg/L)	1,5 ± 0,3
Phosphate (mg/L)	9 ± 2
TS (mg/L)	6,8 ± 0, 1

2.3 Fungal treatment

Eight batch tests were assayed with permeate inoculated with *P. chrysosporium* to study the effect of different operational parameters on the efficacy of fungal treatment. Firstly, the influence of pH during the fungal treatment was evaluated. Considering the parameters established by other authors who have previously treated landfill leachates by white-rot fungi [10,14], the pH of permeate was initially adjusted to 6.0 using NaOH 1M and HCl 1M. Afterwards, **F1** and **F2** tests were carried out without pH control and with pH controlled around pH 6 during the fungal treatment, respectively.

Tests from **F3** to **F6**, were carried out to study the influence of the addition of easily assimilable carbon and nitrogen sources on the fungal treatment using glucose and ammonium tartrate as co-substrates. Taking into account the optimal glucose concentration obtained in a previous work, where an recalcitrant effluent from anaerobic digestion of sewage sludge was treated by *P. chrysosporium* [13], the supplementation with 1 g/L, 3 g/L, and 5 g/L of glucose was tested, corresponding to the tests **F3**, **F4**, and **F5**, respectively. Besides, an additional test, **F6,** was assayed with permeate supplemented with 1 g/L of glucose and 0.5 g/L of ammonium tartrate. In all cases, the pH of the permeate was initially adjusted to 6.0 and controlled between a range of 5.5-6.2 during the fungal treatment using NaOH 1M and HCl 1M.

Finally, to assess the influence of the permeate dilution, test **F7** was carried out using as raw material the permeate mixed with distilled water in a ratio 1:1. As for undiluted effluent, the supplementation with glucose (1 g/L) and ammonium tartrate (0.5 g/L) for the diluted permeate was investigated, corresponding to test **F8**. In both cases, the pH was controlled during the treatment between values around 5.5-6.1.

In all cases, the experiments were performed in 1 L Erlenmeyer flasks containing 200 mL of sample inoculated with 3 g/L (dry matter) of *P. chrysosporium* fungus. Erlenmeyer flasks were incubated at 26ºC for 15 days in an orbital shaking at 135 rpm. For all the conditions tested, control experiments without fungus inoculation were assayed to evaluate the effect of *P. chrysosporium* in relation with the activity of the endogenous microbiota. These control experiments correspond to tests from **C1** to **C8**. All the experiments were carried out in duplicate. The data shown are the average of the experimental data. A detailed description of each experiment is shown in Table 4.8.

Tabla 4. 8. *Experimental design for the fungal treatments (F1-F8) and control tests (C1-C8)*

Sample	pH control	*P. chrysosporium*	Glucose	Ammonium tartrate	Dilution
C1	-	Non-inoculated	-	-	-
C2	5,8-6,1	Non-inoculated	-	-	-
C3	5,7-6,1	Non-inoculated	1 g/L	-	-
C4	5,8-6,0	Non-inoculated	3 g/L	-	-
C5	5,6-6,1	Non-inoculated	5 g/L	-	-
C6	5,8-6,0	Non-inoculated	1 g/L	0,5 g/L	-
C7	5,7-6,2	Non-inoculated	-	-	50%
C8	5,8-6,1	Non-inoculated	1 g/L	0,5 g/L	50%
F1	-	3 g/L (dry matter)	-	-	-
F2	5,7-6,1	3 g/L (dry matter)	-	-	-
F3	5,5-6,0	3 g/L (dry matter)	1 g/L	-	-
F4	5,5-6,1	3 g/L (dry matter)	3 g/L	-	-
F5	5,5-6,1	3 g/L (dry matter)	5 g/L	-	-
F6	5,5-6,0	3 g/L (dry matter)	1 g/L	0,5 g/L	-
F7	5,6-6,1	3 g/L (dry matter)	-	-	50%
F8	5,7-6,1	3 g/L (dry matter)	1 g/L	0,5 g/L	50%

2.4 Analytical methods

Samples was taken periodically each 24 h, centrifuged at 10000 g during 10 min, filtered by 0.45 μm filter (Millipore) and stored at -20 ºC until being analysed. All analytical measurements were done at least in triplicate.

The concentration of soluble chemical oxygen demand (sCOD) and soluble biochemical oxygen demand ($sBOD_5$), as well as the biodegradability index (BI) and the change in the colour, indicated by colour number (CN), were assessed according to Díaz et al. [13]. For the analysis of LiP and MnP enzymatic activities the methodology described by Lisboa et al. [15] was followed.

The moisture of the fungus was analysed by oven-drying (105ºC) a determined amount of fungus pellets to constant weight. Finally, total solids (TS) of permeate were measured according to Standard Methods [16] and the value of pH was measured by means of a pH-meter.

3. RESULTS AND DISCUSSION

3.1 Effect of pH control

The evolution of the sCOD concentration and colour during the fungal treatment for tests F1 and F2, as well as the pH evolution for F1 and C1 tests are shown in Fig. 4. 25.

For the inoculated tests, F1 and F2, the pH adjustment during the treatment proved to be a key parameter for the degradation of sCOD and colour. Therefore, as can be seen in Fig 4.25.A, the best degradations were achieved in the F2 test, which was performed at pH controlled around 6.0, reaching final sCOD and colour removals of 12% and 13%, respectively. On the contrary, for F1 test, where the pH value increased from 6.0 to 7.8 during the first 2 days (see Fig. 1B), the colour remained almost constant and only 3% of sCOD degradation was obtained. Hu et al. [17], who studied the effect of pH on landfill leachate treatment by *P. chrysosporium*, reported a maximum total organic carbon (TOC) removal efficiency around 73% at pH 6. Lu et al. [18], also achieved the higher removal rates of COD and phenolics compounds at pH between 5.0-6.0 during the fungal treatment of a recalcitrant wastewater from the coke industry by *P. chrysosporium*. The low sCOD removal efficiencies obtained in this

study could be since the treated effluent came from a previous biological process, so the organic matter in the effluent is highly recalcitrant. Similar data were reported by Saetang et al. [19] who achieved COD removals around 15% after 12 days of landfill leachate treatment by the white-rot fungus *Trametes versicolor*.

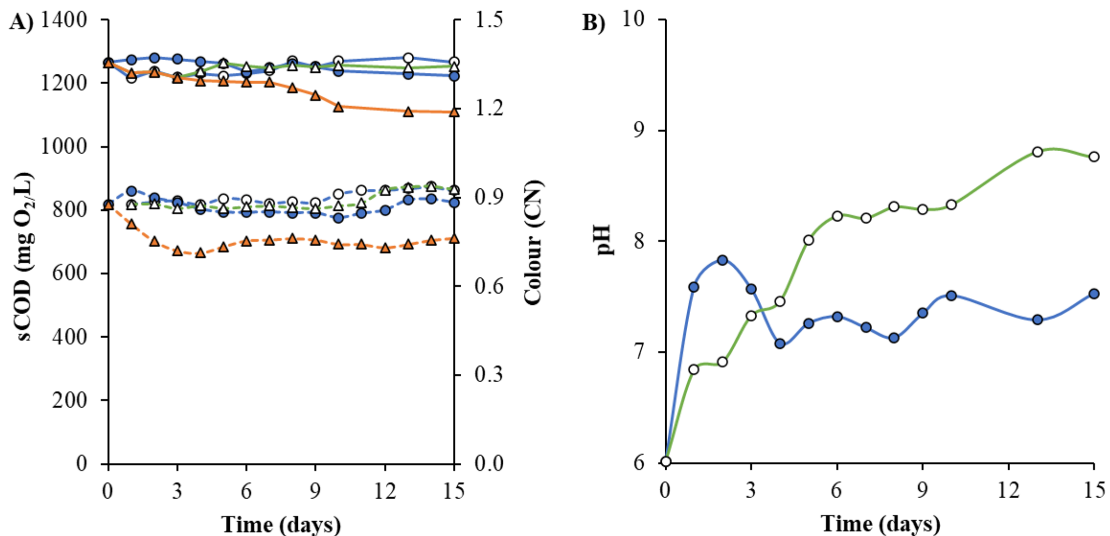


***Figura 4. 25**. **A)** sCOD (solid lines) and CN (dashed lines) evolution during the fungal treatment. Filled markers symbolize the inoculated tests F1 (●), F2 (▲) and empty markers symbolize the non-inoculated tests C1 (○) and C2 (△), used as control. **B)** pH evolution during the fungal treatment for C1 (○) and F1 tests (●).*

As can be seen in Fig 4.25.B, although good results were not obtained after fungal treatment without pH control, the different evolution of pH between the F1 and C1 tests is noteworthy. In the C1 test (non-inoculated), the pH showed a progressive increase during the treatment, reaching values close to pH 9. However, for F1 test, a maximum pH value of 7.8 was reached after two days of treatment. Afterwards, the pH values decreased for 48 h until reaching a pH value of 7. For times longer than 4 days, the pH remained between a pH range of 7-7.5. Several authors have reported the capacity of fungi to synthesize different organic acids [20,21]. Comparing the evolution of the pH in C1 with the data obtained in F1, it seems that, although not significant changes were observed in colour and sCOD concentration, the fungus *P. chrysosporium* was able to produce compounds that acidified the environment, reducing the pH of the permeate during fungal treatment.

Despite the increase in pH in C1, no significant differences were shown in terms of sCOD, and colour removal compared to C2. In both cases, the sCOD remained

almost constant and the colour increased after treatment. These results were expected since, as mentioned above, the permeate sample comes from an ultrafiltration stage, where the effluent is filtered by a membrane system with a pore size of 0.02 µm. Previous works have been reported a microbial concentration lower than 10 UFL/ml for ultrafiltration permeates coming from a landfill leachate treatment plant [2,22]. As most microorganisms were retained during the ultrafiltration stage, the impact of the endogenous microbiota on treatment was not significant.

The capacity of white-rot fungi to remove colour and recalcitrant organic matter is very linked to its ability to synthetize extracellular enzymes such as MnP, LiP or Lac [23]. As can be seen in Fig. 4.26, MnP and LiP enzymatic activities in F2 test were quite higher than those obtained in F1, achieving a maximum activity of 72 ± 3 U/L for LiP and 79 ± 2 U/L for MnP, after 7 and 6 days, respectively. Thus, pH values higher than 6.0 showed not to be optimal for ligninolytic enzymes production, since in F1 test the production of MnP and LiP was very low, which considerably reduced the removals of sCOD and colour. Islam et al. [23], who investigated the effect of fungal enzymes on the treatment of mature landfill leachate reported MnP and LiP of 12.78 U/L and 8.59 U/L, after 12 days of treatment by *P. chrysosporium*, respectively. The enzymatic activities measured in this work were quite higher than those previously reported. It should be noted that the ammonia concentration in the permeate used in this work was around 21 mg/L, since it was biologically pre-treated through a nitrification-denitrification process. High concentrations of ammonia have been associated with significant reductions in fungal enzyme activities [24], which could explain the highest values here obtained.

Regarding $sBOD_5$ concentration, data were only measured at intermediate (8 days) and final (15 days) of treatment. As can be seen in Table 4.9, not important changes were observed for both control and inoculated tests after 15 days the fungal treatment. However, it should be noted that for F2 test, the $sBOD_5$ values after 8 days were higher than the initial ones, reaching values of 52 ± 1 mg/L and giving a treated effluent with a BI of 0.04. After this time, the $sBOD_5$ values decreased to 23 ± 2 mg/L, which indicates that the fungus not only transformed non-biodegradable organic matter into biodegradable but also consumed it simultaneously with its generation.

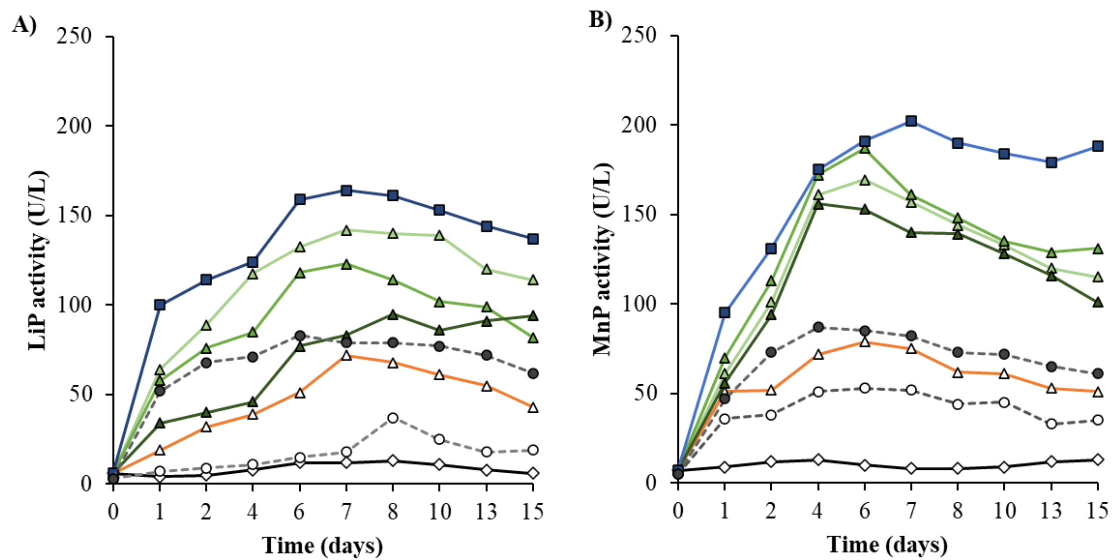


Figura 4. 26. *Evolution of LiP* ***(A)*** *and MnP* ***(B)*** *enzymatic activities during the treatment of permeate by P. chrysosporium. Solid lines correspond to tests carried out with undiluted permeate, whereas dashed lines show the data for the tests performed with diluted permeate. Besides, empty markers show the non-supplemented tests F1 (◇), F2 (△) and F7 (○), whereas solid lines and filled markers show the supplemented tests F3 (▲), F4 (▲), F5 (▲), F6 (■) and F8 (●). The percentage of standard deviation (SD) of the experimental data were in all cases less than 6% of mean value.*

Razarinah et al. [25], who evaluated the treatment of landfill leachate by immobilized *Trametes menziesii*, reported that fungus can not only reduce 50% of BOD_5 but it also degraded 32% of COD after 7 days in a batch reactor at 28ºC and 150 rpm. The fungi immobilization in different supports have been related with best decolourization and organic matter removal efficiencies [17,26]. The fungal treatment in this work was performed with free fungal granules, which could explain the lower removal efficiencies obtained in F2 compared to those reported by other authors who treat recalcitrant effluents.

To evaluate the impact of pH on biomass accumulation, TS was measured at the beginning of the experiments and after 15 days (See Table 4.9). Due to the increase in TS for control tests (non-inoculated) were in all cases lower than 2% (data not shown), the increase of TS was directly related with fungal growth, dismissing the endogenous microbiota contribution. Hence, comparing the data obtained for the F1 and F2 tests, the TS concentration was approximately 4 times higher when the experiment was carried out at pH between 5.7-6.1. Taking all the above into account, subsequent studies focused on determining the effect of carbon and nitrogen supplementation as well as the influence of permeate dilution were carried out keeping the pH at a value around 6.

Tabla 4. 9. *Effect of fungal treatment on sCOD, colour, sBOD$_5$, BI and TS at intermediate (8 days) and final times (15 days) of treatment. Negative percentages of degradation indicate that the sample suffered an increase in the value of the parameter studied.*

Test	Time	sCOD		Colour		sBOD$_5$	BI	TS	
	(days)	(mg/L)	*% red.*	(CN)	*% red.*	(mg/L)		(g/L)	% incr.
F1	0	1265,5 ± 9,6		0,88 ± 0,01		28 ± 3	0,022 ± 0,001	3,02 ± 0,01	
	8	1241,4 ± 7,4	*1,9 ± 0,3*	0,85 ± 0,02	*3,31 ± 0,01*	25 ± 1	0,020 ± 0,002		
	15	1225,5 ± 5,4	*3,1 ± 0,2*	0,88 ± 0,02	*-0,68 ± 0,05*	23 ± 2	0,020 ± 0,002	3,10 ± 0,02	*2,64 ± 0,02*
F2	0	1265,5 ± 9,6		0,88 ± 0,01		28 ± 3	0,022 ± 0,001	2,98 ± 0,03	
	8	1217,6 ± 6,3	*3,8 ± 0,1*	0,75 ± 0,06	*14,37 ± 0,05*	52 ± 1	0,043 ± 0,001		
	15	1110,0 ± 15,1	*12,3 ± 0,5*	0,73 ± 0,03	*16,53 ± 0,11*	26 ± 3	0,023 ± 0,005	3,31 ± 0,01	*11,07 ± 0,02*
F3	0	2440,8 ± 13,2		0,87 ± 0,01		603 ± 3	0,247 ± 0,002	3,01 ± 0,05	
	8	1072,2 ± 8,5	*15,3 ± 0,4*	0,65 ± 0,07	*25,28 ± 0,12*	73 ± 2	0,068 ± 0,001		
	15	979,2 ± 9,4	*22,6 ± 0,7*	0,69 ± 0,03	*20,82 ± 0,09*	25 ± 2	0,026 ± 0,004	3,96 ± 0,06	*31,56 ± 0,12*
F4	0	4551,1 ± 8,3		0,87 ± 0,01		1804 ± 5	0,396 ± 0,010	3,07 ± 0,04	
	8	1247,2 ±10,8	*1,5 ± 0,1*	0,63 ± 0,04	*27,58 ± 0,01*	78 ± 4	0,062 ± 0,002		
	15	998,4 ± 7,5	*21,1 ± 0,9*	0,68 ± 0,05	*22,08 ± 0,08*	29 ± 1	0,029 ± 0,003	4,19 ± 0,04	*36,47 ± 0,03*

Table 4.9. *(continued)*

Test	Time	sCOD		Colour		$sBOD_5$	BI	TS	
	(days)	(mg/L)	*% red.*	(CN)	*% red.*	(mg/L)		(g/L)	% incr.
F5	0	6589,1 ± 13,8		0,87 ± 0,01		2513 ± 4	0,381 ± 0,005	2,99 ± 0,04	
	8	1347,5 ± 9,7	*-6,5 ± 0,3*	0,63 ± 0,01	*23,68 ± 0,06*	272 ± 5	0,202 ± 0,002		
	15	1064,3 ± 9,6	*15,9 ± 0,5*	0,72 ± 0,03	*17,96 ± 0,18*	31 ± 2	0,029 ± 0,002	4,36 ± 0,04	*45,82 ± 0,11*
F6	0	2512,1 ± 7,9		0,84 ± 0,01		615 ± 3	0,245 ± 0,001	3,01 ± 0,06	
	8	917,1 ± 6,1	*27,5 ± 0,1*	0,63 ± 0,04	*25,03 ± 0,01*	112 ± 2	0,067 ± 0,001		
	15	845,2 ± 7,4	*33,2 ± 0,9*	0,53 ± 0,05	*37,72 ± 0,16*	41 ± 1	0,047 ± 0,003	4,02 ± 0,08	*33,55 ± 0,15*
F7	0	618,2 ± 4,1		0,54 ± 0,03		13 ± 3	0,021 ± 0,002	3,01 ± 0,05	
	8	557,6 ± 6,7	*9,8 ± 0,4*	0,47 ± 0,02	*2,61 ± 0,08*	18 ± 4	0,032 ± 0,001		
	15	553,3 ± 5,2	*10,5 ± 0,7*	0,47 ± 0,08	*12,87 ± 0,12*	14 ± 3	0,025 ± 0,001	3,13 ± 0,01	*3,98 ± 0,02*
F8	0	1790,5 ± 9,8		0,54 ± 0,01		586 ± 4	0,327 ± 0,001	3,02 ± 0,06	
	8	512,4 ± 6,6	*17,1 ± 0,1*	0,46 ± 0,05	*14,74 ± 0,11*	36 ± 2	0,070 ± 0,003		
	15	508,0 ± 11,3	*17,8 ± 0,2*	0,44 ± 0,06	*17,54 ± 0,11*	18 ± 2	0,035 ± 0,003	3,21 ± 0,01	*6,29 ± 0,05*

3.2 Effect of supplementation with carbon and nitrogen sources

As can be seen in Table 4.7, the initial $sBOD_5$ and ammonium concentrations in the permeate used in this work were 28 ± 5 mg O_2/L and 21 ± 3 mg/L, respectively. These values are very low due to this effluent has been previously subjected to a nitrification-denitrification process. Considering the low removal efficiencies obtained for F2 test, and the scarcity of easily assimilable carbon and nitrogen sources in the permeate, the addition of glucose and ammonium tartrate as co-substrates was evaluated.

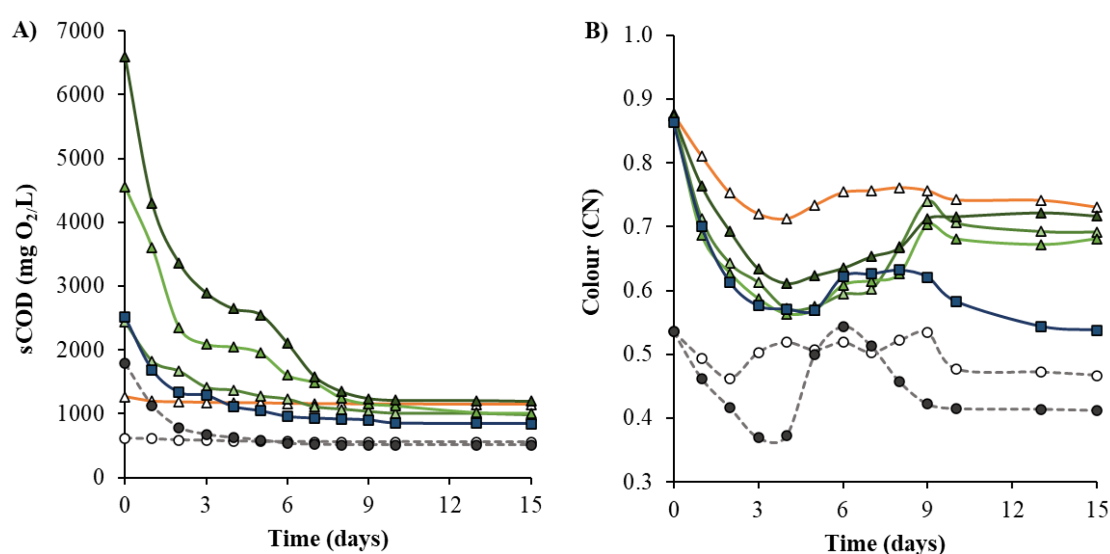


Figura 4. 27. *Evolution of sCOD concentration **(A)** and colour **(B)** during the treatment of permeate effluent by P. chrysosporium at controlled pH. In both cases, solid lines correspond to tests carried out with undiluted permeate, whereas dashed lines show the data for the experiments performed with diluted permeate. Besides, empty markers show the non-supplemented tests, F2 (△) and F7 (○), whereas solid lines and filled markers show the supplemented tests F3 (▲), F4 (▲), F5 (▲), F6 (■) and F8 (●). In F3, F4 and F5 tests, 1 g/L, 3 g/L and 5 g/L of glucose was added as co-substrate, respectively. In F6 and F8 tests, 1 g/L of glucose and 0,5 g/L of ammonium tartrate were supplied. The percentage of standard deviation (SD) of the experimental data were in all cases less than 8% of mean value.*

The supplementation with different carbon and nitrogen sources to improve the fungal treatment has been widely employed by previous authors to treat recalcitrant effluents [13,27,28]. As can be seen in Fig. 4.27.A, for the undiluted permeate, the evolution of sCOD concentration during the fungal treatment was very similar regardless the glucose concentration added. In all cases, the sCOD degradation was progressive until 9th day of treatment, and from this time on, concentration remained almost constant. For both F3 and F4 tests, final sCOD removal was very similar,

achieving values of 979 ± 9 mg/L and 998 ± 8 mg/L after 15 days of treatment, which corresponds with a 23% and 21% of sCOD degradation, respectively (See Table 4.9). This percentage was lower when 5 g/L of glucose were added, which indicated that concentration higher than 3 g/L of glucose was unfavourable for the fungal treatment.

For colour removal, maximum decolourization efficiency (22%) was achieved when 3 g/L of glucose was added, however this decolourization efficiency is almost similar to that obtained using 1 g/L of glucose. Again, the supplementation of 5 g/L gave the worse results, removing only 18% of the colour in F5 test. In addition, it must be considered that in just 8 days, the addition of 1 g/L in the F3 test achieved sCOD and colour removals higher than those obtained after 15 days in F5, where the added glucose was not consumed at 8 days, since the sCOD values were higher than the initial ones.

Regarding the MnP and LiP enzymatic activities, significant differences were observed according to the amount of glucose added, especially in the case of LiP. As can be seen in Fig. 4.26, the LiP activity measured during the treatment was inversely proportional to the concentration of glucose added, obtaining the highest values in F3 test, which was reflected in the higher sCOD removals. This fact might explain the greater increase in the colour compared to F4 and F5, since the degradation of recalcitrant organic matter can give rise to by-products that increase the colour of the effluent [29]. In the case of MnP activity, mainly related to decolourization processes, F4 test showed the highest values, which was correlated with the highest colour removals obtained. Saetang et al. [19], reported a maximum LiP activity of 193 U/L and MnP activity 437 U/L, when a landfill leachate was treated without glucose addition, reaching a 12% of decolourization. Meanwhile, when the leachate was supplied with 3 g/L of glucose, the enzymatic activities were increased until values of 384 U/L and 1241 U/L for LiP and MnP, respectively. Consequently, a 40% of COD removal and 58% of decolourization was achieved. In this work, for the tests where only glucose was added, the maximum enzymatic activity for LiP was measured in F3 test with values 142 ± 3 U/L after 7 days, whereas the maximum MnP value was obtained in F4 test with a 187 ± 4 U/L after 6 days, which could explain the lower degradations of sCOD, and colour achieved.

In both cases, the supplementation with 5 g/L of glucose was not effective, decreasing the enzymatic activity of the fungus. Radha et al. [30], who investigated the

treatment of an effluent from dye-based industries by *P.chrysosporium*, reported that glucose concentrations higher than 5 g/L decreased the decolourization rate. Results here obtained seem to indicate that when glucose concentrations are higher than 3 g/L, *P. chrysosporium* only consumes glucose and does not break down more complex compounds. This fact was reflected in a greater growth of the fungus when glucose concentrations were increased (see Table 4.9), without significantly improving of colour or sCOD removals, or even worsening fungal treatment, as in the case of F5.

Regarding BOD_5 concentration (Table 4.9), the behaviour was similar to that obtained without supplementation (F2). As soon as fungus broke recalcitrant compounds into by-products more biodegradable, they were consumed by the microorganisms. Final BI were also very low with final values between 0.02-0.03. However, it should be noted that if the values measured after 8 days are considered, the addition of 1 g/L allowed to obtain a treated permeate with a biodegradability index 3.5 times higher than the initial one. In view of these results, the concentration of 1 g/L was considered the best option to enhance the fungal treatment.

In addition to carbon sources, previous studies have reported that nitrogen and phosphorus concentrations in the effluent also play a key role in the production of fungal ligninolytic enzymes [24,30]. As can be seen in Fig. 4.26 and Fig. 4.27, the main differences observed between F3 test, where only 1 g/L of glucose was used, or F6, supplemented with 1 g/L of glucose and 0.5 g/L of ammonium tartrate, were observed with respect to the colour degradation and enzymatic activities synthesis, especially for MnP.

For F6 test, decolourizations and sCOD degradations obtained in only 8 days were higher than those obtained for F3 after 15 days, achieving final removals of 38% and 33%, for colour and sCOD, respectively. So that, to supply the permeate with 0.5 g/L of ammonium tartrate almost duplicated the final decolourization rate. Unlike F3 test, where MnP activity abruptly decreased after 6 days of treatment, in F6 the concentration of MnP activity was slightly reduced. This fact could explain the slight increase in colour observed in F6 between the 6th and 9th days of fungal treatment. Moreover, it is important to note that although the $sBOD_5$ values were also reduced during the fungal treatment, for F6 test a treated permeate with a BI of 0.07 and 0.05 was obtained, after 8 and 15 days, respectively.

Finally, comparing the experiments where an external nutrient source was added (F3, F4, F5 and F6) with F2 test, where no carbon or nitrogen source was used, results indicate that some easily degradable carbon or nitrogen source is required to enhance the growth of *P. chrysosporium* and the release of ligninolytic enzymes; otherwise, growth and fungal activity are limited, and degradation efficiency is low.

3.3 Effect of permeate dilution

Landfill leachate can contain a large load of highly toxic compounds [31], so a fungal treatment with a diluted permeate was tested with the aim of reducing the concentration of some compounds that could hinder biological treatment. Results showed that the dilution of permeate did not affect the biodegradation process, achieving similar efficiencies than those obtained for F2 test, which was performed with undiluted effluent. Better results were showed when the diluted permeate was supplied with 1 g/L of glucose and 0.5 g/L of ammonium tartrate, showing higher enzymatic activity than those observed for F2 test, with final decolourizations and sCOD removals around 18%. However, these removal percentages were lower than those obtained in F6, which was carried out under the same operational conditions and using undiluted permeate.

4. CONCLUSIONS

In this work, an attempt to treat a biologically and physical-chemically pre-treated landfill leachate with *P. chrysosporium* was carried out. Results showed that the addition of external nutrient sources were necessary for an optimal growth of the fungus as well as to improve the synthesis of ligninolytic enzymes. In this sense, it was shown that the addition of 1 g/L of glucose as a co-substrate was enough to significantly improve the sCOD and colour removals, while the use of a concentration of 5 g/L abruptly decreased the effectiveness of the fungal treatment. Moreover, when 0.5 g/L of ammonium tartrate were added, the decolourization rate was almost doubled. Finally, the dilution of the permeate did not show best results in sCOD and colour removal. Therefore, the treatment of the undiluted permeate supplemented with 1 g/L of glucose and 0.5 g/L of ammonium tartrate reported the best results, achieving final removals of 38% and 33%, for colour and sCOD, respectively.

Consequently, the use of fungi may be hopeful technology for the removal of colour and recalcitrant organic matter of these type of effluents. Data obtained in this study could be used as basis for future research in order to optimize the most suitable operational parameters for the fungal treatment of ultrafiltration permeates from leachate treatment plants

ACKNOWLEDGMENTS

This work was co-financed by Spanish MINECO (Project CTM2015-63864-R) and Foundation for the Promotion of Applied Scientific Research and Technology in Asturias (Project FC-GRUPIN-IDI/2018/000127). Ana Isabel Díaz acknowledges an FPI grant from Spanish MICINN (BES-2016-077963).

REFERENCES

[1] K. Wang, L. Li, F. Tan, D. Wu, Treatment of Landfill Leachate Using Activated Sludge Technology: A Review, Archaea. 2018 (2018) 1–10. https://doi.org/10.1155/2018/1039453.

[2] A.I. Díaz, P. Oulego, A. Laca, J.M. González, M. Díaz, Metagenomic Analysis of Bacterial Communities from a Nitrification–Denitrification Treatment of Landfill Leachates, CLEAN – Soil, Air, Water. 47 (2019) 1900156. https://doi.org/10.1002/clen.201900156.

[3] I.A. Tałałaj, P. Biedka, I. Bartkowska, Treatment of landfill leachates with biological pretreatments and reverse osmosis, Environ. Chem. Lett. 17 (2019) 1177–1193. https://doi.org/10.1007/s10311-019-00860-6.

[4] E.R. Bandala, A. Liu, B. Wijesiri, A.B. Zeidman, A. Goonetilleke, Emerging materials and technologies for landfill leachate treatment: A critical review, Environ. Pollut. 291 (2021) 118133. https://doi.org/10.1016/J.ENVPOL.2021.118133.

[5] V. Torretta, N. Ferronato, I. Katsoyiannis, A. Tolkou, M. Airoldi, Novel and Conventional Technologies for Landfill Leachates Treatment: A Review, Sustainability. 9 (2016) 9. https://doi.org/10.3390/su9010009.

[6] P. Oulego, S. Collado, A. Laca, M. Díaz, Tertiary treatment of biologically pre-treated landfill leachates by non-catalytic wet oxidation, Chem. Eng. J. 273 (2015) 647–655. https://doi.org/10.1016/j.cej.2015.03.089.

[7] P. Ghosh, I.S. Thakur, Treatment of Landfill Leachate Using Fungi: An Efficient and Cost-Effective Strategy, in: Dev. Fungal Biol. Appl. Mycol., Springer Singapore, Singapore, 2017: pp. 341–357. https://doi.org/10.1007/978-981-10-4768-8_18.

[8] S. Collado, P. Oulego, O. Suárez-Iglesias, M. Díaz, Leachates and natural organic matter. A review of their biotreatment using fungi, Waste Manag. 96 (2019) 108–120. https://doi.org/10.1016/J.WASMAN.2019.07.018.

[9] P. Ghosh, I.S. Thakur, Biosorption of landfill leachate by *Phanerochaete* sp. ISTL01: isotherms, kinetics and toxicological assessment, Environ. Technol. 38 (2017) 1800–1811. https://doi.org/10.1080/09593330.2016.1244218.

[10] L. Hu, Y. Liu, G. Zeng, G. Chen, J. Wan, Y. Zeng, L. Wang, H. Wu, P. Xu, C. Zhang, M. Cheng, T. Hu, Organic matters removal from landfill leachate by immobilized Phanerochaete chrysosporium loaded with graphitic carbon nitride under visible light irradiation, Chemosphere. 184 (2017) 1071–1079. https://doi.org/10.1016/j.chemosphere.2017.06.065.

[11] M. Islam, Q. Yuan, Fungal treatment of mature landfill leachate utilizing woodchips and wheat-straw as co-substrates, Biodegradation. 31 (2020) 109–122. https://doi.org/10.1007/s10532-020-09897-9.

[12] F. Spina, V. Tigini, A. Romagnolo, G.C. Varese, Bioremediation of landfill leachate with fungi: Autochthonous vs. allochthonous strains, Life. 8 (2018). https://doi.org/10.3390/life8030027.

[13] A.I. Díaz, A. Laca, M. Díaz, Fungal treatment of an effluent from sewage sludge digestion to remove recalcitrant organic matter, Biochem. Eng. J. 172 (2021) 108056. https://doi.org/10.1016/j.bej.2021.108056.

[14] Y.K. Kim, S.K. Park, S. Do Kim, Treatment of landfill leachate by white rot fungus in combination with zeolite filters, J. Environ. Sci. Heal. - Part A

Toxic/Hazardous Subst. Environ. Eng. 38 (2003) 671–683. https://doi.org/10.1081/ESE-120016932.

[15] D.S. Lisboa, C. Santos, R.N. Barbosa, O. Magalhães, L.M. Paiva, K.A. Moreira, N. Lima, C.M. Souza-Motta, Requalification of a Brazilian Trichoderma collection and screening of its capability to decolourise real textile effluent, Int. J. Environ. Res. Public Health. 14 (2017) 1–16. https://doi.org/10.3390/ijerph14040373.

[16] APHA;, AWWA;, WEF, Standard Methods for Examination of Water and Wastewater (23th ed.), Am. Public Heal. Assoc. Washingt. DC. (2005).

[17] L. Hu, G. Zeng, G. Chen, H. Dong, Y. Liu, J. Wan, A. Chen, Z. Guo, M. Yan, H. Wu, Z. Yu, Treatment of landfill leachate using immobilized Phanerochaete chrysosporium loaded with nitrogen-doped TiO 2 nanoparticles, J. Hazard. Mater. 301 (2016) 106–118. https://doi.org/10.1016/j.jhazmat.2015.08.060.

[18] Y. Lu, L. Yan, Y. Wang, S. Zhou, J. Fu, J. Zhang, Biodegradation of phenolic compounds from coking wastewater by immobilized white rot fungus Phanerochaete chrysosporium, J. Hazard. Mater. 165 (2009) 1091–1097. https://doi.org/10.1016/j.jhazmat.2008.10.091.

[19] J. Saetang, S. Babel, Effect of glucose on enzyme activity and color removal by Trametes versicolor for high strength landfill leachate, Water Sci. Technol. 62 (2010) 2519–2526. https://doi.org/10.2166/wst.2010.552.

[20] S. Vylkova, Environmental pH modulation by pathogenic fungi as a strategy to conquer the host, PLoS Pathog. 13 (2017). https://doi.org/10.1371/JOURNAL.PPAT.1006149.

[21] N. Liaud, C. Giniés, D. Navarro, N. Fabre, S. Crapart, I.H.- Gimbert, A. Levasseur, S. Raouche, J.-C. Sigoillot, Exploring fungal biodiversity: organic acid production by 66 strains of filamentous fungi, Fungal Biol. Rev. 1 (2014). http://linkinghub.elsevier.com/retrieve/pii/S1749461309000177.

[22] M. Sancha, A. Laca, A. Laca, J.M. González, B. Mayo, M. Díaz, Approaches for microbiological characterization of a landfill leachate treatment, 11 (2014) 39–

44.

[23] M. Islam, A. Wai, G. Hausner, Q. Yuan, Effect of lignocellulosic enzymes on the treatment of mature landfill leachate, J. Environ. Manage. 233 (2019) 400–409. https://doi.org/10.1016/J.JENVMAN.2018.12.045.

[24] M. Ellouze, F. Aloui, S. Sayadi, Effect of high ammonia concentrations on fungal treatment of Tunisian landfill leachates, Desalination. 246 (2009) 468–477. https://doi.org/10.1016/J.DESAL.2008.03.068.

[25] W. Abdul Razak Wan Razarinah, M. Noor Zalina, N. Abdullah, Treatment of landfill leachate by immobilized Ganoderma australe and crude enzyme, ScienceAsia. 40 (2014) 335. https://doi.org/10.2306/scienceasia1513-1874.2014.40.335.

[26] M. Sedighi, A. Karimi, F. Vahabzadeh, Involvement of ligninolytic enzymes of Phanerochaete chrysosporium in treating the textile effluent containing Astrazon Red FBL in a packed-bed bioreactor, J. Hazard. Mater. 169 (2009) 88–93. https://doi.org/10.1016/j.jhazmat.2009.03.070.

[27] A. Bardi, Q. Yuan, G. Siracusa, I. Chicca, M. Islam, F. Spennati, V. Tigini, S. Di Gregorio, D.B. Levin, G. Petroni, G. Munz, G. Petroni, G. Munz, Effect of cellulose as co-substrate on old landfill leachate treatment using white-rot fungi, Bioresour. Technol. 241 (2017) 1067–1076. https://doi.org/10.1016/J.BIORTECH.2017.06.046.

[28] W.D.H. Schneider, R.C. Fontana, S. Mendonça, F.G. de Siqueira, A.J.P. Dillon, M. Camassola, High level production of laccases and peroxidases from the newly isolated white-rot basidiomycete Marasmiellus palmivorus VE111 in a stirred-tank bioreactor in response to different carbon and nitrogen sources, Process Biochem. 69 (2018) 1–11. https://doi.org/10.1016/j.procbio.2018.03.005.

[29] N. Junnarkar, N. Pandhi, N. Raiyani, N. Bhatt, R. Raiyani, Production of LiP by Phanerochaete chrysosporium MTCC 787 Through Solid State Fermentation of Wheat Straw and Assessing its Activity Against Reactive Black B, Int. J. Adv. Res. J. 4 (2016) 812–819. http://www.journalijar.com/uploads/275_IJAR-8575.pdf.

[30] K. V. Radha, I. Regupathi, A. Arunagiri, T. Murugesan, Decolorization studies of synthetic dyes using Phanerochaete chrysosporium and their kinetics, Process Biochem. 40 (2005) 3337–3345. https://doi.org/10.1016/j.procbio.2005.03.033.

[31] S. Renou, J.G. Givaudan, S. Poulain, F. Dirassouyan, P. Moulin, Landfill leachate treatment: Review and opportunity, J. Hazard. Mater. 150 (2008) 468–493. https://doi.org/10.1016/j.jhazmat.2007.09.077.

4.3. Tratamiento de residuos agropecuarios

En este apartado se recogen todas las investigaciones relacionadas con el tratamiento de residuos de origen agropecuario, concretamente de descartes alimentarios (tomate, patata y pimiento) y residuos agroalimentarios (alperujo y purines). Todos estos residuos destacan por su contenido en materia orgánica compleja que dificulta su tratamiento por métodos convencionales. Los estudios recogidos en este apartado, se han enfocado en la aplicación de tratamientos adicionales que permitan la degradación de estos compuestos complejos y/o valorización de los residuos para obtener productos de interés, como es el caso de los descartes alimentarios

Por ello, esta sección está compuesta por tres apartados, en el **4.3.1** se ha llevado a cabo la evaluación de diferentes tratamientos de hidrólisis para la obtención de bioetanol empleando como materia prima descartes alimentarios, considerando los volúmenes de producción y descarte de un supermercado de la región. Estos resultados se recogen en un artículo publicado cuya cita se destaca al inicio del apartado. En el punto **4.3.2**, se ha evaluado el empleo del hongo de pudrición blanca *P. chrysosporium*, para el tratamiento biológico del alperujo, un efluente procedente de la industria del aceite de oliva. Los resultados obtenidos muestran la capacidad del hongo para degradar compuestos fenólicos y amplían las aplicaciones biotecnológicas del mismo. Estos resultados se han publicado en un artículo cuya cita se destaca al inicio del apartado. Finalmente, en el apartado **4.3.3.** se ha llevado a cabo un estudio sobre la aplicación del hongo en el tratamiento de purines procedentes de la una granja de cerdos, así como de dos purines pretratados biológicamente mediante digestión anaerobia, mostrando la posibilidad de acoplar este tratamiento fúngico a otros tratamiento convencionales.

4.3.1. Treatment of supermarket vegetable wastes to be used as alternative substrates in bioprocesses

Ana Isabel Díaz, Amanda Laca, Adriana Laca, and Mario Díaz*

Department of Chemical and Environmental Engineering. University of Oviedo.

C/ Julián Clavería s/n. 33071 Oviedo. Spain

*Corresponding author: mariodiaz@uniovi.es

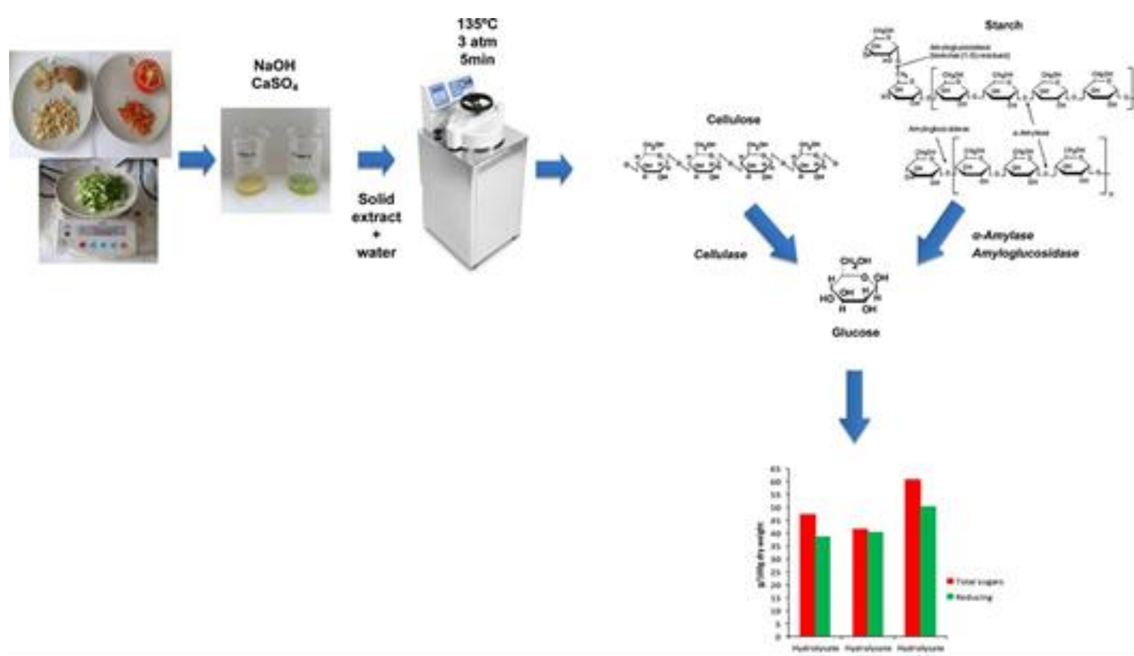


Figura 4. 28. *Resumen gráfico del trabajo 6*

*Artículo publicado en la revista "**Waste Management**"

Cita: Ana Isabel Díaz, Amanda Laca, Adriana Laca and Mario Díaz. *Treatment of supermarket vegetable wastes to be used as alternative substrates in bioprocesses*. Waste Management, Volume 67, **2017**, Pages 59-66, ISSN 0956-053X, https://doi.org/10.1016/j.wasman.2017.05.018.

ABSTRACT

Fruits and vegetables have the highest wastage rates at retail and consumer levels. These wastes have promising potential for being used as substrates in bioprocesses. However, an effective hydrolysis of carbohydrates that form these residues has to be developed before the biotransformation.

In this work, vegetable wastes from supermarket (tomatoes, green peppers, and potatoes) have been separately treated by acid, thermal and enzymatic hydrolysis processes in order to maximise the concentration of fermentable sugars in the final broth. For all substrates, thermal and enzymatic processes have shown to be the most effective. A new combined hydrolysis procedure including these both treatments was also assayed, and the enzymatic step was successfully modelled. With this combined hydrolysis, the percentage of reducing sugars extracted was increased, in comparison with the amount extracted from non-hydrolysed samples, approximately by 30% in the case of tomato and green peeper wastes. For potato wastes this percentage increased from values lower than 1% to 77%. In addition, very low values of fermentation inhibitors were found in the final broth.

Keywords: Thermal hydrolysis, enzymatic hydrolysis, vegetable food wastes, cellulase, starch, reducing sugars, fermentation inhibitors

1. INTRODUCTION

One-third of all food produced globally for human consumption, about 1.3 billion tons, is lost each year according to the Food and Agriculture Organization (Gustavsson et al., 2011). Food is wasted throughout the food supply chain, from initial agricultural production down to final household consumption. Actually, in industrialized countries more than 40% of the food losses occur at retail and consumer levels. In the European Union this loss of produced food is attributed as: 5.5% by improper post-harvest handling, 20% by supermarkets and food industries, 7.5% due to expiring best-before-date, and 13% as consumer household garbage for not being consumed (Nanda et al., 2015). In fact, nowadays, food waste represents worldwide the single largest component of municipal solid waste reaching landfills (Kosseva, 2013).

In this context, fruits and vegetables have the highest wastage rates and, specifically, in the European Union, about 50% of all fruits and vegetables go to waste throughout the entire food chain (Gustavsson et al., 2011; Nanda et al., 2015). Food wastes are a significant global problem for economic, environmental and food security reasons. Therefore, government efforts have focused on diverting waste away from landfill through regulation, taxation, and public awareness. According to the European Landfill Directive (1999/31/EC), the amount of biodegradable waste sent to landfills in member countries by 2016 must be 35% of the levels reached in 1995 (Kosseva, 2013). The solid extract of these wastes is mainly constituted by carbohydrates, proteins, lipids and minor amounts of vitamins and minerals. Indeed, carbohydrates are the main component of fruit and vegetables and represent 70-90% of their dry weight. So, due to their high polysaccharide content, waste fruits and vegetable have promising potential for being converted into value-added products, such as fuels or chemicals, through thermochemical and biological pathways (Nanda et al., 2015).

Several works have been carried out to transform these wastes to value added products like enzymes, organic acids, flavouring compounds, food colorants, bioethanol, bio-methane, etc. (Panda et al., 2016; Ravindran and Jaiswal, 2016). In the past decade, important issues about the world climate change, along with the rising demand for renewable energy, have led to the development of alternative technologies for the production of biofuels like ethanol or butanol (Aguiar et al., 2013). Current production of bioethanol relies on ethanol from starch and sugars but there has been considerable debate about its sustainability (Alvira et al., 2010). For this reason, biofuels produced from food vegetable wastes is an interesting alternative (Kennes et al., 2016). Many of vegetal carbohydrates are in polymeric form, so production of fuels and/or chemicals from these waste materials need to be accomplished by hydrolysing the starch, cellulose and/or hemicellulose to soluble sugars, which can be fermented to the desired products (Thulluri et al., 2013), such as ethanol and hydrogen (Han et al., 2015; Han et al., 2017). Thereof, the pre-treatment of the wastes to facilitate the polysaccharides hydrolysis is a key step in the biotechnological procedure of revalorization.

Nowadays various pre-treatment methods have been developed which include physical, chemical, biological, thermal approaches and even combined processes (Thulluri et al., 2013; Vavouraki et al., 2014; Wu et al., 2016). In addition, it is well

known that hydrolysis that require severe conditions (high temperatures, low pH…), may be limited to be used as pre-treatment for bioprocesses because of the formation of fermentation inhibitory by-products like furfural or 5-hydroxymethylfurfural (HMF) (Khawla et al., 2014; Wu et al., 2016). Hence, it can be concluded that a pre-treatment of vegetable wastes to facilitate the hydrolysis of polysaccharides into monomeric sugars results to be fundamental for revalorization processes via microbial ways. In this work, vegetable supermarket wastes (tomatoes, green peppers and potatoes) have been treated by different hydrolysis procedures with the purpose to maximise the concentration of fermentable sugars in the final broth.

The assayed treatments were evaluated by means of monitoring the evolution of sugar concentrations and analysing the possible formation of fermentation inhibitors (furfural, HMF and acetic acid). The last aim was to optimise the hydrolysis process in order to use vegetable wastes as raw material for fermentation processes.

2. MATERIALS AND METHODS

2.1 Raw materials

Vegetable wastes (tomatoes, green peppers, and potatoes) was selected as model vegetables taking into account three factors:

i) These wastes represent a considerable percentage of total vegetable wastes generated at retail (the three usually sum up approximately 40% of total wastes in the local supermarket that supplied the raw materials).

ii) The carbohydrates of these wastes are mainly complex polymers that need to be hydrolysed.

iii) These vegetables are not seasonal products, and their wastes are available along all the year.

Wastes were supplied by a local market and once in the laboratory they were washed with distilled water and stored at 4ºC during a maximum of three days until being treated. Additionally, nutritional composition and moisture content of these vegetables are shown in Table 4.10.

Table 4. 10. *Nutritional composition of the vegetables employed as substrate (given per 100 g of fresh product).*

	Carbohydrates (g)*	**Lipids (g)***	**Proteins (g)***	**Water content (g)****	**Vitamins (mg)***	**Minerals (mg)***
Tomato	3.9	0.2	0.9	93.0	15.7[1]	272[1]
Pepper	4.6	0.2	0.9	93.5	133[2]	218[2]
Potato	17	0.1	2.0	63.5	21.5[3]	521[3]

*Average values adapted from USDA (United States Department of Agriculture)

**Average values of own data

[1]Vitamins (A, B_1, B_3, B_6, C, E, K) / Minerals (Mg, Mn, P, K)

[2]Vitamins (A, B_1, B_2, B_3, B_6, C, E, K) / Minerals (Ca, Fe, Mg, P, K, Na, Zn)

[3]Vitamins (B_1, B_2, B_3, B_6, C) / Minerals (Ca, Fe, Mg, P, K, Na)

2.2 Determination of sugars in raw materials

2.2.1 Potential reducing sugars

For the determination of potential reducing sugars, samples were treated according to Lenihan et al. (2011). Samples were first ground in pieces under 2 mm. An amount of 0.3 g of minced sample was introduced into a test tube and 3 ml of 85% H_2SO_4 that has been cooled to 15ºC was added. Samples were stirred thoroughly before being placed in a water bath at 30ºC. This temperature was maintained for 2 h, stirring the samples every 10 minutes. After this time, the mixture was washed from the tube into an Erlenmeyer flask and distilled water was added to 89.11 g. The dilute solution was then autoclaved at 121ºC and 1 atm for 1 h. Finally, sample was cooled to room temperature, then bigger solids were removed passing the sample through a 1 mm mesh sieve and the liquid was centrifuged at 20ºC and 5000 rpm during 5 min (Kubota 6500 High Speed Refrigerated Centrifuge) to remove the remaining particles. Supernatant was frozen and potential reducing sugars were determined in these samples according to the DNS method described in 2.4.1 section.

2.2.2 Soluble sugars

In order to determine the amount of total sugars and reducing sugars that can be extracted from the considered wastes just by using water, they were treated as follows. Distilled water was added to waste in a relation 1:2 (100 g of waste and 200 mL of water) and the mixture was homogenized in a kitchen blender during 3 min. Solids were removed with a sieve and the liquid phase was centrifuged at 20ºC and 5000 rpm during 5 min (Kubota 6500 High Speed Refrigerated Centrifuge). The pellet was discarded, and the supernatant were frozen until being analysed, as described in section 2.4.

2.3. Hydrolysis treatments

Each treatment was assayed with each kind of waste separately carried out at least in triplicate using different batches of each residue. All reagents employed in the different hydrolysis were supplied by Sigma-Aldrich.

2.3.1 Thermal hydrolysis

A) This treatment was modified from Del Campo et al. (2006) as follows, 100 g of each waste were minced in pieces smaller than 2 cm, and then this material was dried at 55ºC during 24 h in an incubator (Heidolph Unimax 2010). The dried material was grinded in a kitchen robot (Moulinex Minirobot D81) obtaining a particle size below 2 mm. Distilled water was added to the grinded samples in a relation of 5% (w/v) in 250 mL Pyrex bottles, which were treated in an autoclave at 110ºC and 1.5 atm during 5 min. Solids were removed with a sieve and the liquid phase was adjusted to pH 6.5-7 with 6 M NaOH or 1M HCl. Finally, samples were centrifuged at 20ºC and 5000 rpm during 5 min (Kubota 6500 High Speed Refrigerated Centrifuge) and supernatants were frozen until being analysed.

B) According to Correa et al. (2012) procedure, distilled water was added to 10 g of each waste in a relation 1:1 (w/v) and the mixture was homogenized in a kitchen blender. The samples were then treated in an autoclave at 135ºC and 3 atm during 5 min. After that, samples were treated in the same way as described in A) section.

2.3.2 Acid hydrolysis

Lignin is the most recalcitrant component of the plant cell wall, and the higher the proportion of lignin, the higher the resistance to chemical and enzymatic degradation (Taherzadeh and Karimi, 2008). Indeed, lignin acts as a physical barrier that protects polysaccharides from enzyme action, so lignin removal increases enzyme effectiveness by eliminating non-productive adsorption sites and by increasing access to cellulose and hemicellulose (Kumar et al., 2009; Sun et al., 2011). Hence, with the aim of removing the lignin from the samples before the acid hydrolysis, 10 g of each sample was minced in the kitchen robot (Moulinex Minirobot D81) until a particle size below 2 mm was obtained. Then, samples were immersed in 20 mL of NaOH 0.1N and, after 15 min 0.8 g of $CaSO_4$ were added and the mixtures were let stand for 3 hours at room temperature. After this time, liquid was removed employing a sieve and solid residue was washed twice with distilled water.

Solid material was mixed with 5% H_2SO_4 in a relation 2:1 (w/v) and then it was treated in an autoclave at 125ºC and 2 atm during 15 min. Finally, solids were removed

with a sieve and the liquid phase was neutralized to pH 6.5-7 with NaOH 6M, centrifuged and supernatants were frozen until being analysed (Monsalve et al., 2006).

2.3.3 Enzymatic hydrolysis

The first step in the enzymatic hydrolysis consisted on the delignification of samples as it was described in section 2.3.2. After that, distilled water was added to solid residue in a 6% relation (w/v), pH was adjusted to 4.5 and the mixture was incubated in a bath at 75ºC during 5 min. Once samples were cooled to room temperature, the combination of enzymes was added as follows: 83 µl of cellulase from *Trichoderma reesei* (C2730) (enzymatic activity ≥ 700 Beta-Glucanase units/g, density 1.10-1.30 g/mL), 50 µl of α-amylase from *Aspergillus oryzae* (A8220) (enzymatic activity ≥ 800 Fungal Alpha Amylase units/g, density 1.10-1.30 g/mL) and 8 µl of amyloglucosidase from *Aspergillus niger* (A7095) (enzymatic activity ≥ 260 units/mL, density 1.2 g/mL). Then, 250 mL Erlenmeyer flasks containing the samples were shaken to homogenize and incubated at 60ºC in static during 60 min. Afterwards, bottles were subjected to 95ºC during 5 min in a water bath in order to stop the enzymatic reaction. Finally, bottles were shaken and samples were taken and sieved, centrifuged and frozen as it was explained above for thermal hydrolyses.

The mixture of enzymes was selected according to the nature of the complex polymers contained in the vegetable wastes (mainly cellulose in tomatoes and red peppers and starch in potatoes) (Van Dyk et al., 2013). Cellulase catalyses the breakdown of cellulose into glucose, cellobiose, and higher glucose polymers. α-amylase and amyloglucosidase hydrolyze the α-(1,4) glucosidic bonds in starch into glucose. The pH and incubation conditions were set with the aim to be accurately for all the employed enzymes.

2.3.4 Thermal-enzymatic hydrolysis

In order to optimise the pre-treatment, a procedure that combined thermal and enzymatic hydrolysis was assayed. Lignin was removed from the samples as it was described in 2.3.2. Once solid material was obtained, water was added to solid residue in a relation 1:1 (w/v) and samples were then treated in an autoclave at 135ºC and 3 atm during 5 min. Afterwards, the mixture was cooled to room temperature and pH was adjusted to 4.7, then enzymes were added in the same way described above and the rest

of the procedure was the same as explained in the enzymatic hydrolysis section. Additionally, samples taken during the enzymatic step were subjected to 95ºC during 5 min in a water bath in order to stop the reaction and then were centrifuged and supernatants were frozen until being analysed. Before taken each sample, Erlenmeyer flasks were shaken to homogenize. Along this process some weight of solids is lost, remaining a solid residue after extracting the reducing sugars. So, in order to establish the losses registered, samples were taken, and dry extract were analysed in triplicate. Solid weighed throughout the successive steps are shown in Table 4.11.

Table 4. 11. *Solids weights throughout the thermal-enzymatic hydrolysis expressed on dry weight basis (%).*

	Initial solid weight	Remaining solid weight after delignification process	Remaining solids weight after thermal-enzymatic hydrolysis
Tomato	100	94.9	48.7
Pepper	100	82.1	30.5
Potato	100	83.5	47.5

2.4. Analytical methods

All reagents were supplied by Sigma-Aldrich and analyses were carried out in triplicate.

2.4.1 Dinitrosalicylic acid (DNS) method

The percentage of total reducing sugars was determined with 1% dinitrosalicylic acid reagent (DNS) according to the Miller method (Zhang et al., 2011). This method has been selected because it is widely used to estimate the reducing sugars content of different samples. In this procedure, 0.5 mL of DNS reagent was added to 0.5 mL of sample to be analysed and the mixture was vortexed and incubated in a boiling water bath for 5 min. Afterwards, 5 mL of distilled water was added to the tubes and samples

were cooled down in ice bath to quench the oxidation reaction. The absorbance of samples was recorded at 540 nm against a reagent blank. Analyses were performed with an UV-Spectrophotometer (ThermoScientific Heλios γ). The concentration of reducing sugars was determined according to the standardisation performed on glucose.

2.4.2 Phenol-sulfuric acid method

To determine the amount of total sugars, the DuBois phenol-sulfuric acid assay modified as follows was employed (Hall et al, 2016). In this method, 0.5 mL of 5% phenol and 2.5 mL of 96% H_2SO_4 were added to 1 mL of sample and the mixture was incubated at room temperature for 1 h. Finally, the absorbance was recorded at 492 nm against a reagent blank. Measurements were performed with an UV-Spectrophotometer (ThermoScientific Heλios γ). The concentration of total sugars was determined according to the standardisation performed on glucose.

2.4.3 Determination of fermentation inhibitors

Concentrations of fermentation inhibitors, i.e., furfural, HMF and acetic acid, were analysed by high performance liquid chromatography (HPLC). To determine acetic acid concentration, the Agilent 1200 chromatograph (Agilent Technologies) was equipped with an ICSep ICE-ION-300 column (Transgenomic) coupled to a refractive index detector (RID). The mobile phase employed was sulphuric acid (0.450 mM, pH 3.1) at a flow rate of 0.3 mL/min with the column temperature set at 75 C (Alonso et al., 2014). To determine furfural and HMF, the method was modified from Abu-Bakar et al. (2014) and De Andrade et. al. (2016), the instrument was equipped with a Gemini-NX 5µm C18 110A column (Phenomenex) coupled to a diode-array detection (DAD) system and the flow was fixed at 1 mL/min. The mobile phase was acetonitrile/water (20:90) and final UV detection was carried out at 260 nm for furfural, whereas methanol/water (10:90) and 285 nm were employed for HMF determination. Data acquisition and analysis were performed with ChemStation software (Agilent Technologies). All compounds were determined employing as reference external analytical standards (Sigma-Aldrich).

3. RESULTS AND DISCUSSION

3.1 Soluble and potential reducing sugars in raw wastes

Firstly, the amount of sugars that could be extracted from raw materials just by using distilled water, i.e. soluble sugars, was determined. Figure 4.29 shows a comparison between reducing and total sugars extracted from several batches of vegetable wastes supplied by a local market at different dates. It is remarkable the small differences observed between batches. In fact, values are within the following ranges: 21-25, 20-27 and 0.05-2 for soluble reducing sugars and 31-40, 24-31 and 1-3 for soluble total sugars in tomato, pepper and potato, respectively (given as g per100 g of waste dry weight). It can be observed that values of total and reducing sugars in case of tomato and pepper are in the same order of magnitude. On the contrary, the amount of sugars extracted from potato is in both cases much lower. Choi et al. (2016) described that the amount of total sugars (including, fructose, glucose and sucrose and excepting starch) found in potato was 0.9-1.5 g/100 g dry weight, whereas the amount of reducing sugars (fructose and glucose) was within the range 0.3-0.9 g/100 g dry weight. These values were in accordance with those extracted with water from potato waste in the present work as total and reducing sugars, as expected since potato native starch, the main carbohydrate in this vegetable, is not easily solubilized in water at room temperature (Hong et al., 2016). Likewise, values of available sugars of approximately 28 g/100 g dry weight were reported by Guil-Guerrero et al. (2006) for pepper, almost the same than that obtained in our work for total sugars.

Smoleń et al. (2015) found values of 22.8 g/100 g dry weight of fructose and glucose in tomato, values that are very similar to those we found for soluble reducing sugars (23.3 g/100 g dry weight), as fructose and glucose are the reducing sugars present in tomato. Regarding pepper, a value of 56 g/100 g dry weight of total sugars was found by Guil-Guerrero et al. (2006), value slightly higher than that reported in this work for potential reducing sugars (around 48 g/100 g dry weight).

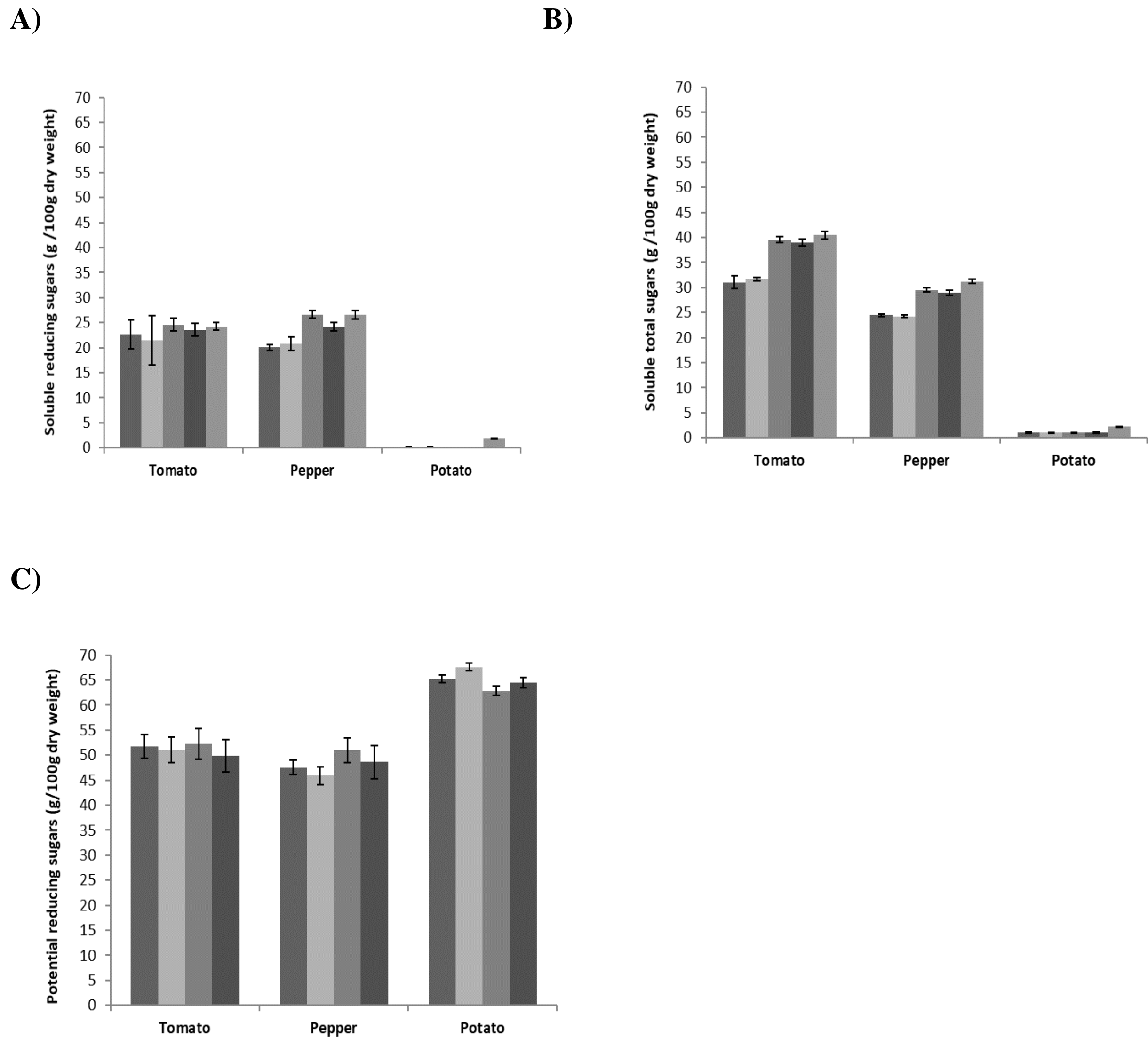


Figure 4. 29. *Soluble reducing sugars (A), soluble total sugars (B) and potential reducing sugars (C) extracted from different batches of vegetable wastes*

Additionally, it was observed that more than a half of soluble total sugars were reducing sugars in case of tomato and pepper (around 64% and 86%, respectively), whereas only slightly more than a third (approximately 36%) of soluble total sugars were reducing sugars in case of potato. With the aim to determine the maximum amount of reducing sugars that could be recovered from these vegetable wastes by means of hydrolysis treatments, potential reducing sugars were quantified. Values obtained from several batches of tomato, pepper and potato, were within the ranges: 50-52, 46-51 and 63-65 (g/100 g dry weight), respectively. Again, small differences were found between batches. In this case, the values obtained were in the same order of magnitude for all the tested wastes. Del campo et al. (2006) described a total content of carbohydrates in

tomato of around 56% (w/w) (4% sucrose and 35% lignin), value similar to that here measured for potential reducing sugars.

It also should be noticed that the potential reducing sugars for tomato and pepper were approximately twofold the soluble reducing sugar values. With regards to potato, value of potential sugars was more than 60 times the value obtained in potato for soluble reducing sugars.

Potential sugar values determined here for potato wastes is within the range (60-80 g/100 g dry weight) reported by Raatz et al. (2016) for starch content in fresh potatoes. This means that employing only water as extraction agent nearly half of potential reducing sugars are extracted in case of tomato and pepper, however less than 1% of potential reducing sugars are recovered in case of potato. Hence, these results showed the importance of pretreat these wastes as a key step in their revalorisation by fermentation processes, mainly in the case of potato wastes.

3.2. Performance of hydrolysis methods

Thermal, acid and enzymatic hydrolysis have been tested in order to maximise the amount of reducing sugars that can be obtained from wastes. In Fig 4.30 it is shown the comparison between reducing and total sugar extracted from the vegetable wastes after the different hydrolyses treatments.

For each substrate, similar values of total and reducing sugars were obtained employing 110 ºC thermal and acid hydrolyses (Figure 4.30.A and 4.30.C). Del Campo et al. (2006), employing a 110 ºC hydrothermal hydrolysis with similar procedure as that developed in this work, recovered an amount of single sugars (glucose and fructose) of 35 g/100g for tomato and 50 g/100g for red pepper, values much higher than those obtained in this work for reducing sugars of tomato and green pepper (around 18 and 14 g/100g, respectively).

Summoogum-Utchanah and Swami (2015) employed fruit and vegetable wastes treated by dilute acid hydrolysis achieving a maximum extraction of 23 g/100 g (dry weight) of reducing sugars. This value is slightly higher than those obtained in this work with acid treatment for tomato and pepper (19 and 15 g/100 g, respectively). Better results were obtained with 135 ºC thermal and enzymatic treatments (Figure 4.30.B and

4.30.D). with these hydrolysis methods, the amount of reducing sugars recovered from tomato wastes were 25-30 g per 100 g of initial waste (dry weight) and around 37 g/100 g for pepper.

Table 4.12 shows the percentage of reducing sugars extracted with respect to the potential reducing sugars contained in each substrate. The higher percentages were obtained with 135 ºC thermal and enzymatic treatments for tomato and pepper. In case of pepper the amounts extracted were above 75% for both treatments.

Concerning potato, it should be remarked that 110 ºC thermal and acid hydrolysis (Figure 4.30.A and 4.30.B) does not seem to be efficient enough to break down the starch contained in this substrate. In addition, 135 ºC thermal hydrolysis increased the total sugars extracted from potato but not the amount of reducing sugars. Higher temperatures favour the solubilisation of starch, as reported (Fuentes-Zaragoza et al., 2010). However, this treatment is not efficient enough to convert this polymer into reducing sugars.

Finally, with enzymatic treatment an increase in total and reducing sugars in potato hydrolysate were achieved. Nevertheless, the amount of reducing sugars extracted was still far from the potential reducing sugars of potato wastes, and the percentage extracted hardly achieved 5% (see Table 4.12).

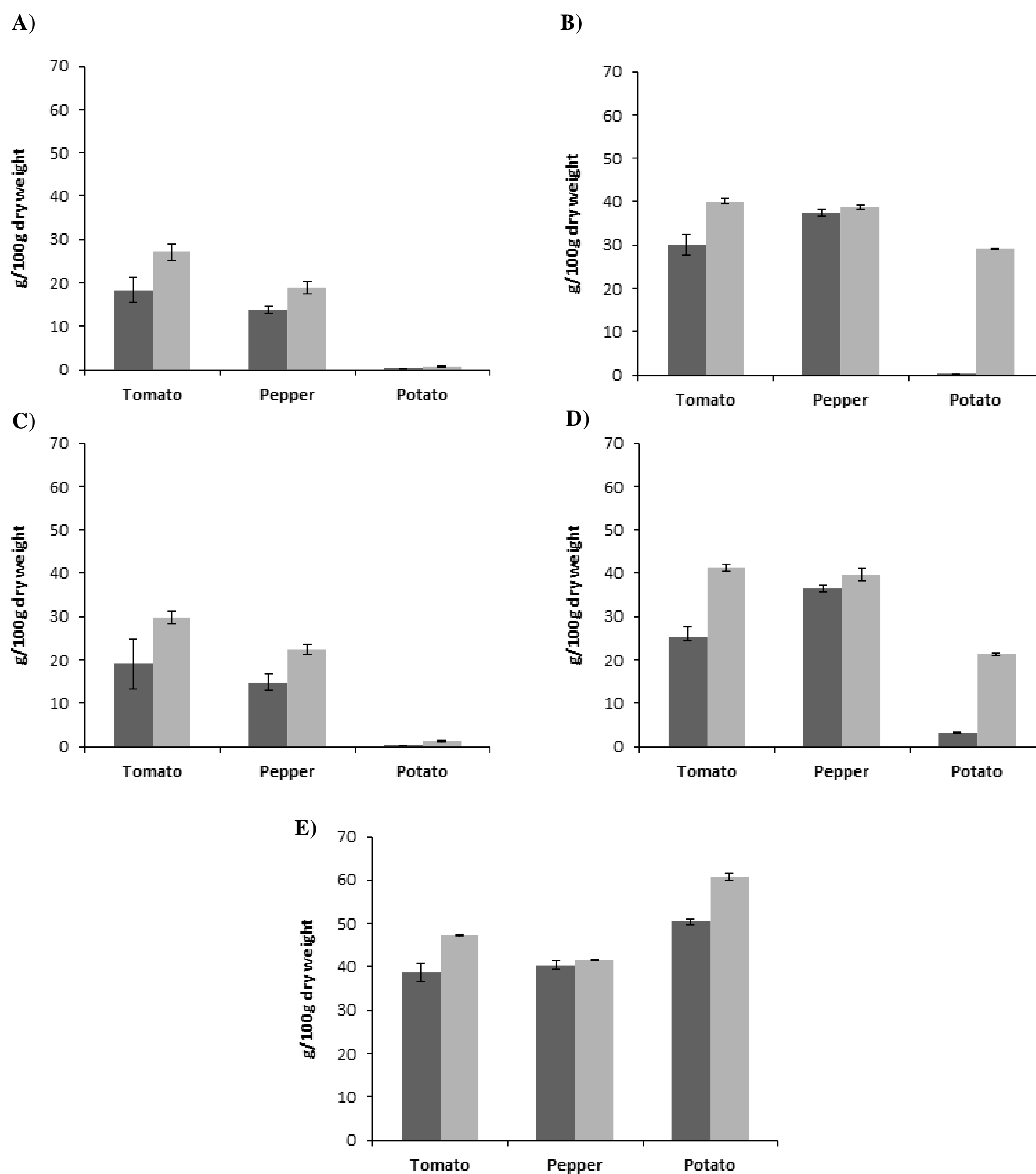


Figura 4. 30. *Reducing and total sugars extracted from vegetable wastes after the hydrolyses: (A) thermal 110ºC, (B) thermal 135ºC, (C) acid, (D) enzymatic and (E) thermal-enzymatic.*

Tabla 4. 12. *Comparison of reducing sugars yield extraction of the different hydrolysis assayed. Average values are shown.*

Reducing sugars extracted with respect to reducing potential sugars (%)*			
	Tomato	**Pepper**	**Potato**
Non-hydrolysed samples	45.5±0.0	49.0±0.1	0.72±0.01
Hydrolysed samples			
• ***Thermic (110°C)***	37.2±5.9	30.8±2.0	0.12±0.05
• ***Thermic (135°C)***	58.8±2.3	77.5±0.8	0.18±0.02
• ***Acid***	35.9±3.0	28.7±0.9	0.09±0.02
• ***Enzymatic***	49.5±1.0	75.5±0.7	4.93±0.02
• ***Thermic-Enzymatic***	75.4±2.1	83.7±1.0	77.4±0.7

*Average values were used for reducing potential sugars.

3.3. Thermic-enzymatic hydrolysis

Considering data shown in Table 4.12 (excepting for potato thermally treated), the best results were achieved for all wastes with thermal (135 ºC) and enzymatic hydrolysis. For this reason, both treatments were combined in a two-step hydrolyses. Indeed, in all cases, the highest amount of reducing and also total sugars were achieved employing the thermal-enzymatic hydrolyses (Figure 4.30). Furthermore, this increment was notably higher in case of potato wastes (Figure 4.30.E). Moreover, as shown in Table 4.12, the highest percentages of extracted reducing sugars with respect to potential reducing sugars were obtained with the thermic-enzymatic hydrolyses for tomato, pepper and potato (in all cases higher than 75%).

Cöpür et al. (2012) employed a mixture of tomato, pepper, and eggplant stalks as substrates for reducing sugars production. Stalks were chemically pre-treated with sodium borohydrate and then enzymatically hydrolysed. Best results achieved sugar

yields of 30% (w/w). This value is similar to those obtained here for tomato and pepper wastes in enzymatic treatment, whereas it is lower than the values obtained in the combined thermal-enzymatic treatment for these vegetables residues (around 40 g /100 g dry weight).

Potato residues can be compared with vegetable residues with high content in starch such as cassava. Indeed, tapioca starch factories generate a large amount of solid waste that contains a high level of starchy-lignocellulosic biomass, especially cassava pulp. This waste can be hydrolysed enzymatically achieving values of approximately 50 g / 100 g dry extract (Virunanon et al., 2013), values very similar to those obtained in our work for thermal-enzymatic treatment of potato residues.

In the hydrolysis treatments by chemical and/or physical technologies the temperature and time of reaction are determinant factors. Furthermore, the total content of hydrolysable biopolymers and the nature of these polysaccharides are also key factors. However, it is important to highlight that, in general, for vegetable wastes, specially, those that contain high levels of starch, best results are obtained with enzymatic processes with a previous pre-treatment (Patle and Lal, 2007; Preeti et al., 2012; Virunanon et al., 2013).

The differences observed in this work between enzymatic and thermal-enzymatic results are due to the fact that a thermal treatment at 135 ºC previous to enzymatic treatment contributes to the solubilisation of carbohydrates making easier to the enzymes to break the complex polysaccharides. These differences found between these treatments were notably greater in case of potato waste. As it has been commented above, potato carbohydrates are mainly constituted by starch and starch in potatoes is generally 70-80% amylopectin with the remainder being amylose (Raatz et al., 2016). As in enzymatic hydrolyses a combination of cellulose, α-amylase and amyloglucosidase were employed, amylopectin and amylose can be easily degraded. It is difficult to solubilise potato native starch (Hong et al., 2016), nevertheless, the structure and properties of starch are altered upon hydrothermal treatment induced in excess water. This phenomenon is well recognised as gelatinisation which is an irreversible order-disorder transition (Somboonchan et al., 2016). Gelatinised starch is easily accessible to enzymes that can break the starch’s structural units, amylose and amylopectin, into glucose.

In order to acquire a deeper knowledge of the enzymatic step, samples were taken during the combined treatment and the evolution with time of extracted reducing and total sugars were measured. It is noticeable that the initial amount of reducing sugars after the thermic step was much lower in potato compared to tomato and pepper. As can be seen in Figure 4.31, along the enzymatic hydrolysis, the concentration of reducing sugars in tomato and pepper wastes increased slowly, whereas in case of potato residue this increment was notably sharper. During the enzymatic step the concentration of reducing sugars in the broth increased in all cases, just 21% and 15% for tomato and pepper (respectively), whereas for the potato the reducing sugars concentration increases 11 times.

After the 60 min that the enzymatic step lasted, a percentage around 25, 16 and 23 for tomato, pepper, and potato, respectively, of the potential reducing sugars initially measured for the wastes remained without being hydrolyzed into reducing sugars. It was observed that, in case of tomato and potato wastes a maximum content in reducing sugars was achieved at 45 min. On the contrary, in case of potato waste, the trend of the curve indicated that a higher amount of reducing sugars could be achieve if the enzymatic reaction goes on for some more time.

The possible formation of fermentation inhibitors, i.e. furfural, HMF and acetic, was investigated by analyzing the final products and in all cases the amount of these sugar degradation products was below the detection limit (< 1 mg/L). For furfural or HMF, a concentration of 4 g/L has been reported to inhibit sugar fermentation by yeast. In addition, for acetic acid the reported inhibition level for *S. cerevisiae* was 6 g/L (Zheng et al., 2013). According to the results obtained in this work it can be assured that furfural, HMF and acetic acid concentrations are quite below those concentrations described as necessary to cause fermentation inhibition problems.

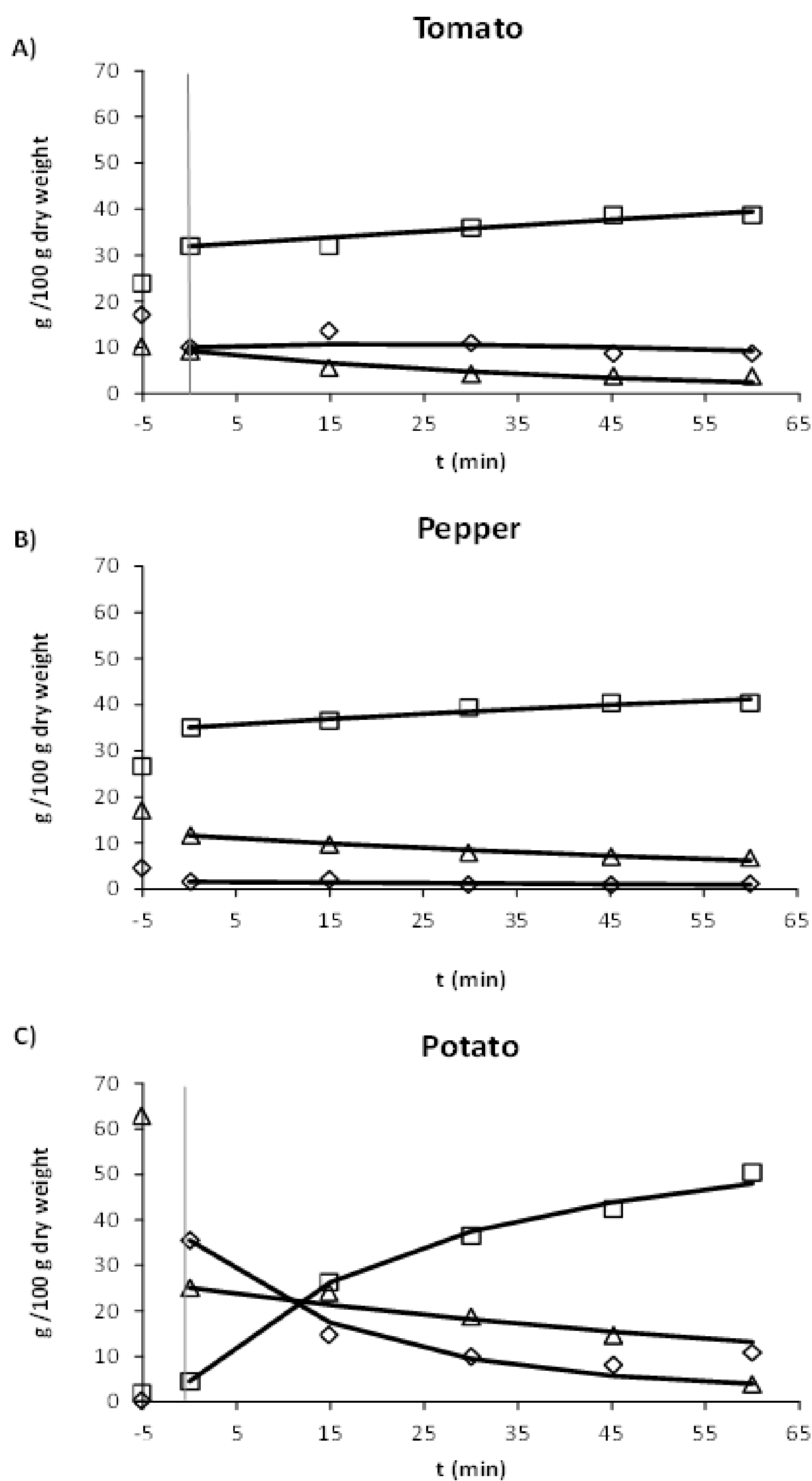
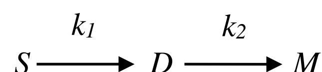


Figura 4. 31. *Evolution of solubilised reducing sugars (M:□), solubilised non-reducing sugar intermediates (D:◇) and potential reducing sugars in solid phase (S:△) with time. Symbols correspond to experimental data (or calculated from experimental data) and lines correspond to model results. All concentrations are expressed as equivalent glucose. Vertical grey line divides the thermic and the enzymatic steps. In all cases, SD < 2.0 g/100 g.*

3.4. Modelling

A model based on irreversible first-order reactions was developed for the enzymatic step. It considers that solid carbohydrates are hydrolyzed into soluble intermediates that are subsequently degraded to monomers of glucose. So, *S* is the polymers in solid phase (estimated as potential reducing sugars minus dissolved total sugars), *D* is the dissolved non-reducing-sugar intermediates (estimated as dissolved total sugars minus dissolved reducing sugars) and *M* is the dissolved reducing sugars, all of them expressed as equivalent glucose. k_1 is the solubilisation rate of polymers in solid phase and k_2 is the hydrolysis rate of soluble intermediates into glucose. The inhibitors formation was not included in the model because, as it was commented above, the amount formed during the enzymatic hydrolysis is very low and can be considered negligible for modelling purpose.

Parameters were determined by fitting the model to experimental data using Microsoft Excel software. Model reactions are represented below.



$$S \xrightarrow{k_1} D \xrightarrow{k_2} M$$

The comparison between experimental and model data is shown in Figure 4.31. It can be observed that the fitting is very good in all cases. Furthermore, in Table 4.13 are shown the values of kinetic constants k_1 and k_2 and also the r^2 obtained from fitting by a linear regression experimental data and model data. It should be noticed that the correlations represented by r^2 values were in all cases above 0.98 which indicated the high level of accuracy achieved by the model. Although all the constants values are of the same order, the solid polymers are solubilized to non-reducing-sugar intermediates in half time for tomato with respect to pepper and potato. However, the transformation of the solubilised non-reducing-sugar intermediates to solubilised reducing sugars takes place faster in pepper and potato residues than in case of tomato wastes. Lenihan et al. (2011), employed a similar model to analyze the kinetics of acid hydrolysis of lignocellulosic biomass and reported that the reaction rate of hydrolysis depends on a

number of variables, i.e.: temperature, time, substrate concentration and substrate composition.

Tabla 4. 13. *Kinetic constant calculated from experimental data*

	k_1 (min^{-1})	k_2 (min^{-1})	r^2
Tomato	0.0221	0.0121	0.993
Pepper	0.0106	0.0804	0.999
Potato	0.0108	0.0577	0.987

r^2 values were obtained from fitting by a linear regression experimental and model data.

The particular behavior of potato wastes is determined by starch content which is the main carbohydrate found in potato. On the contrary, the principal polysaccharides contained in tomato and pepper are pectin and cellulose, hemicellulose is also present but in lower concentrations, around 2% (w/w) (Egüés et al., 2013; Deinychenko and Yudicheva; 2016). As the composition of these vegetables is very similar, the different behavior during the combined hydrolysis process may be defined by the specific structure and interactions between their compounds. In recent works, it was reported that the organization and interactions of vegetable wall components is not known with certainty, so the effect of treatments are determined by chemical components, physical properties and supermolecular structures (Cöpür et al., 2012; Sun et al., 2016).

4. CONCLUSIONS

The high content of carbohydrates in vegetable wastes indicates that these materials may be valuable recourses to be used as substrates for fermentation purpose. A pre-treatment to facilitate the polysaccharides solubilisation and hydrolysis is recommended for cellulose-containing materials, i.e. tomato and peppers, and essential for starchy wastes, i.e. potato. The combined thermic-enzymatic hydrolysis turned out as the most efficient method of the assayed procedures to obtain monomeric sugars from tomato, pepper and potato wastes. Specifically, with this combined pre-treatment the percentage of reducing sugars extracted (with respect to the reducing potential sugars) was increased in approximately 1.7 times for tomato and green peeper wastes

and in 107 times for potato wastes. It is remarkable that the obtained hydrolysates are suitable to be directly used as fermentation media due to the low concentrations of growth inhibitors, which allow avoiding the purification step. Additionally, it should be highlighted that, according to results reported here, and for the assayed enzymes, 45 min was time enough to achieve the maximum concentration of reducing sugars for tomato and pepper wastes, whereas, in the case of potato waste, 1 hour did not allow to obtain the maximum yield of reducing sugars. So, the duration of the enzymatic process resulted to be an important parameter that should be adjusted depending on the specific composition of vegetable wastes mixture. The kinetic model developed for the enzymatic step showed a good fitting, so it could be employed in future technical studies.

ACKNOWLEDGEMENTS

The authors are grateful for financial support from the Economy and Employment Office of Principality of Asturias (Spain) through project GRUPIN14-140.

REFERENCES

[1] Abu-Bakar, N.B., Makahleh, A., Saad, B., 2014. Vortex-assisted liquid-liquid microextraction coupled with high performance liquid chromatography for the determination of furfurals and patulin in fruit juices. Talanta 120, 47-54.

[2] Aguiar, R.S., Silveira, M.H.L., Pitarelo, A.P., Corazza, M.L., Ramos, L.P., 2013. Kinetics of enzyme-catalyzed hydrolysis of steam-exploded sugarcane bagasse. Bioresource Technol., 147, 416-423.

[3] Alvira, P., Tomás-Pejó, E., Ballesteros, M., Negro, M.J. Pre-treatment technologies for an efficient bioethanol production process based on enzymatic hydrolysis: A review, 2010. Bioresource Technol. 101 (13), 4851-4861.

[4] Alonso, S., Herrero, M., Rendueles, M., Díaz, M., 2014. Physiological heterogeneity in *Lactobacillus casei* fermentations on residual yoghurt whey. Process Biochem. 49 (5), 732-739.

[5] Choi, S.-H., Kozukue, N., Kim, H.-J., Friedman, M., 2016. Analysis of protein amino acids, non-protein amino acids and metabolites, dietary protein, glucose, fructose, sucrose, phenolic, and flavonoid content and antioxidative properties of potato tubers, peels, and cortexes (pulps). J. Food Comp. Anal. 50, 77-87.

[6] Çöpür, Y., Özyürek, O., Tozluoglu, A., Kütük, S., 2013. Enzymatic digestibility of tomato, pepper, and eggplant stalks mixture. BioResources 7 (3), 3252-3261.

[7] Correa, J.F., Ruiz, A.A., Garrote, G., 2012. Hydrothermal treatment study of banana fruit. Afinidad LXIX (559), 191-196.

[8] De Andrade, J.K., Komatsu, E., Perreault, H., Torres, Y.R., da Rosa, M.R., Felsner, M.L., 2016. In house validation from direct determination of 5-hydroxymethyl-2-furfural (HMF) in Brazilian corn and canesyrups samples by HPLC-UV. Food Chem. 190, 481-486.

[9] Deinychenko, G., Yudicheva, O., 2016. Chemical composition of frozen biofortified pepper and pumpkin. EUREKA: Physics and Engineering, 3, 56-62.

[10] Del Campo, I., Alegría, I., Zazpe, M., Echeverría, M., Echeverría, I., 2006. Diluted acid hydrolysis pre-treatment of agri-food wastes for bioethanol production. Ind. Crop. Prod. 24 (3), 214-221.

[11] Egüés, I., Eceiza, A., Labidi, J., 2013. Effect of different hemicelluloses characteristics on film forming properties. Ind. Crop. Prod. 47, 331-338.

[12] Fuentes-Zaragoza, E., Riquelme-Navarrete, M.J., Sánchez-Zapata, E., Pérez-Álvarez, J.A., 2010. Resistant starch as functional ingredient: A review. Food Res. Int. 43 (4), 931-942.

[13] Guil-Guerrero, J.L., Martínez-Guirado, C., Del Mar Rebolloso-Fuentes, M., Carrique-Pérez, A., 2006. Nutrient composition and antioxidant activity of 10 pepper (*Capsicum annuun*) varieties. Eur. Food Res. Technol. 224 (1), 1-9.

[14] Gustavsson, J., Cederberg, C., Sonesson, U., van Otterdijk, R., Meybeck, A., 2011. Global food losses and food wastes: extent, causes and prevention. FAO.

[15] Hall, M.B., 2016. Efficacy of reducing sugar and phenol-sulfuric acid assays for analysis of soluble carbohydrates in feedstuffs. Anim. Feed Sci. Tech. 185 (1-2), 94-100.

[16] Han, W., Ye, M., Zhu, A.J., Zhao, H.T., Li, Y.F., 2015. Batch dark fermentation from enzymatic hydrolyzed food waste for hydrogen production. Bioresource Technol. 191, 24-29.

[17] Han, W., Hu, Y., Li, S., Huang, J., Nie, Q., Zhao, H., Tang, J., 2017. Simultaneous dark fermentative hydrogen and ethanol production from waste bread in a mixed packed tank reactor. J. Clean. Prod. 141, 608-611.

[18] Hong, J., Chen, R., Zeng, X.-A., Han, Z., 2016. Effect of pulsed electric fields assisted acetylation on morphological, structural and functional characteristics of potato starch. Food Chem. 192, 15-24.

[19] Kennes, D., Abubackar, H.N., Diaz, M., Veiga, M.C., Kennes, C., 2016. Bioethanol production from biomass: Carbohydrate vs syngas fermentation. J. Chem. Technol. Biot. 91 (2), 304-317.

[20] Khawla, B.J., Sameh, M., Imen, G., Donyes, F., Dhouha, Raoudha, E.G., Oumèma, N.E., 2014. Potato peel as feedstock for bioethanol production: A comparison of acidic and enzymatic hydrolysis. Ind. Crop. Prod. 52, 144-149.

[21] Kosseva, M.R., 2013. Introduction: causes and challenges of food wastage, in Kosseva, M.R. and Webb, C. (Eds.), Food industry wastes: assessment and recuperation of commodities. Londres, Academic Press (Elsevier) pp. xiii-xxiii.

[22] Kumar , P., Barrett, D.M., Delwiche, M.J., Stroeve, P., 2009. Methods for pre-treatment of lignocellulosic biomass for efficient hydrolysis and biofuel production. Ind. Eng. Chem. Res. 48, 3713-3729.

[23] Lenihan, P., Orozco, A., O'Neill, E., Ahmad, M.N.M., Rooney, D.W., Mangwandi, C., Walker, G.M., 2011. Kinetic modelling of dilute acid hydrolysis of lignocellulosic biomass, in M.A. Dos Santos (Ed.), Biofuel Production-Recent Developments and Prospects. InTech, pp. 293-308 pp. DOI: 10.5772/17129.

[24] Monsalve J.F., Medina, V.I., Ruiz A.A., 2006. Ethanol production of banana shell and cassava starch. Dyna 73, 21-27.

[25] Nanda, S., Reddy, S.N., Hunter, H.N., Dalai, A.J., Kozinski, J.A., 2015. Supercritical water gasification of fructose as a model compound for waste fruits and vegetables. J. Supercrit. Fluids 104, 112-121.

[26] Panda, S.K., Mishra, S.S., Kayitesi, E., Ray, R.C., 2016. Microbial-processing of fruit and vegetable wastes for production of vital enzymes and organic acids: Biotechnology and scopes. Environ. Res. 146, 161-172.

[27] Patle, S., Lal, B., 2007. Ethanol production from hydrolysed agricultural wastes using mixed culture of *Zymomonas mobilis* and *Candida tropicalis*. Biotechnol. Lett. 29, 1839-1843.

[28] Preeti, V.E., Sandhya, S.V., Kuttiraja, M., Sindhu, R., Vani, S., Kumar, S.R., Pandey, A., Binod, P., 2012. An evaluation of chemical pre-treatment methods

for improving enzymatic saccharification of chili postharvest residue. Appl. Biochem. Biotech. 167 (6), 1489-1500.

[29] Raatz, S.K., Idso, L., Johnson, L.K., Jackson, M.I., Combs, G.F., 2016. Resistant starch analysis of commonly consumed potatoes: Content varies by cooking method and service temperature but not by variety. Food Chem. 208, 297-300.

[30] Ravindran, R., Jaiswal, A.K., 2016. Exploitation of food industry waste for high-value products. Trends Biotechnol. 34 (1), 58-69.

[31] Smoleń, S., Wierzbińska, J., Sady, W., Kołton, A., Wiszniewska, A., Liszka-Skoczylas, M., 2015. Iodine biofortification with additional application of salicylic acid affects yield and selected parameters of chemical composition of tomato fruits (*Solanum lycopersicum* L.). Sci. Hortic.-Amsterdam 188, 89-96.

[32] Somboonchan, S., Lubbers, S., Roudaut, G., 2016. Water and temperature contribution to the structuration of starch matrices in the presence of flavour. Food Chem. 195, 79-86.

[33] Summoogum-Utchanah, S.L., Swami, J., 2015. Investigating the extraction of alcohol from agricultural wastes in Mauritius. JECE 3, 2255-2262.

[34] Sun, R., Song, X., Sun, R., Jiang, J., 2011. Effect of lignin content o enzymatic hydrolysis of furfural residues. BioResources 6, 317-328.

[35] Sun, F.F., Zhao, X., Hong, J., Tang, Y., Wang, L., Sun, H., Li X., Hu, J., 2016. Industrially relevant hydrolyzability and fermentability of sugarcane bagasse improved effectively by glycerol organosolv pre-treatment. Biotechnol. Biofuels 9:59, 1-13.

[36] Taherzadeh, M.J., Karimi, K., 2008. Pre-treatment of lignocellulosic wastes to improve ethanol and biogas production: A Review. Int. J. Mol. Sci. 9, 1621-1651.

[37] Thulluri, C., Goluguri, B.R., Konakalla, R., Shetty, P.R., Addepally, U., 2013. The effect of assorted pre-treatments on cellulose of selected vegetable waste and enzymatic hydrolysis. Biomass Bioenerg. 49, 205-213.

[38] Vavouraki, A.I., Volioti, V., Kornaros, M.E., 2014. Optimization of thermo-chemical pre-treatment and enzymatic hydrolysis of kitchen wastes. Waste Manage. 34 (1), 167-173.

[39] Van Dyk, J.S., Gama, R., Morrison, D., Swart, S., Pletschke, B.I., 2013. Food processing waste: Problems, current management and prospects for utilisation

of the lignocellulose component through enzyme synergistic degradation. Renew. Sust. Energ. Rev. 26, 521-531.

[40] Virunanon, C., Ouephanit, C., Burapatana, V., Chulalaksananukul, W., 2013. Cassava pulp enzymatic hydrolysis process as a preliminary step in bio-alcohols production from waste starchy resources. J. Clean. Prod. 39, 273-279.

[41] Wu, M., Yan, Z.Y., Zhang, X.M., Xu, F., Sun, R.C., 2016. Integration of mild acid hydrolysis in γ-valerolactone/water system for enhancement of enzymatic saccharification from cotton stalk. Bioresource Technol. 200, 23-28.

[42] Zhang, J., Tuomainen, P., Siika-aho, M., Viikari, L., 2011. Comparison of the synergistic action of two thermostable xylanases from GH families 10 and 11 with thermostable cellulases in lignocellulose hydrolysis. Bioresource Technol. 102 (19), 9090-9095.

[43] Zheng, Y., Lee, C., Yu, C., Cheng, Y.-S., Zhang, R., Jenkins, B.M. VanderGheynst, J.S., 2013. Dilute acid pre-treatment and fermentation of sugar beet pulp to ethanol. Appl. Energy 105, 1-7.

4.3.2 Biodegradation of olive mill effluent by white-rot fungi

Ana Isabel Díaz, Marta Ibañez, Adriana Laca * and Mario Díaz

Department of Chemical and Environmental Engineering, Faculty of Chemistry, University of Oviedo, C/Julián Clavería, s/n, 33071 Oviedo, Spain;

*Correspondence: lacaadriana@uniovi.es; Tel.: +34-985-10-29-74

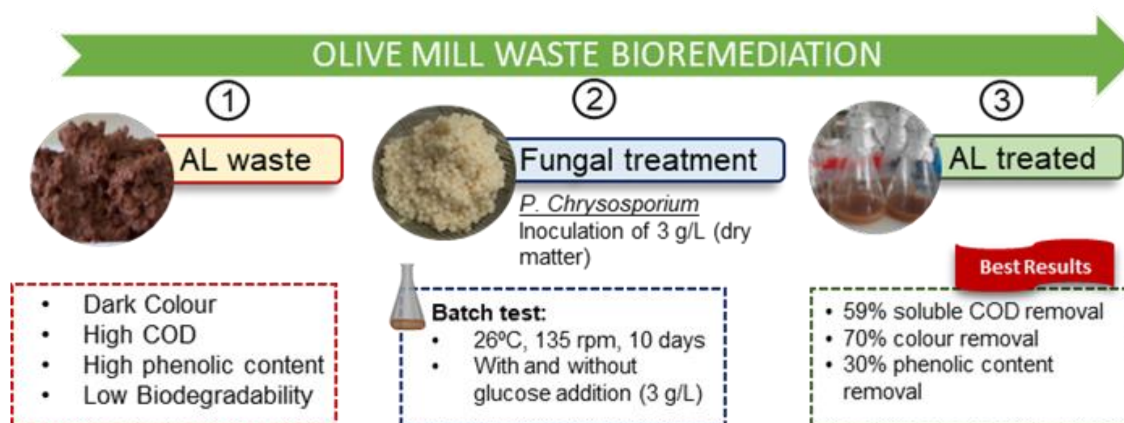


Figura 4. 32. *Resumen gráfico del trabajo 7*

*Artículo publicado en la revista "**Applied Sciences**"

Cita: Díaz, A. I., Ibañez, M., Laca, A., Díaz, M., Biodegradation of olive mill effluent by fungi. *Appl. Sci. 2021, 11, 9930.*

https://doi.org/10.3390/app11219930

ABSTRACT

The liquid fraction from the two-phase extraction process in the olive industry (alperujo), is a waste that contains lignocellulosic organic matter and phenolic compounds, difficult to treat by conventional biological methods. Lignocellulosic enzymes from white-rot fungi can be an interesting solution to break down these recalcitrant compounds and advance the treatment of that waste. In the present work the ability of *Phanerochaete chrysosporium* to degrade the abovementioned liquid waste (AL) was studied. Experiments were carried out at 26 °C within the optimal pH range 4–6 for 10 days and with and without the addition of glucose, measuring the evolution of COD, BOD_5, biodegradability index, reducing sugars, total phenolic compounds, and colour. The results obtained in this study revealed the interest of *Phanerochaete chrysosporium* for an economical and eco-friendly treatment of alperujo, achieving COD and colour removals around 60%, and 32% of total phenolic compounds degradation, regardless of glucose addition.

Keywords: alperujo; olive mill waste; bioremediation; *Phanerochaete chrysosporium*; fungal treatment

1. INTRODUCTION

The Mediterranean region is the main producer of olive oil, concentrating more than 95% of the world's olive trees. Within this area, Spain represents around half of total world manufacturing, considering this industry as one of the most important agri-food sectors for this country [1,2].

Presently, the main industrial process to obtain olive oil is continuous extraction by two-phase or three-phase systems. Depending on the oil extraction system used, considerable amounts of solid and liquid waste as final products are generated. Although the three-phase extraction process mainly generates alpechin and pomace as final residues, the main waste stream from two-phase extraction system is the alperujo (AL) [3]. The pomace is a solid waste composed of the pulp and pits of the olive, commonly employed as fertilizer, biofuel production, or animals feed. The alpechin corresponds with the liquid effluent, composed of water and minerals and characterized

by a high organic matter load. This waste stream is considered, together with the AL, a highly polluting residue, for which reuse is not an easy task so its revalorization is still being investigated [4,5]. Due to the global increase of olive oil demand, the excessive amount of waste streams generated throughout the olive oil industry is a growing problem that poses an environmental challenge [6].

In Spain, the two-phase olive oil extraction system is used in approximately 90% of olive mills [4]. The application of this extraction system generates about 800 kg of AL per ton of processed olive, which represents an annual production of around four million tons for the Spanish oil industry [3]. The AL obtained is a semi-solid waste stream composed of vegetable water and olive pomace with high moisture content (60%) that still contains a certain amount of oil [7]. The AL is subjected to a second centrifugation, to obtain a pomace oil. The resulting residue is usually dried in rotary heat dryers at high temperatures and the by-product is subjected to an extraction with hexane to recover more oil, which requires a large amount of energy and incurs high costs [8,9]. Dried or wet AL can also be used in composting processes. However, due to its low porosity, the addition of bulking agents such as bark chips or cotton gin is necessary [9,10]. In addition, its high content of lignocellulosic compounds and polyphenols, which are toxic to animal cells, plants, insects, and microorganisms, is an important drawback [11]. Discharging these wastes without treatment would cause serious damage to aquatic systems, such as the reduction of soluble oxygen. Furthermore, its strong odour would also cause serious problems for the population living near the discharge area [12,13]. Therefore, the removal of the pollutant compounds of the AL, and therefore favouring of the subsequent biological/physical treatment, is one of the main problems that the olive oil industry must confront.

The literature has been mainly focused on the treatment of olive mill wastes coming from the three-phase extraction system, with the aim of removing the organic and phenolic compounds and improving the biodegradability of the effluent. Regarding biological methods, anaerobic digestion or aerobic activated sludge processes have mainly been applied for the treatment of olive mill wastewater (OMW). However, these methods are not usually applied directly to the effluent due to the presence of recalcitrant molecules, polyphenols, the low nutrient load, and the acidic pH of the waste, which make treatment difficult [14]. Previous studies have reported an improvement in the efficiency of these processes reducing the acidity, adding nutrients

such as cobalt, or extracting polyphenols before biodegradation [15]. Due to the high level of antimicrobial compounds present in OMW, the acclimatization of biomass or the use of physical $CaCO_3$ supports has also been required to improve the biodegradation and methanization process [16]. Therefore, the traditional biological methods are not as effective as would be desirable. Physical–chemical methods such as nanofiltration, ultrafiltration, ultrasound, hydrothermal carbonization, and different advances oxidation processes have been reported for the treatment of OMW reducing its chemical oxygen demand (COD) and phenol content [17,18]. However, these techniques have several drawbacks, such as the addition of chemicals, fouling of the membrane, and high pressure and temperature conditions [5]. Therefore, the search for alternative methods that allow the treatment of OMW and AL in an economic and eco-friendly way is crucial.

The use of fungi has been described as a promising alternative over the use of bacteria for OMW treatment due to its ability to grow under adverse conditions and to produce a great variety of extracellular enzymes that make possible the degradation of recalcitrant compounds [19]. In this way, fungi can break down the complex recalcitrant compounds making them more assimilable to be used by themselves or by the bacteria in a subsequent treatment [20,21]. The OMW treatment by fungi has mainly focused of removing COD, phenolic content, and colour, as well as obtaining by-products with biotechnological interest, such as fungal enzymes [22,23]. White-rot fungi can degrade the lignin present in lignocellulosic wastes due to the release of enzymes, mainly lignin peroxidase and manganese peroxidase [24]. These fungi have been investigated for the treatment of recalcitrant compounds and colour degradation of OMW, obtaining good results. For example, Ntougias et al. [25], who studied the capacity of several strains of *Pleurotus* and *Ganoderma* fungi to treat OMW, reported significant removals of COD, TOC, and phenolic compounds, as well as a reduction of the toxicity of the effluent. COD degradations around 50% have been achieved by the fungus *Phanerochaete chrysosporium* immobilized on loofah [26]. Great removals of colour, phenolic compounds and COD have also been obtained when OMW was treated with fungi from genus *Aspergillus* [22,27].

As far as we know, the bioremediation with fungi has been mainly applied to treat OMW or pomace coming from the three-extraction system. However, its application to AL waste, obtained from the two-phase extraction system, has been

hardly studied. Therefore, the main objective of this study was to investigate the capability of the white-rot fungus *Phanerochaete chrysosporium* to treat AL waste, to reduce its COD, colour, and phenolic compounds.

2. MATERIALS AND METHODS

2.1. Sample Description

The AL used for this work corresponds with the semi-solid effluent generated during olive oil extraction by a two-phase extraction system.

The sample was collected from an olive oil factory sited in Sevilla, Spain. For the fungal treatment, the sample was mixed with distilled water in a ratio 1:20 (*p*/*v*). The mix was filtered using a 1.5 mm mesh sieve to remove the rest of the peel and pit of the olives. After that, the effluent was centrifuged for 10 min at 9000 g and the supernatant was filtered by a cellulose filter (10–20 μm). This diluted AL was used for the subsequent fungal treatments. The characteristics of diluted AL are shown in Table 4.14.

Tabla 4. 14. *Characteristics of the diluted AL.*

Parameter	Value
pH	4.6 ± 0.01
sCOD (mg O_2/L)	4854 ± 19
sBOD (mg O_2/L)	408 ± 14
Biodegradability Index (B.I.)	0.080 ± 0.003
Reducing sugars (mg/L)	578 ± 24
Total phenolic compounds (mg/L)	134 ± 4
Colour index (C.I.)	1.60 ± 0.04
Total Suspended Solids (mg/L)	2475 ± 21
Fixed Suspended Solids (mg/L)	375 ± 12
Volatile Suspended Solids (mg/L)	2100 ± 28

2.2. Fungal Pellet Obtention

The white-rot fungus, *Phanerochaete chrysosporium* Burdsall 1974 was used. The freeze-dried strain (CECT 2798 from Spanish Type Culture Collection) was recovered in aseptic conditions by adding 100 µl of the resuspended fungus to 10 mL of malt extract (ME). Then, a Petri plate of 1.5% malt extract agar (MEA) was inoculated with 100 µl of this suspension and incubated at 26 °C for 6 days. Two subcultures of the fungus were necessary before use in the biological treatment. Fungal subcultures were routinely made every month to conserve the strain.

The methodology described by Díaz et al. [28] was followed to obtain the fungus pellets. To this aim, five cylinders of 1 cm diameter from the growing zone of inoculated plates were used to inoculate 500 mL Erlenmeyer flasks containing 150 mL of sterilised malt extract broth (VWR Chemicals BDH), with a pH between 4.5 and 5. The inoculated flasks were incubated at 26 °C and 135 rpm for 6 days. The fungal mycelial obtained after this process was separated with a sieve and homogenized with 0.8% NaCl (*w*/*v*) in a ratio of 1:3 (*w*/*v*). An amount of 600 µL of resulting suspension was used to inoculate a 1 L Erlenmeyer flask with 250 mL of sterilised ME. Finally, the inoculated ME was incubated at 26 °C and 135 rpm for 6 days. After that time, pellets were obtained, removed with a sieve, and preserved in 0.8% NaCl (*w*/*v*) solution at 4 °C until use.

2.3. Fungal Treatment

Several batch tests were carried out to treat diluted AL with *P. chrysosporium*. All the experiments were performed using 1 L Erlenmeyer flask with 250 mL of AL effluent.

Test E1 and E2 were inoculated with the fungus pellet (3 g/L of dry matter), with the only difference that E2 was supplied with 3 g/L of glucose.

Test C1 and C2 were used as control without fungus inoculation, without and with glucose addition, respectively.

The flasks were incubated at 26 °C and in an orbital shaking (150 rpm) for 10 days. During the treatment, the pH values were maintained within the range 5–7 by adding NaOH 0.5 M or HCl 0.5 M to ensure the optimal range for the fungus enzymatic system. Samples taken periodically were centrifuged at 15,000 g for 15 min and the supernatant were conserved at 4 °C until analysed. The experiments were carried out in duplicate. Data shown in Results and Discussion section are the average values of both experiments. In all cases, standard deviations were lower than 15% with respect to average value.

2.4. Analytical Methods

2.4.1. Determination of sCOD, sBOD$_5$, and Biodegradability Index

The concentration of soluble COD (sCOD) was spectrophotometrically measured (at 600 nm) by dichromate method according to Standard Methods [29], using a DR2500 spectrophotometer (Hach Company). Soluble biochemical oxygen demand (sBOD$_5$) was determined using a manometric respirometry measurement system (Lovibond Water Testing BD 600) and biodegradability index (BI) was calculated as the ratio of sBOD$_5$ over sCOD.

2.4.2. Determination of Colour and pH

The change in the colour of the AL was determined by means of the colour

$$CI=\frac{SAC_{436}^2+SAC_{525}^2+SAC_{620}^2}{SAC_{436}+SAC_{525}+SAC_{620}} \quad 1)$$

index (CI), which is defined according to Equation (1) [30].

Spectral absorbance coefficients (SAC) are defined as the ratio of the values of the respective absorbance over the cell thickness. The absorbances were measured at 436, 525 and 620 nm using a UV/vis spectrophotometer (Thermo Scientific, Heλios γ). The value of pH was measured by means of a pH-meter (Basic-20 Dilabo).

2.4.3. Determination of Total Reducing Sugars

The total reducing sugars concentration was determined by the dinitrosalicylic acid (DNS) method with glucose as standard, according to the Miller's method [31]. The absorbance of samples was measured at 540 nm. The glucose was used as standard.

2.4.4. Determination of Total Phenolic Compounds

The total phenolic compounds were determined by the Folin–Ciocalteu method in dark conditions, according to Moussi et al. [32], using gallic acid as standard. In this procedure, 400 μL of sample were mixed with 3 mL of Folin–Ciocalteu reagent (previously diluted 1:10 with distilled water). This mixture was maintained at 22 °C for 5 min. After that, 3 mL of sodium bicarbonate ($NaHCO_3$ 6 g/100 mL) were added, and the sample was again incubated at 22 °C for 90 min. After incubation, the absorbance was measured at 725 nm.

2.4.5. Determination of Moisture, TSS, FSS, and VSS.

Total suspended solids (TSS), fixed suspended solids (FSS) and moisture were measured according to Standard Methods [29]. The volatile suspended solids were calculated as the difference between TSS and FSS.

3. RESULTS AND DISCUSSION

3.1. Removal of Organic Matter

The evolution of sCOD concentration during the fungal treatments is shown in Figure 4.33.

The initial sCOD value in the AL effluent was 4854 mg/L, which increased to 9243 mg/L after glucose addition. For C1 and C2 test, which were carried out without fungal inoculation, minor sCOD removals were observed. This degradation was carried out by the endogenous microbiota present in the effluent. With respect to test C1, no change was observed during the first two days and a 27% elimination of sCOD was achieved after four days of incubation. Afterwards, the sCOD value remained almost constant, reaching a final sCOD degradation of 30%. In the case of C2, where glucose

where added, sCOD degradation did not occur until the 4th day. The final percentage of sCOD removal was similar to that achieved with C1.

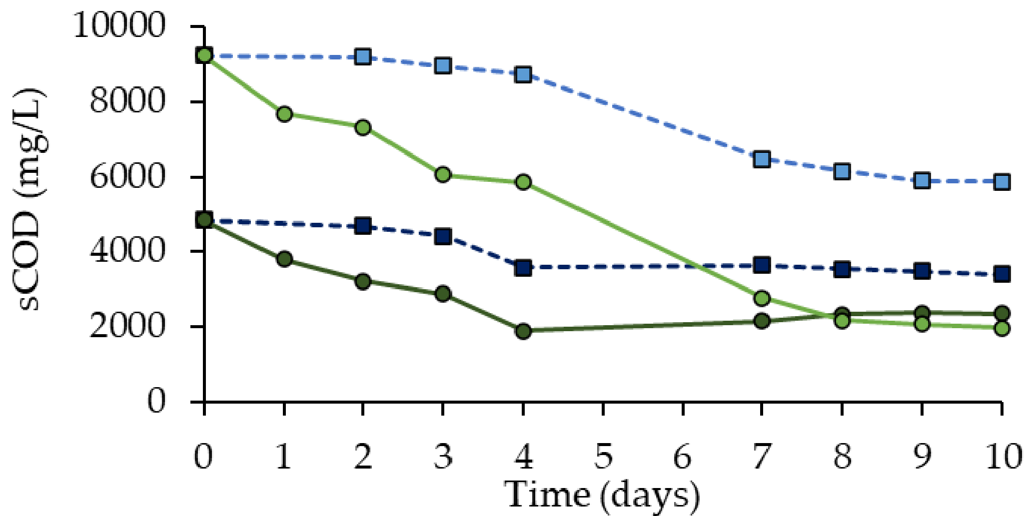


Figura 4. 33. *Changes in sCOD concentration during biological treatment. The dashed lines shown the non-inoculated tests C1 (■) and C2 (■), used as controls. The solid lines shown the inoculated tests E1 (●) and E2 (●). The standard deviation (SD) of the experimental data were in all cases less than 6.5% of mean value.*

However, it is necessary to point out that in C2 the final sCOD concentration was higher than the sCOD of AL before being supplemented with glucose. Therefore, the endogenous microorganisms were not able to assimilate even the sCOD provided by glucose added.

The addition of *P. chrysosporium* in E1 and E2, caused a fast decrease in sCOD from the beginning of the treatment, obtaining sCOD removals of 51% and 59%, respectively, after 10 days of treatment. In the experiment E1, carried out without the addition of glucose, 61% of the initial sCOD was degraded in only 4 days of treatment, which duplicate the degradation efficiency reached in C1 by endogenous microorganisms.

Thus, the fungal inoculation gave an average rate of sCOD degradation of 0.51 mg/(L min) during the first 4 days, whereas the average rate in C1 was only 0.22 mg/(L min). Regarding test E2, again higher sCOD removals were obtained compared with the

non-inoculated test C2. As in E1, the inoculation of the fungus duplicated the sCOD removal rate, which increased up to 0.61 mg/(L min) during first 8 days, whereas in C2 it was only of 0.27 mg/(L min).

To estimate the fungus growth, the TSS at the beginning of the experiments and after 10 days were measured. Data are shown in Table 4.15. As can be seen, in the control tests (C1 and C2), the supplementation with glucose increased the growth of the endogenous microbiota. Moreover, in the inoculated tests (E1 and E2), the TSS increases were higher than in the controls, which can be explained by the fungus growth.

Comparing the increase in TSS observed in controls and inoculated tests, it can be estimated that fungus growth was similar in E1 and E2, around 0.6–0.7 g/L (dry matter), which is in agreement with the fact that final sCOD removals were similar to (E2) and without (E1) glucose addition. Therefore, in this case, the addition of glucose was not effective for the AL treatment.

Tabla 4. 15. *TSS for control (C1 and C2) and inoculated (E1 and E2) tests at initial and final times of the fungal treatment.*

Sample	Initial TSS (g/L)	Final TSS (g/L)	Increase (g/L)
C1	2.47 ± 0.01	3.06 ± 0.01	0.61 ± 0.01
E1	6.87 ± 0.01	8.21 ± 0.01	1.34 ± 0.01
C2	2.47 ± 0.01	5.16 ± 0.01	2.71 ± 0.01
E2	6.87 ± 0.02	10.16 ± 0.02	3.29 ± 0.02

Results for sCOD removals here obtained were higher than have been previously reported. Aloui et al. [33], reported that a 44% of COD removal was achieved by a solid-state fermentation of AL using *P. chrysosporium* in a support of sugarcane bagasse. Ahmadi et al. [26] achieved a COD degradation around 50% using this fungus

immobilized on loofah. Nogueira et al. [34] reported COD removals efficiencies lower than 44% for *P. chrysosporium* for an OMW pre-treat by photocatalytic oxidation.

The initial concentration of $sBOD_5$ was 408 mg/L, with a biodegradability index of 0.08 (See Figure 4.34), which means that AL effluent has very low biodegradability. In the experiments C2 and E2, the initial biodegradability index (BI) was higher (0.13), as a consequence of the glucose addition.

In all cases, the $sBOD_5$ concentration decreased throughout the fungal treatment, with final values lower than 100 mg/L. Moreover, the BI decreases with the treatment since biodegradable matter was consumed. The P. chrysosporium inoculated in E1 and E2 released enzymes able to break down recalcitrant organic matter into compounds more biodegradable. However, the fungus, as well as the endogenous microorganisms, consumed these compounds as they were produced, reducing the sCOD, the sBOD5, and the BI.

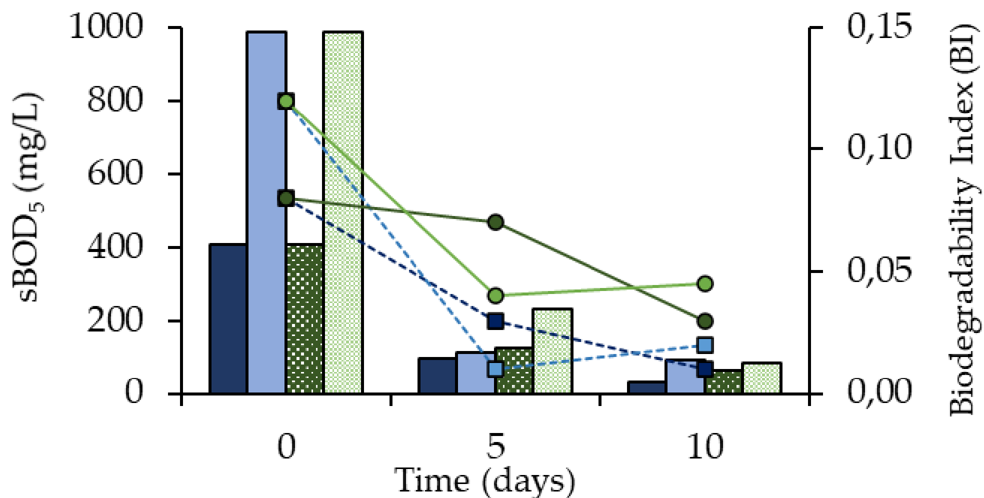


Figura 4. 34. *Changes in $sBOD_5$ concentration and biodegradability index for the different experiments at initial, intermediate, and final time of the treatment. Bars corresponds with $sBOD_5$ concentration for non-inoculated tests C1 (■) and C2 (■), and inoculated tests E1 (■) and E2 (■). The dashed lines shown the biodegradability index (BI) for non-inoculated tests C1 (■) and C2 (■), used as controls, whereas the solid lines represent the inoculated tests E1 (●) and E2 (●).*

Regardless, the addition of the fungus gave final BI higher than in the controls, even though it was low. If the enhancing of biodegradability were the objective, for example, as the previous step for the biomethanization process, an alternative could be to directly use the enzymes produced by the fungus instead of inoculating the fungus strain. In this way, the recalcitrant compounds present in the AL effluent could be broken down without the fungus using this organic matter as a nutrient source [35,36]. A sterilisation process may also be necessary to inactivate the endogenous microflora.

The evolution of reducing carbohydrates has been also measured, and results are shown in Figure 4.35. The reducing sugar concentration of the initial sample was 563 mg/L, and the ratio $sBOD_5$/reducing sugars was 0.7, indicating that a great part of the $sBOD_5$ measured is due to the reducing sugars. As expected, in the samples supplemented with 3 g/L of glucose, the initial concentration increased until 3662 mg/L. For the non-inoculated sample C1, the amount of reducing sugars remained practically stable during the treatment. In contrast, the inoculated samples (E1 and E2) showed a significant decrease in the reducing sugars concentration, with final values of 176 and 140 mg/L, respectively.

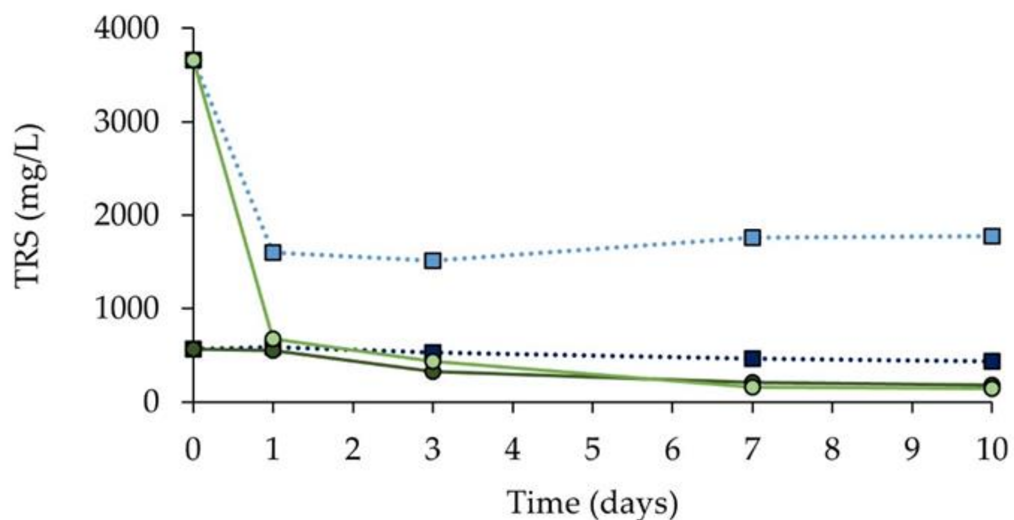


Figura 4. 35. *Changes in reducing sugars concentration during biological treatment. The dashed lines shown the non-inoculated tests C1 (■) and C2 (■), used as controls. The solid lines shown the inoculated tests E1 (●) and E2 (●). The standard deviation (SD) of the experimental data were in all cases less than 11% of mean value.*

The initial reducing sugars concentration dropped abruptly in the experiments supplemented with glucose, especially in the one that had been inoculated with the fungus. In this sense, all the glucose that was practically added to test E2 was consumed during the first 24 h, whereas in the supplemented control (C2), the amount of reducing sugars dropped from 3663 mg/L to 1772 mg/L in 24 h, and afterwards remained almost constant, indicating that the endogenous microbiota was not able to degrade all the glucose added. Although the enzymatic activities of the fungus were not measured in this study, the literature has widely reported that the addition of glucose favours the synthesis of fungal enzymes, which in turn are related to the elimination of colour, COD and recalcitrant compounds [37–40]. This fact was reflected in E2, which showed a rapid degradation of reducing sugars, whereas sCOD removal was slower. Probably the fungus decomposed recalcitrant compounds that increased sCOD and, simultaneously, consumed them.

3.2. Removal of Phenolic Compounds

The concentration of total phenolic compounds in the initial AL effluent and in AL effluent after fungal treatment was analysed. The phenolic compounds cause severe pollution of surface and ground water, soils, and vegetation. Its presence has a negative effect on microorganisms due to its high antibacterial activity [35,36].

As is shown in Figure 4.36, the best efficiencies of phenolic compound removal were reached with the inoculation of *P. chrysosporium* in E1, where around 30% of phenolic compounds were degraded after 10 days of treatment reaching values of 91 mg/L. This percentage of removal was slightly lower when glucose was added (E2), obtaining final removals of 25%. For the non-inoculated samples with fungus (C1 and C2), the amount of phenolic compounds removed was lower, with removal percentage of 12% in both cases. Results proved that the fungus inoculation increases the degradation of phenolic compounds with removal percentages almost three times greater than in the non-inoculated samples. However, higher efficiencies have been reported by other authors when the AL effluent was previously sterilised.

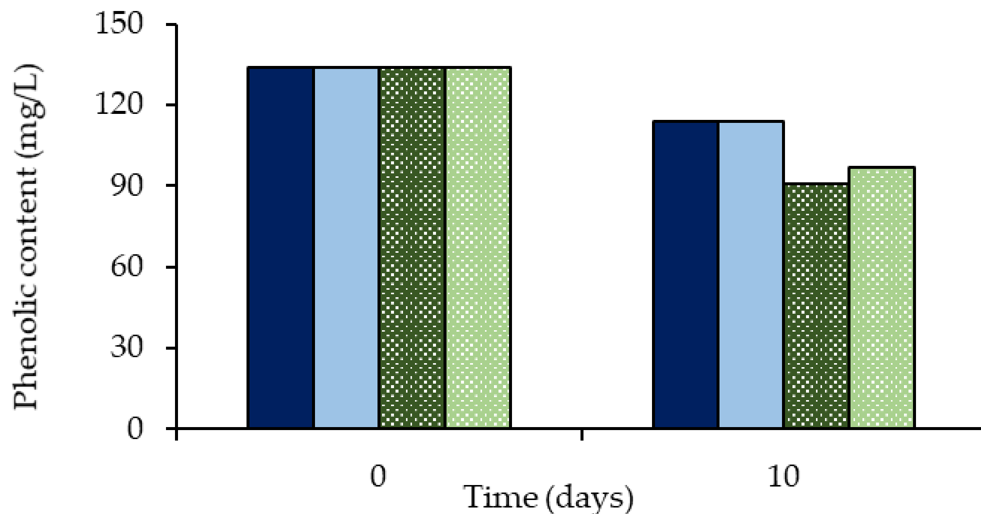


Figura 4. 36. *Changes in total phenolic content for non-inoculated tests C1 (■) and C2 (■), and inoculated tests E1 (■) and E2 (■). The standard deviation (SD) of the experimental data were in all cases less than 4% of mean value.*

Elisashvili et al. [23], who treated a diluted and sterilised olive pomace effluent by submerged fermentation with *Cerrena unicolor*, reported a removal of phenolic content of around 80%. Additionally, this fungus showed a good capacity to release laccases, which are involved in the degradation of phenolic compounds. The low removals achieved in this study it could be because a non-sterilised AL effluent. Moreover, low laccase activity has been reported for *P. chrysosporium* [24]. García et al. [35] reported a 92% total phenol degradation using *P. chrysosporium* to treat a sterilised OMW supplied with a nitrogen source. Additionally, great phenolic removals were obtained when AL was dried and the concentrate was treated. For example, Sampedro et al. [37] reported removals around 85% using the fungus *Phlebia* sp. immobilized in polyurethane sponge, while 43% was achieved when the effluent was treated by free mycelia.

3.3. Removal of Colour

AL waste has a dark brown colour, so its decolourization is important to avoid negative environmental and visual effects. Highly coloured wastewater reduces the passage of light through the water, causing a reduction in photosynthetic activity and, therefore, altering the flora and fauna of the water [38]. The colour index profile is shown in Figure 4.37.

Greater removals were obtained when the fungus was added. In the non-inoculate experiments (C1 and C2), the colour index was also reduced by endogenous microflora, especially during the first 24 h. Afterwards, the C1 slightly changed up and down, reaching final percentages of removal around 20%. When the fungus was inoculated (E1 and E2) the colour index decreased more abruptly especially during the first 24 h. Removals around 70% were obtained in both cases after 4 days. Then, the colour removal slightly increased and remained approximately stable, finally reaching a 60% reduction. The obtained results were in accordance with those found in the literature regarding the colour removals in recalcitrant wastewaters using white-rot fungi. Pakshirajan and Kheria [39] reported colour degradations of 64% after continuous fungal treatment with *P. chrysosporium* of industrial textile wastewaters. Ntougias et al. [25] reported colour removals around 60–65% in OMW using basidiomycetes fungus *Pleurotus* spp. Similar reductions were reported for olive mill effluent treated by an adapted strain of *Trametes versicolor* [40].

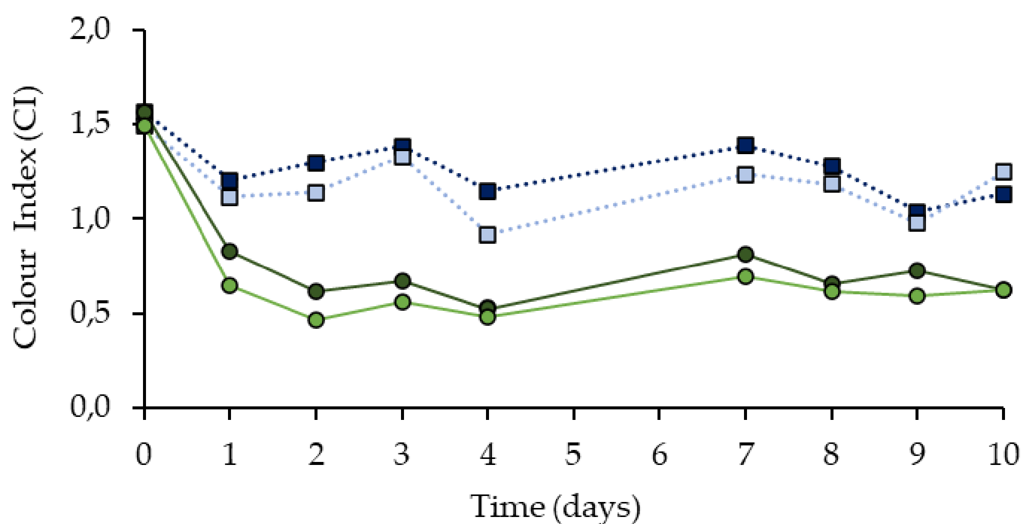


Figura 4. 37. *Changes in colour index during biological treatment. The dashed lines shown the non-inoculated tests C1 (■) and C2 (■), used as controls. The solid lines shown the inoculated tests E1 (●) and E2 (●). The standard deviation (SD) of the experimental data were in all cases less than 11.5% of mean value.*

Taking into account data reported by the literature and results obtained in this work, the use of white-rot fungi to treat AL could be considered to be a promising treatment technology. Although the removal efficiencies of sCOD, colour and phenolic compounds after treatment with fungi were slightly lower than those reported for OMW treated with physical–chemical treatments, it should be considered that these processes

usually present serious drawbacks such as their high cost, bad odour, addition of chemicals, or fouling of the membrane [4,18]. Additionally, chemical oxidation treatments can produce more recalcitrant or toxic intermediate compounds, reducing the effectiveness of the treatment. In contrast, the use of white-rot fungus allows the degradation of a wide range of recalcitrant contaminants due to its ability to release extracellular enzymes, as well as lead to the detoxification of wastewater [25].

4. CONCLUSIONS

Biotreatment with white-rot fungus *Phanerochaete chrysosporium* is useful to degrade AL waste. When the non-inoculate AL was incubated at 26 °C, 27% of the sCOD was removed in 4 days, whereas the inoculation of fungus allowed the achievement of a sCOD degradation of 60% over the same time. The addition of glucose as an easy carbon source did not enhance the sCOD degradation. The addition of *P. chrysosporium* also allowed a reduction of the colour index of the residue close to 60%. In all the conditions tested, including a control test without inoculation, a reduction in the $sBOD_5$ and in the reducing sugar content was observed after the treatment. However, the biodegradability index decreased, more highly in the inoculated effluent than those in the absence of inoculation. Finally, the treatment of the diluted AL with the fungus allowed degradation of 32% of the total phenols initially present in the effluent, whereas the endogenous microflora could only degrade around 15% of phenolic content. Results obtained in this study open the possibility of using *P. chrysosporium* fungus in the bioremediation of low-biodegradable wastes from the olive oil industry.

AUTHOR CONTRIBUTIONS

Conceptualization: A.I.D., M.I., A.L. and M.D.; methodology: A.I.D., M.I., A.L. and M.D.; validation: A.I.D., M.I. and A.L.; formal analysis: A.I.D., M.I., A.L.; investigation: A.I.D. and M.I.; data curation: A.I.D. and M.I.; writing—original draft preparation: A.I.D.; writing—review and editing: A.L. and M.D.; visualization: A.I.D. and M.I.; supervision: A.L. and M.D.; project administration, M.D.; funding acquisition, M.D. All authors have read and agreed to the published version of the manuscript.

FUNDING

This work was co-financed by Spanish MINECO (Project CTM2015-63864-R) and Foundation for the Promotion of Applied Scientific Research and Technology in Asturias (Project FC-GRUPIN-IDI/2018/000127). Ana Isabel Díaz acknowledges an FPI grant from Spanish MICINN (BES-2016-077963).

REFERENCES

[1] Messineo, A.; Maniscalco, M.P.; Volpe, R. Biomethane recovery from olive mill residues through anaerobic digestion: A review of the state of the art technology. *Sci. Total Environ*. **2020**, *703*, 135508, https://doi.org/10.1016/j.scitotenv.2019.135508.

[2] International Olive Council, Economic Affairs & Promotion Unit. EU olive oil figures. 2020. Available online: https://www.internationaloliveoil.org/wp-content/uploads/2020/12/OT-CE-901-23-11-2020-P.pdf (accessed on 23 March 2021).

[3] Alburquerque, J. Agrochemical characterisation of "alperujo", a solid by-product of the two-phase centrifugation method for olive oil extraction. *Bioresour. Technol.* **2004**, *91*, 195–200, https://doi.org/10.1016/S0960-8524(03)00177-9.

[4] Dermeche, S.; Nadour, M.; Larroche, C.; Moulti-Mati, F.; Michaud, P. Olive mill wastes: Biochemical characterizations and valorization strategies. *Process Biochem*. **2013**, *48*, 1532–1552, https://doi.org/10.1016/j.procbio.2013.07.010.

[5] Roig, A.; Cayuela, M.L.; Sánchez-Monedero, M.A. An overview on olive mill wastes and their valorisation methods. *Waste Manag*. **2006**, *26*, 960–969, https://doi.org/10.1016/j.wasman.2005.07.024.

[6] Benavente, V.; Fullana, A. Torrefaction of olive mill waste. *Biomass Bioenergy* **2015**, *73*, 186–194, https://doi.org/10.1016/j.biombioe.2014.12.020.

[7] Sampedro, I.; Aranda, E.; Rodríguez-Gutierrez, G.; Lama-Muñoz, A.; Ocampo, J.A.; Fernández-Bola, J.; García-Romera, I. Effect of a New Thermal Treatment in Combination with Saprobic Fungal Incubation on the Phytotoxicity Level of Alperujo. *J. Agric. Food Chem.* **2011**, *59*, 3239–3245, https://doi.org/10.1021/jf2003305.

[8] Göğüş, F.; Maskan, M. Air drying characteristics of solid waste (pomace) of olive oil processing. *J. Food Eng.* **2006**, *72*, 378–382, https://doi.org/10.1016/j.jfoodeng.2004.12.018.

[9] Galliou, F.; Markakis, N.; Fountoulakis, M.S.; Nikolaidis, N.; Manios, T. Production of organic fertilizer from olive mill wastewater by combining solar greenhouse drying and composting. *Waste Manag.* **2018**, *75*, 305–311, https://doi.org/10.1016/j.wasman.2018.01.020.

[10] Alburquerque, J.A.; Gonzálvez, J.; García, D.; Cegarra, J. Effects of a compost made from the solid by-product ("alperujo") of the two-phase centrifugation system for olive oil extraction and cotton gin waste on growth and nutrient content of ryegrass (Lolium perenne L.). *Bioresour. Technol.* **2007**, *98*, 940–945, https://doi.org/10.1016/j.biortech.2006.04.014.

[11] Justino, C.I.; Duarte, K.; Loureiro, F.; Pereira, R.; Antunes, S.C.; Marques, S.M.; Gonçalves, F.; Rocha-Santos, T.A.P.; Freitas, A.C. Toxicity and organic content characterization of olive oil mill wastewater undergoing a sequential treatment with fungi and photo-Fenton oxidation. *J. Hazard. Mater.* **2009**, *172*, 1560–1572, https://doi.org/10.1016/j.jhazmat.2009.08.028.

[12] Lee, Z.S.; Chin, S.Y.; Lim, J.W.; Witoon, T.; Cheng, C.K. Treatment technologies of palm oil mill effluent (POME) and olive mill wastewater (OMW): A brief review. *Environ. Technol. Innov.* **2019**, *15*, 100377, https://doi.org/10.1016/j.eti.2019.100377.

[13] Smeti, E.; Kalogianni, E.; Karaouzas, I.; Laschou, S.; Tornés, E.; de Castro-Català, N.; Anastasopoulou, E.; Koutsodimou, M.; Andriopoulou, A.; Vardakas, L.; et al. Effects of olive mill wastewater discharge on benthic biota in Mediterranean streams. *Environ. Pollut.* **2019**, *254*, 113057, https://doi.org/10.1016/j.envpol.2019.113057.

[14] Gonçalves, C.; Lopes, M.; Ferreira, J.P.; Belo, I. Biological treatment of olive mill wastewater by non-conventional yeasts. *Bioresour. Technol.* **2009**, *100*, 3759–3763, https://doi.org/10.1016/j.biortech.2009.01.004.

[15] Pinto-Ibieta, F.; Serrano, A.; Jeison, D.; Borja, R.; Fermoso, F.G. Effect of cobalt supplementation and fractionation on the biological response in the biomethanization of Olive Mill Solid Waste. *Bioresour. Technol.* **2016**, *211*, 58–64, https://doi.org/10.1016/j.biortech.2016.03.031.

[16] Hanafi, F.; Belaoufi, A.; Mountadar, M.; Assobhei, O. Augmentation of biodegradability of olive mill wastewater by electrochemical pre-treatment: Effect on phytotoxicity and operating cost. *J. Hazard. Mater.* **2011**, *190*, 94–99, https://doi.org/10.1016/j.jhazmat.2011.02.087.

[17] Kuscu, O.S.; Eke, E. Oxidation of olive mill wastewater by a pulsed high-voltage discharge using oxygen or air. *J. Environ. Chem. Eng.* **2021**, *9*, 104701, https://doi.org/10.1016/j.jece.2020.104701.

[18] Domingues, E.; Fernandes, E.; Gomes, J.; Castro-Silva, S.; Martins, R.C. Olive oil extraction industry wastewater treatment by coagulation and Fenton's process. *J. Water Process Eng.* **2021**, *39*, 101818, https://doi.org/10.1016/j.jwpe.2020.101818.

[19] Rodríguez-Couto, S. Industrial and environmental applications of white-rot fungi. *Mycosphere* **2017**, *8*, 456–466, https://doi.org/10.5943/mycosphere/8/3/7.

[20] Collado, S.; Oulego, P.; Suárez-Iglesias, O.; Díaz, M. Biodegradation of dissolved humic substances by fungi. *Appl. Microbiol. Biotechnol.* **2018**, *102*, 3497–3511, https://doi.org/10.1007/s00253-018-8851-6.

[21] Rouches, E.; Herpoël-Gimbert, I.; Steyer, J.P.; Carrere, H. Improvement of anaerobic degradation by white-rot fungi pretreatment of lignocellulosic biomass: A review. *Renew. Sustain. Energy Rev.* **2016**, *59*, 179–198, https://doi.org/10.1016/j.rser.2015.12.317.

[22] Salgado, J.M.; Abrunhosa, L.; Venâncio, A.; Domínguez, J.M.; Belo, I. Combined bioremediation and enzyme production by *Aspergillus* sp. in olive mill and winery wastewaters. *Int. Biodeterior. Biodegrad.* **2016**, *110*, 16–23, https://doi.org/10.1016/j.ibiod.2015.12.011.

[23] Elisashvili, V.; Kachlishvili, E.; Asatiani, M.D. Efficient production of lignin-modifying enzymes and phenolics removal in submerged fermentation of olive mill by-products by white-rot basidiomycetes. *Int. Biodeterior. Biodegrad.* **2018**, *134*, 39–47, https://doi.org/10.1016/j.ibiod.2018.08.003.

[24] Coconi-Linares, N.; Ortiz-Vázquez, E.; Fernández, F.; Loske, A.M.; Gómez-Lim, M.A. Recombinant expression of four oxidoreductases in *Phanerochaete chrysosporium* improves degradation of phenolic and non-phenolic substrates. *J. Biotechnol.* **2015**, *209*, 76–84, https://doi.org/10.1016/j.jbiotec.2015.06.401.

[25] Ntougias, S.; Baldrian, P.; Ehaliotis, C.; Nerud, F.; Antoniou, T.; Merhautová, V.; Zervakis, G.I. Biodegradation and detoxification of olive mill wastewater by selected strains of the mushroom genera *Ganoderma* and *Pleurotus*. *Chemosphere* **2012**, *88*, 620–626, https://doi.org/10.1016/J.CHEMOSPHERE.2012.03.042.

[26] Ahmadi, M.; Vahabzadeh, F.; Bonakdarpour, B.; Mehranian, M.; Mofarrah, E. Phenolic removal in olive oil mill wastewater using loofah-immobilized *Phanerochaete chrysosporium*. *World J. Microbiol. Biotechnol.* **2006**, *22*, 119–127, https://doi.org/10.1007/s11274-005-9006-3.

[27] Abrunhosa, L.; Oliveira, F.; Dantas, D.; Gonçalves, C.; Belo, I. Lipase production by *Aspergillus ibericus* using olive mill wastewater. *Bioprocess Biosyst. Eng.* **2013**, *36*, 285–291, https://doi.org/10.1007/s00449-012-0783-4.

[28] Díaz, A.I.; Laca, A.; Díaz, M. Fungal treatment of an effluent from sewage sludge digestion to remove recalcitrant organic matter. *Biochem. Eng. J.* **2021**, *172*, 108056, https://doi.org/10.1016/j.bej.2021.108056.

[29] Baird, R.B.; Rice, C.E.W.; Eaton, A.D. *Standard Methods for Examination of Water and Wastewater*, 23th ed.; American Public Health Association: Washington, DC, USA, 2005.

[30] Díaz, A.I.; Oulego, P.; González, J.M.; Laca, A.; Díaz, M. Physico-chemical pre-treatments of anaerobic digestion liquor for aerobic treatment. *J. Environ. Manag.* **2020**, *274*, 111189, https://doi.org/https://doi.org/10.1016/j.jenvman.2020.111189.

[31] Díaz, A.I.; Laca, A.; Laca, A.; Díaz, M. Treatment of supermarket vegetable wastes to be used as alternative substrates in bioprocesses. *Waste Manag*. **2017**, *67*, 59–66, https://doi.org/10.1016/j.wasman.2017.05.018.

[32] Moussi, K.; Nayak, B.; Perkins, L.B.; Dahmoune, F.; Madani, K.; Chibane, M. HPLC-DAD profile of phenolic compounds and antioxidant activity of leaves extract of *Rhamnus alaternus L. Ind. Crops Prod.* **2015**, *74*, 858–866, https://doi.org/10.1016/j.indcrop.2015.06.015.

[33] Aloui, F.; Abid, N.; Roussos, S.; Sayadi, S. Decolorization of semisolid olive residues of "alperujo" during the solid state fermentation by *Phanerochaete chrysosporium*, *Trametes versicolor*, *Pycnoporus cinnabarinus* and *Aspergillus niger*. *Biochem. Eng. J.* **2007**, *35*, 120–125, https://doi.org/10.1016/j.bej.2007.01.005.

[34] Nogueira, V.; Lopes, I.; Freitas, A.C.; Rocha-Santos, T.A.P.; Gonçalves, F.; Duarte, A.C.; Pereira, R. Biological treatment with fungi of olive mill wastewater pre-treated by photocatalytic oxidation with nanomaterials. *Ecotoxicol. Environ. Saf.* **2015**, *115*, 234–242, https://doi.org/10.1016/j.ecoenv.2015.02.028.

[35] García, I.G.; Peña, P.R.R.J.; Venceslada, J.L.L.B.; Martín, A.M.; Santos, M.A.M.; Gómez, E.R. Removal of phenol compounds from olive mill wastewater using *Phanerochaete chrysosporium*, *Aspergillus niger*, *Aspergillus terreus* and *Geotrichum candidum*. *Process Biochem*. **2000**, *35*, 751–758, https://doi.org/10.1016/S0032-9592(99)00135-1.

[36] Martínková, L.; Kotik, M.; Marková, E.; Homolka, L.; Markov, E. Biodegradation of phenolic compounds by Basidiomycota and its phenol oxidases: A review. *Chemosphere* **2016**, *149*, 373–382, https://doi.org/10.1016/j.chemosphere.2016.01.022.

[37] Sampedro, I.; Cajthaml, T.; Marinari, S.; Stazi, S.R.; Grego, S.; Petruccioli, M.; Federici, F.; D'Annibale, A. Immobilized Inocula of White-Rot Fungi Accelerate both Detoxification and Organic Matter Transformation in Two-Phase Dry Olive-Mill Residue. *J. Agric. Food Chem.* **2009**, *57*, 5452–5460, https://doi.org/10.1021/jf900243k.

[38] Collivignarelli, M.C.; Abbà, A.; Miino, M.C.; Damiani, S. Treatments for color removal from wastewater: State of the art. *J. Environ. Manag*. **2019**, *236*, 727–745, https://doi.org/10.1016/j.jenvman.2018.11.094.

[39] Pakshirajan, K.; Kheria, S. Continuous treatment of coloured industry wastewater using immobilized *Phanerochaete chrysosporium* in a rotating biological contactor reactor. *J. Environ. Manag.* **2012**, *101*, 118–123, https://doi.org/10.1016/j.jenvman.2012.02.008.

[40] Ergül, F.E.; Sargın, S.; Öngen, G.; Sukan, F.V. Dephenolisation of olive mill wastewater using adapted *Trametes versicolor*. *Int. Biodeterior. Biodegradation.* **2009**, *63*, 1–6, https://doi.org/10.1016/J.IBIOD.2008.01.018.

4.3.3 Evaluation of *Phanerochaete chrysosporium* for swine wastewater treatment

Ana Isabel Díaz[1], Adriana Laca[1], Mercedes Sánchez[2] and Mario Díaz[1]

[1]Department of Chemical and Environmental Engineering. University of Oviedo. C/Julián Clavería, s/n, Oviedo, 33006, Asturias, Spain.

[2]Department of Agroforestry Sciences, ETSIIAA, University of Valladolid, Avenida de Madrid 44, Palencia, 34004, Castilla y León, Spain

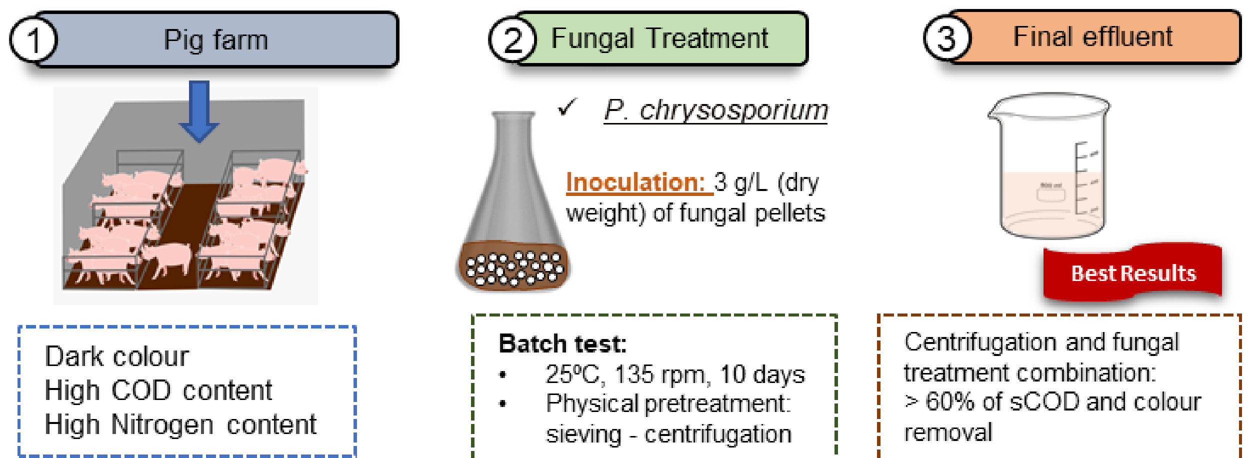


Figura 4. 38. *Resumen gráfico del trabajo 8*

ABSTRACT

The swine slurry is a mixture of manure, urine, and cleaning water from pig livestock farms, characterized by its high chemical oxygen demand (COD) and ammonia concentration. This effluent needs to be treated before being spilled to avoid negative environmental impacts as eutrophication of surface water reservoirs. In this study, an untreated slurry and two pig slurries pre-treated by anaerobic digestion with and without ammonia trapping were treated biologically by *Phanerochaete chrysosporium*. The effect on the biological treatment of pre-treating the raw slurry by sieving and centrifugation was evaluated. Samples were inoculated with 3 g/L (dry matter) of fungus and incubated at 26°C during 10 days in a batch reactor. The activity of ligninolytic enzymes (LiP, MnP and Lac), colour, COD, total nitrogen and BOD_5 evolution during the treatment were analysed. For undigested slurries, the best results were obtained for sieved slurry, reaching removal efficiencies for soluble COD and colour around 68% and 78%, respectively. For digested effluents, the digestate coming from the reactor with ammonia trapping membrane showed the best results with efficiencies of 38% for soluble COD and 35% for colour. These results provide new insights into the application of this fungus as complementary method to treat swine wastewaters.

Keywords: swine wastewater; white-rot fungi; biological treatment; bioremediation; organic removal.

1. INTRODUCTION

The swine industry has expanded rapidly in Spain, currently reaching around 40% of final livestock production [1]. Consequently, the volume of swine wastes generated by this sector has been increased. The slurry is a farming effluent resulting from the mixture of pig manure, urine and the water used to clean the pigsty, which is characterized for its high nitrogen, phosphorus, colour and organic matter concentration [2,3]. Normally, this wastewater is used as biofertilizer, precisely due to its high content of nutrients. However, because of the high slurry production in intensive farms, a large amount of this effluents needs to be managed and stored until be treated. Important environmental problems, such as acidification, soil contamination, water eutrophication, as well as the emission of gases that increase the greenhouse effect, results from a wrong management and discharge into the environment of this effluent [4,5]. This situation has encouraged the development of different technologies to treat this effluent at low costs and without environmental risks.

Different physico-chemical techniques have been evaluated to treat this effluent in order to reduce its COD and nutrients concentration [6,7]. Nevertheless, most of these methods require high energy and the addition of chemicals to the process, increasing operational costs. Thus, the use of anaerobic and aerobic biological methods has drawn extensive attention. In particular, the anaerobic digestion (AD) of slurry to produce biogas has been applied worldwide [8]. However, it is difficult to make the process economically sustainable. The high concentration of pollutants, as well as the complexity of the lignocellulosic materials present in the slurry, make necessary a pre-treatment to improve the final performance of the process. In addition, the digestate resulting from the process contains still high concentration of phosphorus, nitrogen and recalcitrant matter which needs to be treated before being discharged [9]. Besides, the aerobic treatment implies high cost of aeration. Recent research has focused on the use of microorganisms capable of growing under both aerobic and anoxic conditions, such as purple bacteria or phototrophic algae [3,10,11]. However, the problem of the wine slurry and wastewaters are still far to be solved. For this reason, some countries have promoted the creation of thermoelectric power plants using natural gas to dry the slurries. Nevertheless, this solution needs of the economic support of Government.

The use fungi as complement for biological treatments, has several advantages over the use of just bacteria since they can survive in more adverse conditions of pH and nutrient requirements. Fungi are able to synthesize extracellular enzymes such as lignin peroxidase (LiP), manganese peroxidase (MnP) or laccase (Lac), which allow them to degrade complex or recalcitrant compounds that could not be used for bacteria [12,13]. Regarding to the application of fungi for the processing of swine wastewaters and pig manures, research works have focused mainly on its use as pre-treatment to accelerate composting processes [14–16] or enhance biogas production [17–19]. Also, fungi has decribed as promising alternative to remove toxic metals, such as copper or zinc [20]. Other studies open the possibility of using swine wastewaters as growth medium for algae cultivation. In this regard, Liu et al. [21] reported a reduction of the endogenous microbial diversity in a digested piggery wastewater inoculated with *Phanerochaete chrysosporium*. This reduction increased the growth of algae around 70% in comparison to the growth obtained in untreated wastewater. In addition, various authors have employed the swine wastewaters as raw material for the production of fungal enzymes with important biotechnological applications [22,23].

The aim of this work was to evaluate the use of *Phanerochaete chrysosporium* fungus to treat three different effluents, a raw slurry and two anaerobically digested swine wastewaters. The effect of the fungal treatment on the high chemical oxygen demand (COD), colour, biological oxygen demand (BOD_5), and total nitrogen concentration has been studied. Also, the Lac, MnP and LiP enzymatic activities were determined. In addition, the effect of a physical pre-treatment of the slurry on the fungus activity was also evaluated.

2. MATERIAL AND METHODS

2.1. Samples description

Three different swine wastewaters were used for the experiments. Slurry resulting from the mixture of pig manure and pigsty washing water was collected from a pig farm sited in Castilla y Leon (Spain), and used for S1, S2 and S3. A digestate from a conventional anaerobic digestion process of pig slurry and a digestate from an AD process of pig slurry coupled with a gas-permeable membrane made of polytetrafluoroethylene (ePTFE), which allowed to recover ammonia [24], was used for

test S4 and S5, respectively. The initial characterization of these samples was showed in Table 4.16.

Tabla 4. 16. Characteristics of the initial samples subjected to fungal treatment.

	Swine wastewater			Digested slurry	
	S1	**S2**	**S3**	**D4**	**D5**
tCOD (mg O_2/L)	21632	19442	3638	5362	4125
sCOD (mg O_2/L)	3916	3850	3638	3246	2852
$sBOD_5$ (mg O_2/L)	724	695	672	213	234
BI	0.18	0.18	0.18	0.07	0.08
pH	8.63	8.59	8.55	7.58	8.03
Colour (CN)	2.418	2.232	2.240	2.122	2.106

2.2. Microorganisms

The basidiomycete white-rot fungus, *Phanerochaete chrysosporium* Burdsall 1974 (CECT 2798) was used for the biological treatment. The recovery of freeze-dried strain as well as the obtention and conservation of fungus pellets were carried out according to the methodology described by Díaz et al. [12].

2.3. Fungal treatment

Five batch tests were performed to evaluate the potential of *Phanerochaete chrysosporium* on the swine wastewaters bioremediation. Three experiments were carried out with fungus and a slurry directly coming from the pig farm. Firstly, this effluent was subjected to fungal treatment without any physical pre-treatment (**Test S1**). Then, with the aim of assess the influence of sieving or centrifugation processes as physical pre-treatments on subsequent biodegradation, two additional experiments were assayed using as raw materials the slurry sieved by 0.5 cm (**Test S2**) or the supernatant obtained from the slurry centrifugation at 10000 rpm during 15 min (**Test S3**).

Finally, two test were carried out using *P. chrysosporium* to treat an anaerobically digestated slurry from a conventional AD process (**Test D4**) or a digested

slurry from a digester with an ammonium trapping membrane (**Test D5**). Likewise, for all the conditions tested, control experiments without fungus addition were carried out to assess the impact of fungus addition in relation with the activity of the endogenous microbiota. These control experiments correspond to **C1**, **C2**, **C3**, **C4,** and **C5**.

The experiments S1, S2 and S3 were carried out in 1 L Erlenmeyer flasks containing 200 mL of sample, while S4 and S5 were performed in 250 mL Erlenmeyer flasks with 50 mL of sample. Erlenmeyer flasks were incubated at 26ºC for 10 days in an orbital shaking at 135 rpm. In all cases, according to Hu et al. [25], the sample was initially adjusted to pH = 6.0 using 1 M NaOH or 1 M HCl. The amount of fungus inoculated was 3 g/L (dry matter) in all the conditions tested. The degradation tests were carried out in duplicate. The data shown are the average values of both experiments. In all cases, standard deviations were lower than 13% with respect to average value.

The evolution of the following parameters in the supernatant were studied: chemical oxygen demand (COD), biological oxygen demand (BOD_5), pH, colour, total nitrogen, and fungal enzymatic activities (lignin peroxidase, manganese peroxidase and laccase).

2.4. Analytical methods

Samples were taken periodically each 24 h and centrifuged at 10000 rpm during 30 min. The pellet obtained after centrifugation process was dismissed, and the supernatant was used for the analytical measurements, which were done at least in triplicate.

The concentration of soluble chemical oxygen demand (sCOD) was measured by dichromate method according to Standard Methods [26]. The soluble biochemical oxygen demand ($sBOD_5$) was measured using the respirometric method by measuring oxygen pressure decrease by Oxitop bottles. The biodegradability index (BI) was calculated as the ratio of $sBOD_5$ over sCOD.

For the measurement of Lac, LiP and MnP enzymatic activities the methodology described by Lisboa et al. [27] was followed.

Concentrations of nitrogen as nitrites, nitrates, ammonium and organic nitrogen in the supernatant were measured using a Segmented Flow Self-Analyzer, SKALAR SAN PLUS, based on colourimetric reactions.

Finally, the change in the colour of slurry, defined as colour number (CN), was assessed according to Diaz et al. [28] and the value of pH was measured by means of a pH-meter.

3. RESULTS AND DISCUSSION

3.1. Fungal treatment of undigested slurry

The evolution of sCOD concentration during the fungal treatments, is shown in Fig 4.39.A and significant differences can be observed depending on the pretreatment. The highest sCOD removals were obtained for S3 test, where centrifuged slurry was used. In this case, a 59% of sCOD degradation was achieved just after three days of incubation. Then, the sCOD value remained almost constant, reaching final percentages of removal around 65%, whereas only 20% of the initial sCOD was removed in the control samples (without fungus inoculation).

With respect to S1 and S2 tests, final sCOD removals of 41% and 55% were achieved, respectively, in relation with the initial value. As can be seen in Fig 4.39, during the first two days of treatment, an increase of sCOD was observed in both test S1 and test S2, achieving values of 4685 ± 132 mg/L for and 5251 ± 202 mg/L, respectively. This effect was not observed in the control experiments.

Slightly higher concentrations of soluble organic matter were measured in the slurry pre-treated by sieving (test S2). It seems that the removal of the biggest particles allowed a better contact of the remaining particles with the fungus, favoring the solubilization of the solids and increasing the sCOD of the sample [29,30]. However, the fungus, probably with certain contribution of endogenous microbiota, consumed these compounds as they were produced.

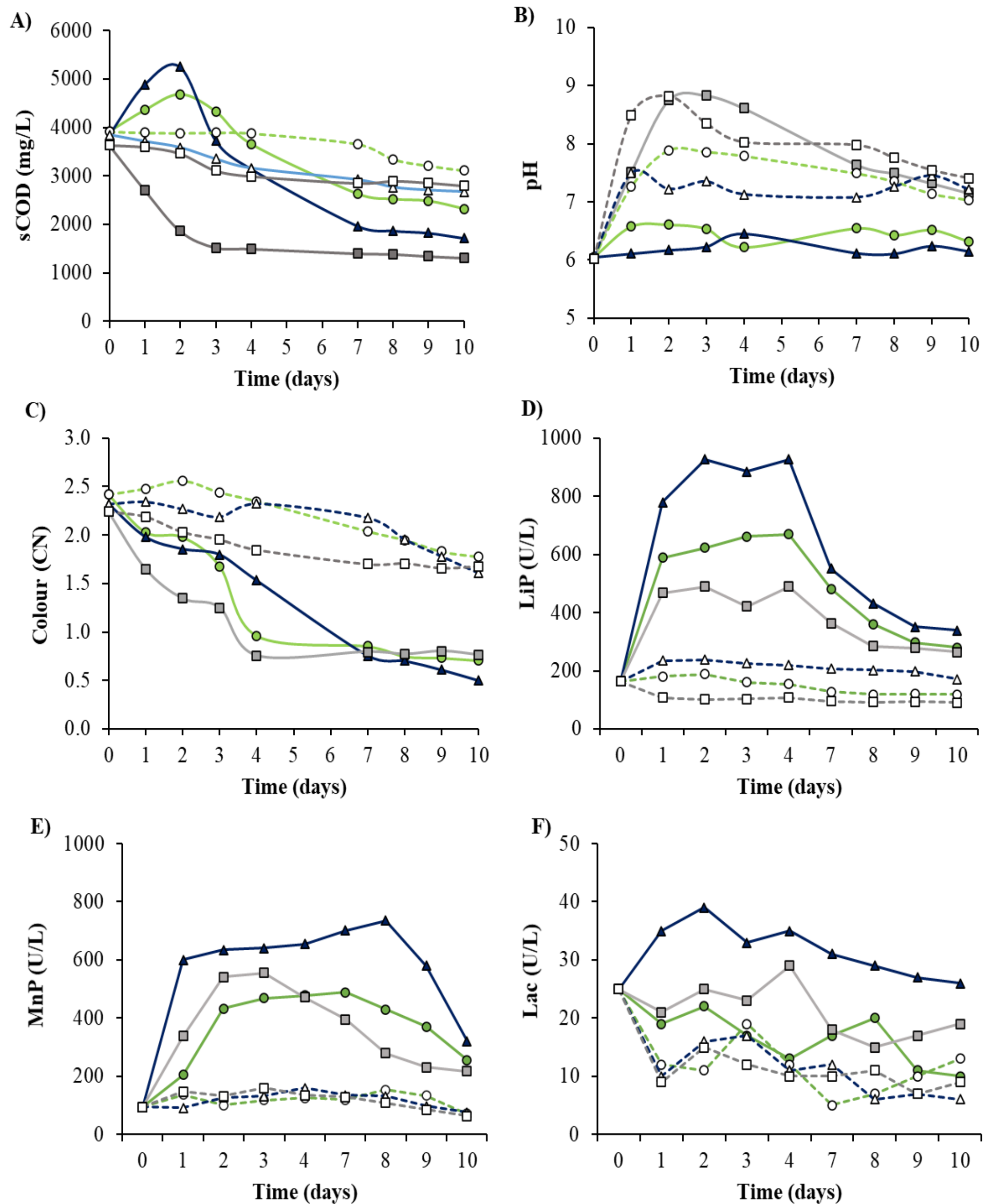


Figura 4. 39. *Evolution of different parameters during the swine wastewater treatment by P. chrysosporium.* ***A)*** *pH* ***B)*** *soluble COD* ***C)*** *Colour number (CN)* ***D)*** *LiP enzymatic activity* ***E)*** *MnP enzymatic activity* ***F)*** *Lac enzymatic activity. The standard deviation (SD) of the experimental data were in all cases less than 10% of mean value. In A, B, and C, empty markers and dashed lines show the control test, C1 ( ), C2 ( ) and C3 ( ) and filled markers and solid lines show the inoculated tests, S1 (●), S2 (▲) and S3 (■). In D, E and F, solid lines show data of S1 (●), S2 (▲) and S3 (■), while columns show data of control samples C1 (green colour), C2 (blue colour) and C3 (grey colour).*

It should be noted that, in S3, in just 5 hours, the fungus was able to drop the sCOD from 5251 mg/L to 1715 mg/L, which corresponds to a sCOD removal of 67%, similar to that obtained in S3. For non-inoculated samples, C2 and C3, sCOD degradation was a 23% and 30%, respectively, which proves that the observed degradation was mainly caused by the inoculated fungus.

Regarding the $sBOD_5$ concentration, a decrease throughout the fungal treatment was observed in all cases. From a value around 700 mg/L (Table 4.16), final values of 244 ± 11 mg/L, 283 ± 17 mg/L and 143 ± 11 mg/L were achieved for S1, S2, and S3, respectively. These concentrations were higher than those reached for the control samples, with final values lower than 80 mg/L in all cases. Consequently, because of biodegradable matter consumption, the BI decreased with the treatment, giving final effluents with a BI values between 0.11 and 0.17 for inoculated tests and below to 0.03 for controls.

Significant differences were also observed in the pH evolution. For tests S1 and S2, where the fungal treatment was carried out in the presence of suspended solids, the pH value showed a slight increase after 24 hours of treatment and then remained almost constant, reaching maximum pH values of 6.6. However, for the S3 experiment, which was carried out with the centrifuged swine wastewater, the increase in pH was significantly greater reaching pH values around 9.0 after 48 hours of treatment, which progressively dropped to a pH value of 7.2. Regarding the control samples C1, C2 and C3, an increase in pH values was also observed, greater for the C3 sample, which reached values close to 9.0 after 48 hours of treatment. It seems that the activity of microorganisms tends to increase the pH values, but this basification is balance by the solubilization of solid that occurred in S1 and S2.

With respect to the changes in CN (see Fig. 4.39.C), decolourizations over 65% were achieved in all the inoculated samples, reaching the highest removals in S2 test (78%), whereas for the control tests the decolourization efficacies were just 30%. These results revealed indicate that there was a synergistic effect between the fungus *P.chrysosporium* and the endogenous microorganisms present in the slurry. The extracellular enzymes released by the fungi allowed to break the complex organic compounds that then, could be degrade by fungus and endogenous microflora, reducing the colour. Lac, Lip and MnP activities have been measured and can be seen in

Fig.4.39. The inoculation of *P. chrysosporium* substantially increased the enzymatic activity. Lac and MnP enzymatic activities have mainly been related with colour removal and LiP with COD degradation processes [31].

In this work, the highest enzymatic production corresponded to LiP. In the case of S1 and S2, where the sample contained solids, an increase in sCOD was observed during the first 2 days of treatment, which corresponded to the high LiP values measured. Surely, the breakdown and solubilization of the complex compounds present in the solids increased the sCOD. In addition, it was observed that from the 7th day on, LiP synthesis was reduced while sCOD degradation remained almost stable. In the case of S3, the highest sCOD removal also corresponded to the highest LiP values.

The fungal enzymes production in the experiments carried out with the sieved slurry (S2) gave the highest values of LiP, MnP and Lac enzymatic activities, with a maximum activity achieved of 927 ± 11 U/L, 736 ± 17 U/L, and 39 ± 8 U/L, respectively. These results indicated that the sieving process was key for optimal enzyme synthesis and agree with the values reported by Meehnian et al [29]. These authors studied the influence of the particle size of cotton stalks on the synthesis of ligninolytic enzymes by the fungus *Daedalea flavida*. They reported higher enzyme activity when the particle size was 0.5 mm, while for particle sizes of 1 mm or 10 mm the activity was lower, which would explain the differences observed in this work for S1 and S2.

Several authors have reported the pH range of 4.5-6 as optimal for the enzymatic synthesis by *P. chrysosporium* [12,25,32]. In this work, S1 and S2 test, remained within this pH values throughout the treatment, whereas in S3 pH was above 7 after the first day of treatment. This fact, together with the absence of solids, could explain the lower enzymatic activity measured in S3.

Finally, regarding the total nitrogen (TN) concentrations in the treated samples (Table 4.17), the lowest concentrations were obtained for S3 with a final value of 487 mg/L. Comparing this value with C3 (1456 mg/L), it can be observed that the inoculation of the fungus allowed a reduction in the final TN concentration of around 67%. When the concentrations of the different nitrogen species are analyzed, it can be observed that the differences between TN concentrations in S3 and C3 were mainly due

to the removal of inorganic nitrogen by *P. chrysosporium*, with ammonium and nitrates concentration 4 times and 10 times lower, respectively, in S3 than in C3. In S1 and S2, final TN concentration were also lower than in the controls, but the differences were less marked. In these cases, the lower TN concentration were due to the degradation of dissolved organic nitrogen. Hu et al. [25], who evaluated the effect of pH on the efficiency of ammonium removal by *P. chrysosporium* for landfill leachates, described greater removals when the fungal treatment was performed at pH values higher than 7.0. This fact could explain the higher NH_3 removals obtained in S3 compared to S1 and S2.

Tabla 4. 17. *Concentrations of nitrites (NO_2^-), nitrates (NO_3^-), ammonium (NH_3), total inorganic nitrogen (TIN), dissolved organic nitrogen (DON) and total nitrogen (TN) for the inoculated (S1-S3) and control (C1-C3) samples after 10 days of swine wastewater treatment.*

Sample	NO_3^- (mg/L)	NO_2^- (mg/L)	NH_3 (mg/L)	TIN (mg/L)	DON (mg/L)	TN (mg/L)
S1	174	72	817	1063	97	1160
S2	117	51	789	957	53	1010
S3	13	0,3	207	221	266	487
C1	87	1,1	910	998	728	1726
C2	32	0,7	859	892	506	1398
C3	163	1,3	874	1039	417	1456

A pretreatment of centrifugation to remove the solids, followed by fungus treatment allowed decreases higher than 60% in COD, TN and colour. These results open the possibility of using *P. chrysosporium* to treat pig slurries in an economical and eco-friendly way. The effluent obtained could be employed for irrigation or being incorporated to aerobic conventional biological treatments Other possible utility of the fungal treatment would be to use it as a pretreatment with the aim of increasing the dissolved organic matter in the effluent so that the biogas production is improved in a subsequent anaerobic digestion process [15,19,33]. As can be observed in Fig 4.39.A, a fungal treatment of sieved slurry (S2) for just 48 h increased the sCOD concentration in around 30%.

3.2. Fungal treatment of digested slurry

The treatment of swine wastewater by anaerobic digestion as well as its co-digestion with other agri-food wastes to produce biogas has been widely studied [19,34,35]. However, the resulting digestate still contains a high recalcitrant organic load that could not be degraded by anaerobic bacteria during the anaerobic digestion process. Considering the ability of *P. chrysosporium* to synthesize fungal enzymes capable of breaking down these recalcitrant compounds, the fungal treatment could be an interesting alternative [12].

Regarding the results obtained for sCOD concentration (Fig.4.40.B), both digestates showed very similar trends, reaching greater degradations in the inoculated samples (D4 and D5) than in the control samples (C4 and C5). With respect to the sCOD removal for D5 test, carried out with the digestate from the membrane reactor, a degradation of 32% was achieved after four days of treatment. Afterwards, the sCOD concentration decreased slowly until achieving a final percentage of removal of 38%, substantially higher than the 11% reached in the control sample (C5). Regarding to the digestate from the conventional AD process, removals of 23% and 9% were achieved for D4 and C4, respectively. These results seem to indicate that the presence of an ammonium trapping membrane in the reactor favors the subsequent fungal treatment process, with final COD concentrations lower than those obtained with the conventional digestate (1780 mg/L for D5 and 2500 mg/L for D4).

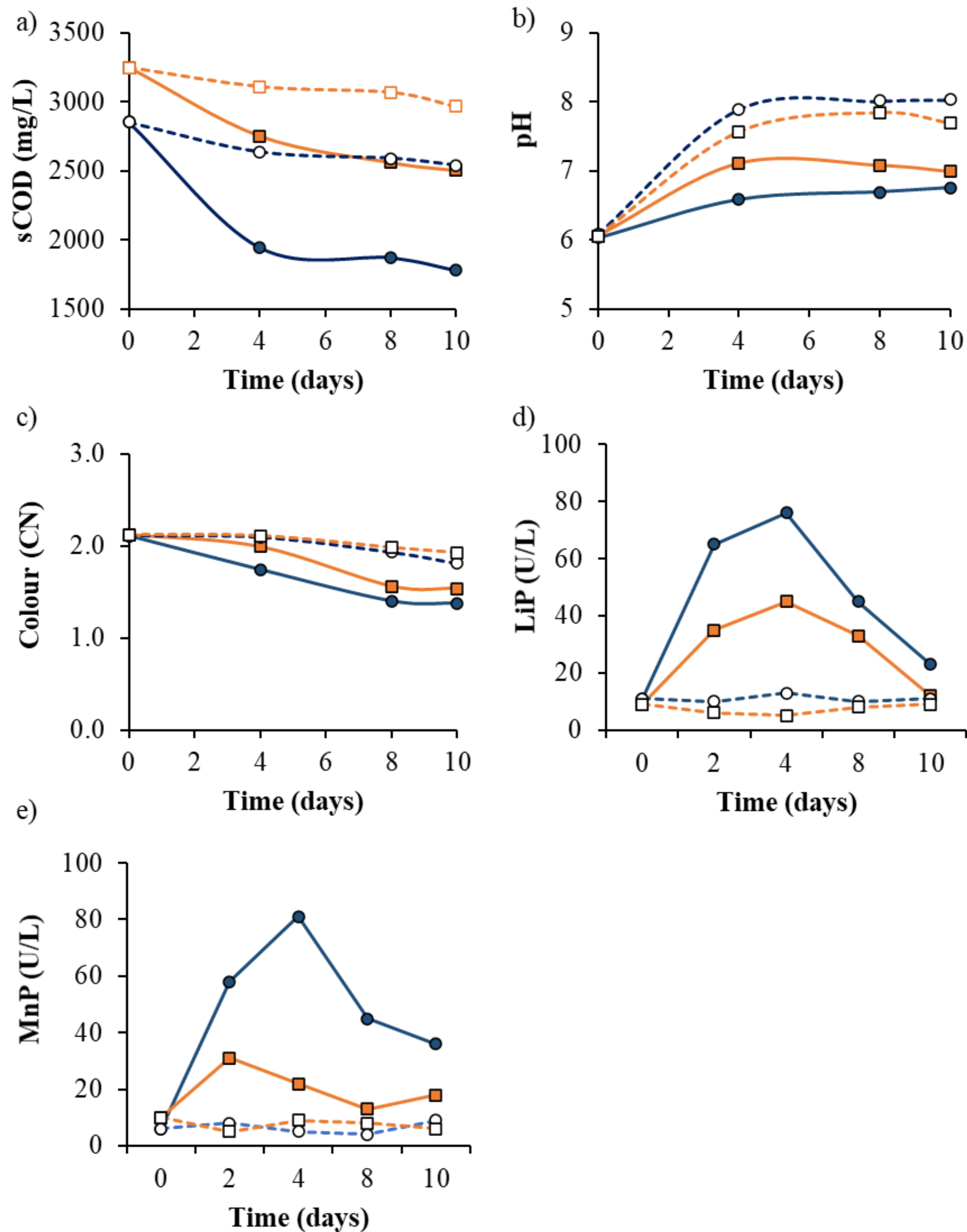


Figura 4. 40. *Evolution of different parameters during the digestated slurry treatment by P. chrysosporium.* ***A)*** *soluble COD* ***B)*** *pH* ***C)*** *Colour number (CN)* ***D)*** *LiP enzymatic activity* ***E)*** *MnP enzymatic activity. The standard deviation (SD) of the experimental data were in all cases less than 13% of mean value. Empty markers and dashed lines show the control test C4 ( ) and C5 ( ), and filled markers and solid lines show the inoculated tests, D4 (■) and D5 (●).*

With respect to the concentration of BOD_5, the values were reduced in all cases, reaching final values around 80 mg/L in the inoculated samples (D4 and D5) and around 170 mg/L in the controls (C4 and C5). In this case, the final inoculated samples showed lower BOD_5 values than the control samples, and in all cases the final effluents are low biodegradable with a BI values lower than 0.07.

Accordingly to the removal of colour (Fig.4.40.C), again higher degradations was obtained when the effluent was treated by *P. chrysosporium*. However, the colour removals achieved with the anaerobic digestates were lower than those obtained for the undigested slurry, with percentages around 30% for D4 and D5.

The LiP and MnP enzymatic activities are shown in Fig 4.40.D and Fig 4.40.E, respectively. For D5, the digestate from the membrane reactor, slightly higher values of LiP and MnP were obtained than for the conventional digestate, whereas Lac enzymatic activity was undetectable for all cases. The values obtained with the digestates were quite lower than those obtained for the undigested slurry. In the treatment of recalcitrant effluent by fungi, the importance of counting with easily assimilable carbon sources to favor enzymatic synthesis has been described by several authors [12,36]. It should be noted that the digested slurries were less biodegradable than the undigested ones, as indicated by the $sBOD_5$ shown in Table 4.16. In addition, pH was not controlled during the treatment, reaching values higher than 6.5 in all cases, which could also decrease the synthesis of fungal enzymes (see Fig. 4.40.B), as pH values were above the range reported as optimal.

4. CONCLUSIONS

Results obtained in this work showed the potential of *P. chrysosporium* to treat swine slurries and wastewaters, either effluents directly from the pig farm or effluents previously subjected to anaerobic digestion processes. A short fungal treatment of the raw slurry (2 days) caused the solubilization of solids, increasing the sCOD concentration for a subsequent anaerobic treatment. If the fungal treatment continues for 7 days, percentages of sCOD removal (calculated with respect to the initial concentration) of 41% were achieved. Additionally, the use of centrifugation as previous step allowed decreases higher than 60% in COD, TN and colour, so that the effluent can be more easily used for irrigation or treated in conventional processes.

Moreover, high enzymatic activities of LiP and MnP were measured after 2 days of fungal treatment, in particular the highest values were obtained when the sieved slurry was treated, which opens the possibility of using this residue as a raw material to synthetize fungal ligninolytic enzymes with large industrial applications. Regarding the treated digestates, even though they were low-biodegradable effluents (BI < 0.1), the fungal treatment was able to eliminate 23-32% of the initial sCOD concentration and around 30% of the colour. The possibilities of coupling fungal treatment with other biological processes are wide, however further specifical research should be carried out.

ACKNOWLEDGEMENTS

This work was co-financed by Spanish MINECO (Project CTM2015-63864-R) and Foundation for the Promotion of Applied Scientific Research and Technology in Asturias (Project FC-GRUPIN-IDI/2018/000127). Ana Isabel Díaz acknowledges an FPI grant from Spanish MICINN (BES-2016-077963) Technical assistance from the School of Agricultural Engineering of Palencia. (University of Valladolid -ETSIIAA) is gratefully acknowledged.

REFERENCES

[1] MAPA, The meat sector in figures. Main economic indicators. Subdirectorate General of Livestock and Hunting Productions. General Directorate of Agricultural Productions and Markets., 2020. https://cpage.mpr.gob.es/.

[2] Z. Luo, D. Wang, J. Yang, W. Zeng, The effect of using pig manure as an internal carbon source in a traditional piggery wastewater treatment system for biological denitrification, Ecol. Eng. 143 (2020) 105638. https://doi.org/10.1016/j.ecoleng.2019.105638.

[3] C.A. Sepúlveda-Muñoz, I. de Godos, D. Puyol, R. Muñoz, A systematic optimization of piggery wastewater treatment with purple phototrophic bacteria, Chemosphere. 253 (2020). https://doi.org/10.1016/j.chemosphere.2020.126621.

[4] Z. Yuan, X. Pan, T. Chen, X. Liu, Y. Zhang, S. Jiang, H. Sheng, L. Zhang, Evaluating environmental impacts of pig slurry treatment technologies with a life-cycle perspective, J. Clean. Prod. 188 (2018) 840–850.

https://doi.org/10.1016/j.jclepro.2018.04.021.

[5] S. Lopez-Ridaura, H. van der Werf, J.M. Paillat, B. Le Bris, Environmental evaluation of transfer and treatment of excess pig slurry by life cycle assessment, J. Environ. Manage. 90 (2009) 1296–1304. https://doi.org/10.1016/j.jenvman.2008.07.008.

[6] A. Makara, Z. Kowalski, Pig manure treatment and purification by filtration, J. Environ. Manage. 161 (2015) 317–324. https://doi.org/10.1016/j.jenvman.2015.07.022.

[7] P. Chelme-Ayala, M.G. El-Din, R. Smith, K.R. Code, J. Leonard, Advanced treatment of liquid swine manure using physico-chemical treatment, J. Hazard. Mater. 186 (2011) 1632–1638. https://doi.org/10.1016/J.JHAZMAT.2010.12.047.

[8] Q. Cheng, C. Xu, W. Huang, M. Jiang, J. Yan, G. Fan, J. Zhang, K. Chen, B. Xiao, G. Song, Improving anaerobic digestion of piggery wastewater by alleviating stress of ammonia using biochar derived from rice straw, Environ. Technol. Innov. 19 (2020) 100948. https://doi.org/10.1016/J.ETI.2020.100948.

[9] D. Nagarajan, A. Kusmayadi, H.W. Yen, C. Di Dong, D.J. Lee, J.S. Chang, Current advances in biological swine wastewater treatment using microalgae-based processes, Bioresour. Technol. 289 (2019) 121718. https://doi.org/10.1016/j.biortech.2019.121718.

[10] D. Li, R. Liu, X. Cui, M. He, S. Zheng, W. Du, M. Gao, C. Wang, Co-culture of bacteria and microalgae for treatment of high concentration biogas slurry, J. Water Process Eng. 41 (2021) 102014. https://doi.org/10.1016/j.jwpe.2021.102014.

[11] Y. Lu, C. Zhuo, Y. Li, H. Li, M. Yang, D. Xu, H. He, Evaluation of filamentous heterocystous cyanobacteria for integrated pig-farm biogas slurry treatment and bioenergy production, Bioresour. Technol. 297 (2020) 122418. https://doi.org/10.1016/j.biortech.2019.122418.

[12] A.I. Díaz, A. Laca, M. Díaz, Fungal treatment of an effluent from sewage sludge

digestion to remove recalcitrant organic matter, Biochem. Eng. J. 172 (2021) 108056. https://doi.org/10.1016/j.bej.2021.108056.

[13] A.F. Ariste, R.A. Batista-García, V.K. Vaidyanathan, N. Raman, V.K. Vaithyanathan, J.L. Folch-Mallol, S.A. Jackson, A.D.W. Dobson, H. Cabana, Mycoremediation of phenols and polycyclic aromatic hydrocarbons from a biorefinery wastewater and concomitant production of lignin modifying enzymes, J. Clean. Prod. 253 (2020) 119810. https://doi.org/10.1016/j.jclepro.2019.119810.

[14] Y.A.Y. Abdellah, T. Li, X. Chen, Y. Cheng, S. Sun, Y. Wang, C. Jiang, H. Zang, C. Li, Role of psychrotrophic fungal strains in accelerating and enhancing the maturity of pig manure composting under low-temperature conditions, Bioresour. Technol. 320 (2021) 124402. https://doi.org/10.1016/j.biortech.2020.124402.

[15] S. Voběrková, M.D. Vaverková, A. Burešová, D. Adamcová, M. Vršanská, J. Kynický, M. Brtnický, V. Adam, Effect of inoculation with white-rot fungi and fungal consortium on the composting efficiency of municipal solid waste, Waste Manag. 61 (2017) 157–164. https://doi.org/10.1016/j.wasman.2016.12.039.

[16] G.M. Zeng, H.L. Huang, D.L. Huang, X.Z. Yuan, R.Q. Jiang, M. Yu, H.Y. Yu, J.C. Zhang, R.Y. Wang, X.L. Liu, Effect of inoculating white-rot fungus during different phases on the compost maturity of agricultural wastes, Process Biochem. 44 (2009) 396–400. https://doi.org/10.1016/J.PROCBIO.2008.11.012.

[17] N. Muradov, M. Taha, A.F. Miranda, D. Wrede, K. Kadali, A. Gujar, T. Stevenson, A.S. Ball, A. Mouradov, Fungal-assisted algal flocculation: Application in wastewater treatment and biofuel production, Biotechnol. Biofuels. 8 (2015). https://doi.org/10.1186/s13068-015-0210-6.

[18] W. Cao, X. Wang, S. Sun, C. Hu, Y. Zhao, Simultaneously upgrading biogas and purifying biogas slurry using cocultivation of Chlorella vulgaris and three different fungi under various mixed light wavelength and photoperiods, Bioresour. Technol. 241 (2017) 701–709. https://doi.org/10.1016/j.biortech.2017.05.194.

[19] C. Vasmara, S. Cianchetta, R. Marchetti, S. Galletti, Biogas production from

wheat straw pre-treated with ligninolytic fungi and co-digestion with pig slurry, Environ. Eng. Manag. J. 14 (2015) 1751–1760. https://doi.org/10.30638/eemj.2015.186.

[20] M.S. Price, J.J. Classen, G.A. Payne, Aspergillus niger absorbs copper and zinc from swine wastewater, Bioresour. Technol. 77 (2001) 41–49. https://doi.org/10.1016/S0960-8524(00)00135-8.

[21] J. Liu, W. Qiu, Y. Wang, Fungal pretreatment of raw digested piggery wastewater enhancing the survival of algae as biofuel feedstock, Bioresour. Bioprocess. 4 (2017). https://doi.org/10.1186/s40643-016-0136-2.

[22] J. Lee, S.G. Shin, J. Ahn, G. Han, K. Hwang, W. Kim, S. Hwang, Use of Swine Wastewater as Alternative Substrate for Mycelial Bioconversion of White Rot Fungi, Appl. Biochem. Biotechnol. 181 (2017) 844–859. https://doi.org/10.1007/s12010-016-2253-y.

[23] S. Shradhdha, D.S. Murty, Production of lignolytic and cellulolytic enzymes by using basidiomycetes fungi in the solid state fermentation of different agro-residues, Res. J. Biotechnol. 15 (2020) 10–17.

[24] B. Molinuevo-Salces, B. Riaño, M.B. Vanotti, M.C. García-González, Gas-Permeable Membrane Technology Coupled With Anaerobic Digestion for Swine Manure Treatment, Front. Sustain. Food Syst. 2 (2018) 1–12. https://doi.org/10.3389/fsufs.2018.00025.

[25] L. Hu, G. Zeng, G. Chen, H. Dong, Y. Liu, J. Wan, A. Chen, Z. Guo, M. Yan, H. Wu, Z. Yu, Treatment of landfill leachate using immobilized Phanerochaete chrysosporium loaded with nitrogen-doped TiO 2 nanoparticles, J. Hazard. Mater. 301 (2016) 106–118. https://doi.org/10.1016/j.jhazmat.2015.08.060.

[26] APHA;, AWWA;, WEF, Standard Methods for Examination of Water and Wastewater (23th ed.), Am. Public Heal. Assoc. Washingt. DC. (2005).

[27] D.S. Lisboa, C. Santos, R.N. Barbosa, O. Magalhães, L.M. Paiva, K.A. Moreira, N. Lima, C.M. Souza-Motta, Requalification of a Brazilian Trichoderma collection and screening of its capability to decolourise real textile effluent, Int. J.

Environ. Res. Public Health. 14 (2017) 1–16. https://doi.org/10.3390/ijerph14040373.

[28] A.I. Díaz, P. Oulego, J.M. González, A. Laca, M. Díaz, Physico-chemical pre-treatments of anaerobic digestion liquor for aerobic treatment, J. Environ. Manage. 274 (2020) 111189. https://doi.org/https://doi.org/10.1016/j.jenvman.2020.111189.

[29] H. Meehnian, A.K. Jana, M. Maiti Jana, Effect of particle size, moisture content, and supplements on selective pretreatment of cotton stalks by Daedalea flavida and enzymatic saccharification, 3 Biotech. 6 (n.d.). https://doi.org/10.1007/s13205-016-0548-x.

[30] S.J.A. van Kuijk, A.S.M. Sonnenberg, J.J.P. Baars, W.H. Hendriks, J.W. Cone, The effect of particle size and amount of inoculum on fungal treatment of wheat straw and wood chips, J. Anim. Sci. Biotechnol. 7 (2016) 1–9. https://doi.org/10.1186/s40104-016-0098-4.

[31] M. Sedighi, A. Karimi, F. Vahabzadeh, Involvement of ligninolytic enzymes of Phanerochaete chrysosporium in treating the textile effluent containing Astrazon Red FBL in a packed-bed bioreactor, J. Hazard. Mater. 169 (2009) 88–93. https://doi.org/10.1016/j.jhazmat.2009.03.070.

[32] S.R. Couto, E. Rosales, M.A. Sanromán, Decolourization of synthetic dyes by Trametes hirsuta in expanded-bed reactors, Chemosphere. 62 (2006) 1558–1563. https://doi.org/10.1016/j.chemosphere.2005.06.042.

[33] A. Folino, D.A. Zema, P.S. Calabrò, Environmental and economic sustainability of swine wastewater treatments using ammonia stripping and anaerobic digestion: A short review, Sustain. 12 (2020). https://doi.org/10.3390/su12124971.

[34] G. Lourinho, L.F.T.G. Rodrigues, P.S.D. Brito, Recent advances on anaerobic digestion of swine wastewater, Int. J. Environ. Sci. Technol. 2020 1712. 17 (2020) 4917–4938. https://doi.org/10.1007/S13762-020-02793-Y.

[35] M. Ros, J. de Souza Oliveira Filho, M.D. Perez Murcia, M.A. Bustamante, R.

Moral, M.D. Coll, A.B. Lopez Santisima-Trinidad, J.A. Pascual, Mesophilic anaerobic digestion of pig slurry and fruit and vegetable waste: Dissection of the microbial community structure, J. Clean. Prod. 156 (2017) 757–765. https://doi.org/10.1016/J.JCLEPRO.2017.04.110.

[36] A. Bardi, Q. Yuan, G. Siracusa, I. Chicca, M. Islam, F. Spennati, V. Tigini, S. Di Gregorio, D.B. Levin, G. Petroni, G. Munz, G. Petroni, G. Munz, Effect of cellulose as co-substrate on old landfill leachate treatment using white-rot fungi, Bioresour. Technol. 241 (2017) 1067–1076. https://doi.org/10.1016/J.BIORTECH.2017.06.046.

4.4. Tratamiento de efluentes de la industria papelera

En este apartado se recogen todas las investigaciones relacionadas con el tratamiento de residuos procedentes de la industria papelera, como es el licor negro. Este efluente se caracteriza por su olor, su elevado grado de color y su alto contenido en lignina, siendo por tanto altamente recalcitrante. La búsqueda de alternativas de tratamiento más sostenibles ha impulsado las investigaciones llevadas a cabo en esta sección.

Por ello, en el apartado **4.4.1** se ha evaluado el empleo del hongo basidiomiceto *P. chrysosporium* y el hongo ascomiceto *A. uvarum* para el tratamiento biológico del licor negro. Los resultados obtenidos mostraron la capacidad de ambos hongos para eliminar los compuestos fenólicos presentes en el licor de biometanización, así como el color y la materia orgánica recalcitrante. Así mismo se ha evaluado la influencia de la presencia de sólidos y del valor del pH en la efectividad del tratamiento biológico y en la capacidad de síntesis enzimática por parte de ambos hongos. Los resultados obtenidos en esta etapa de investigación son prometedores en cuanto a la aplicabilidad de los hongos para procesos de biorremediación de efluentes industriales, especialmente para el hongo *A. uvuram*, del que no se ha encontrado bibliografía previa que describa su influencia en los procesos de degradación de licores procedentes de la industria papelera.

4.4.1. Treatment of kraft black liquor using basidiomycete and ascomycete fungi

Ana Isabel Díaz[1], Adriana Laca[1*], Nelson Lima[2] and Mario Díaz[1]

[1]Department of Chemical and Environmental Engineering. University of Oviedo. C/Julián Clavería, s/n, E-33006, Oviedo, Asturias, Spain.

[2]Centre of Biological Engineering. University of Minho. Campus de Gualtar, 4710-057 Braga, Portugal.

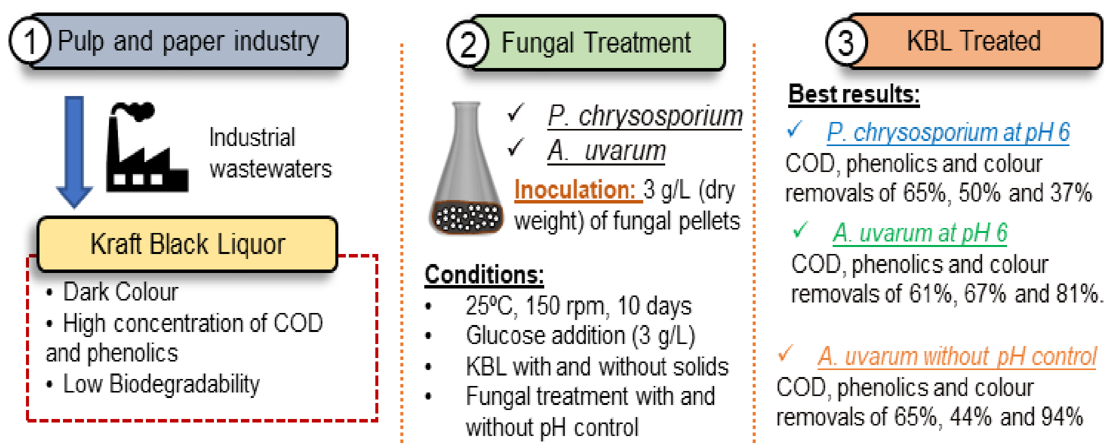


Figura 4. 41. *Resumen gráfico del trabajo 9*

ABSTRACT

The Kraft Black Liquor effluent generated by the pulp and paper industry is a highly alkaline solution with high chemical oxygen demand (COD), phenolic content, toxicity, and low biodegradability. In this work, fungi have been assayed to treat this pollutant wastewater. The experiments were carried out at 25 ºC and 150 rpm for 10 days in a batch reactor. Two fungi capable of releasing suitable enzymes have been tested, i.e., the ascomycete fungus *Aspergillus uvarum* and the basidiomycete fungus *Phanerochaete chrysosporium*. The effluent was treated with and without solids (centrifuged and filtered by 0.45 µm filter), at two different initial pH values (4 and 6) and with and without pH control. In all cases, the evolution of COD, biological oxygen demand (BOD_5), colour index, and the concentration of reducing sugars and phenolic compounds were analysed. Besides, the enzymatic activities manganese peroxidase (MnP), lignin peroxidase (LiP), and laccase (Lac) were measured throughout the experiments. Results showed that the presence or absence of solid did not affect the biodegradation process, achieving similar efficiencies. Bioremediation with *P. chrysosporium* allowed to obtain removals of COD, colour and phenolic compounds of 65%, 37% and 56%, respectively, while *A. uvarum* achieved 61%, 81% and 67%, for the best conditions tested. These results give good perspectives for application of both fungi for problematic industrial wastewaters, such as black liquor. It is especially interesting the good results obtained with *A. uvarum*, which has not been previously tested for the treatment of effluents from the paper industry.

Keywords: kraft black liquor; *Phanerochaete chrysosporium*; *Aspergillus uvarum*; biological treatment; phenolics removal.

Abbreviations: KBL, Kraft black liquor; COD, Chemical oxygen demand; BOD, Biological oxygen demand; MnP, Manganese peroxidase; LiP, Lignin peroxidase; Lac, Laccase; PHAs, Polycyclic aromatic hydrocarbons; MEA, Malt extract agar; SD, Standard deviation; DNS, dinitrosalicylic acid; CN, colour number; SAC, Spectral absorbance coefficient; ABS, absorbance.

1. INTRODUCTION

The pulp and paper production entails huge amounts of water consumption, which depends on the characteristics of the raw material and the type of produced paper. Consequently, huge amounts of complex, toxic and low biodegradable wastewater, that needs to be treated, are produced. Among these wastewaters, the Kraft Black Liquor (KBL) effluent, generated during the kraft pulping process, is considered the most problematic waste stream [1,2].

During the kraft pulping process the wood chips are digested at high temperature and pressure in a solution of sodium hydroxide and sodium sulphide called white liquor. This solution dissociates the lignin and cellulose fibres, giving a solid pulp and a liquid residue as final products. The solid pulp obtained is washed and continues to the manufacture process, while the liquid phase is mixed with the liquid residue from the pulp washing process to form the KBL [3]. This effluent is a dark colour and highly alkaline solution with high chemical oxygen demand and toxicity, and low biodegradability [4]. It is mainly composed by lignin, chlorophenols, tannins, fatty acids, and soluble sodium salts, among others [5]. Hence, this effluent must be adequately treated before being discharged to avoid adverse environmental damages on natural flora, fauna as well as aquatic bodies [6].

The KBL is normally subjected to a chemical process so that inorganic chemicals are recovered and the lignin is used as energy source [7,8]. For this, the KBL is concentrated at least up to 65% of solids and subsequently burned. The ashes are used to form the green liquor by adding water. Then, this solution is causticized and turns into white liquor, which after a filtration process is recirculated to the digestion stage. Although this is the most widely employed method, its use entails important operational and environmental problems, i.e., the increase in KBL viscosity during the first stages that favours the plugging of pipelines, the formation of deposits and fumes of inorganics salts in evaporators and furnaces or the emission of odours and hazardous gases [9,10].

Other physic-chemical techniques such as wet air oxidation, ozonation, electrocoagulation, or membrane technologies have been studied in order to reduce its colour or its concentration of recalcitrant compounds [11–14]. Although the application of these methods has been shown to be effective in removing COD, many disadvantages

are related to their use, mainly associated to the high operating cost. In addition, it has been reported that undesirable by-products such as aliphatic acids or phenolic compounds could be generated during some of these treatments [15]. For these reasons, the interest for searching alternative methods to treat KBL, which allow the removal of recalcitrant pollutants in an economical and eco-friendly way is still at its peak.

It is frequent that biological methods can suitably overcome some of the drawbacks of physic-chemical techniques since the allow the transformation of the toxic chemicals to less harmful forms [16]. Both aerobic and anaerobic treatment techniques have been described previously for the treatment of pulp and paper industry achieving a partial removal of organic matter and phenolics compounds [17–19]. However, the composition of KBL makes impossible to treat it by a conventional biological process. The removal of KBL recalcitrant pollutants in not an easy task for bacteria, mainly because of the presence of complex structures such as lignin or chlorinated lignin compounds [15]. In this sense, some researchers have focused on the research of suitable microorganisms that allow the degradation of these complex compounds, improving the effectiveness of the biological treatment process [20,21].

Fungal treatment has been considered a promising alternative for the treatment of complex industrial effluents due to the capability of some fungi to synthetize extracellular enzymes, including lignin peroxidases (LiP), manganese peroxidases (MnP) and laccases (Lac) [22]. Unlike bacteria, fungi have strong adaptability and can resist adverse conditions of temperature and pH, as well as limited nutrient availability [23]. The release of these nonspecific enzymes allows them to degrade a wide variety of recalcitrant contaminants such as pesticides, fuels, polycyclic aromatic hydrocarbons (PAHs), and synthetic dyes [24].

The use of fungi to treat industrial wastewaters in order to degrade complex compounds such as humic acids, phenols, azo dyes or pharmaceutical products have been deeply studied in the last decades [25–27]. Specifically, some authors have tested the application of white-rot fungi and its enzymes for the treatment of KBL. Font et al. [28], have reported reductions in COD and colour higher than 60% after the treatment of black liquor by *Trametes versicolor* in the form of pellets in aerated reactors. Costa et al. [29] have studied the capacity of *Phanerochaete chrysosporium* and *Bierkandera adusta* to degrade lignin, reaching removal values up to 74% and 97%, respectively, in

synthetic pulp mill wastewater. Moreover, both fungi were able to achieve 100% of delignification when real pulp mill wastewater was used. The fungi within the black *Aspergillus* group have been also described as good degraders for the wastewaters from the paper industry. Regarding phenolic compounds, an elimination around 60% was accomplished using *Aspergillus niger* in batch treatment of real pulp mill effluent [30,31].

The aim of this study was to evaluate the capacity of the ascomycete fungus *Aspergillus uvarum* and the basidiomycete fungus *Phanerochaete chrysosporium* to remove phenolic compounds, reducing sugar, colour, recalcitrant organic matter and/or enhance the biodegradability of KBL. As far as we know, the application of *A. uvarum* to treat this industrial wastewater has not been previously reported. Its behaviour is compared with the use of *P.chrysosporium* that has previously given good results for the treatment of pulp and paper mill effluents. The presence of solids and the influence of pH during the treatment were also evaluated in order to optimize and select the most suitable operating conditions for the bioremediation process.

2. MATERIAL AND METHODS

2.1. Sample description

The kraft black liquor (KBL) corresponds with the waste stream obtained after the cooking step from *Eucalyptus* wood, which was provided by a paper mill located in Asturias (Spain). A detailed information about it physicochemical composition can be seen in Pola et al. [4]. The pH value of the raw KBL was 12.7, for the biological treatment, the raw KBL was diluted with distilled water in a ratio of 1:24 (v/v) and the pH was adjusted to a value of 6.0 (KBL 1) or 4.0 (KBL 2), using NaOH 1M and HCl 1M. The characterization of the diluted and pH adjusted KBL used for the experiments is showed in Table 4.18.

Tabla 4. 18. *Characteristics of KBL diluted 1:24 after pH adjusting.*

Parameter	Value	
	KBL 1	KBL 2
pH	6,03 ± 0,01	4,03 ± 0,03
sCOD (mg O_2/L)	5792 ± 68	3590 ± 44
$sBOD_5$ (mg O_2/L)	443 ± 10	291 ± 13
Biodegradability (BI)	0,08 ± 0,001	0,06 ± 0,001
Soluble reducing sugars (mg/L)	393 ± 16	380 ± 13
Soluble phenolic compounds (mg/L)	629 ± 11	587 ± 8
Colour Number (CN)	3,4 ± 0,1	2,0 ± 0,1
Dry matter (mg/L)	7 ± 0,1	9 ± 0,1

2.2. Microorganisms and culture conditions

Aspergillus uvarum MUM 08.01 and *Phanerochaete chrysosporium* MUM 95.01 were obtained from MUM culture collection (University of Minho, Braga, Portugal). The fungi were grown on 2% of malt extract agar (MEA) at 26 ºC for 7 days.

To obtain the *A. uvarum* fungal pellets, a small portion of fungal biomass from the growing zone of petri dish was scraped with a smear loop and used to inoculate a 250 mL Erlenmeyer flask with 150 mL of Czapek-Yeast medium (1.3 g/L of K_2HPO_4, 5 g/L of yeast extract, 30 g/L of sucrose and 5 mL of a Czapeck concentrate), previously sterilized at 121 ºC for 20 min. The Czapeck concentrate consisted of 300 g/L of $NaNO_3$, 50 g/L of KCl, 50 g/L of $MgSO_4{\cdot}7H_2O$, 1 g/L of $FeSO_4{\cdot}7H_2O$, 1 g/L of $ZnSO_4{\cdot}7H_2O$ and 0.5 g/L of $CuSO_4{\cdot}5H_2O$. After 6 days, the pellets were obtained. For the obtention of *P. chrysosporium* fungal pellets, the procedure described by Díaz et al. [32] was followed. To determine the moisture of the pellets, the fungal biomass was oven-dried to constant weight at 105ºC.

2.3. Batch experimental procedure

Six different batch experiments were carried out to treat non-sterile diluted KBL. In all cases, the media were supplemented with 3 g/L of glucose to promote fungi growth, enzymatic activity and improve the degradation process of recalcitrant matter [32,33]. The operational conditions are shown in Table 4.19. Two experiments were carried out with *P. chrysosporium* using diluted KBL with solids and keeping the pH constant at a value of 4.0 (P4) and 6.0 (P6) by adding NaOH 1M or HCL 1M during the treatments.

The experiments were carried out in 1 L Erlenmeyer flasks containing 200 mL of sample inoculated with 3 g/L (dry matter) of fungus. Erlenmeyer flasks were incubated at 25ºC for 10 days in an orbital shaking at 150 rpm. Two experiments were also carried out with *A. uvarum* in 500 mL Erlenmeyer flasks containing 100 mL of sample at pH 4.0 (U4) and pH 6.0 (U6). In both cases, the pH was controlled during the treatment. The inoculation and incubation conditions were the same as in the case of *P. chrysosporium*.

Finally, with the aim to evaluate the influence of pH control during the treatment, two additional experiments similar to U6 were carried out using diluted KBL adjusted initially to pH 6, with solids (US) and without solids (UL) and without pH control during the treatment. To remove the solids, the sample was centrifuged for 20 min at 9000 rpm and filtered by 0.45 µm filter (Millipore).

For all the conditions tested, control experiments without fungus addition were assayed with the aim of evaluating the effect of fungus addition in relation with the activity of the endogenous microbiota. These control tests correspond to C-P4, C-P6, C-U4, C-U6, CL and CS.

In all cases, the experiments were carried out in duplicate. The data shown are the average of the experimental data. In each particular case, the percentage of standard deviation (SD) with respect to the mean value is shown. Periodically, samples were taken for the analysis of COD, total phenolic content, reducing sugars concentration, colour number, pH, BOD_5 and enzymatic activities (Lac, MnP and LiP).

Tabla 4. 19. *Experimental design for the biological treatments. In all cases, the medium used was diluted KBL and the amount of inoculated fungus was 3 g/L (dry matter).*

Test	Fungus	Initial pH	Glucose	pH control	Solids
P4	*P. chrysosporium*	4.0	3 g/L	Yes	Yes
C-P4	Non inoculated	4.0	3 g/L	Yes	Yes
P6	*P. chrysosporium*	6.0	3 g/L	Yes	Yes
C-P6	Non inoculated	6.0	3 g/L	Yes	Yes
U4	*A. uvarum*	4.0	3 g/L	Yes	Yes
C-U4	Non inoculated	4.0	3 g/L	Yes	Yes
U6	*A. uvarum*	6.0	3 g/L	Yes	Yes
C-U6	Non inoculated	6.0	3 g/L	Yes	Yes
US	*A. uvarum*	6.0	3 g/L	No	Yes
CS	Non inoculated	6.0	3 g/L	No	Yes
UL	*A. uvarum*	6.0	3 g/L	No	No
CL	Non inoculated	6.0	3 g/L	No	No

2.4. Analytical methods

Samples were centrifuged at 9000 rpm during 10 min, filtered by 0.45 μm filter (Millipore) and stored at -20ºC until being analysed. All analytical measurements were done at least in triplicate.

Soluble biochemical oxygen demand ($sBOD_5$) was determined using a manometric respirometry measurement system (Lovibond® Water Testing BD 600).

For soluble COD (sCOD) determination, the test kit Hach Lange LCK 514 was used, and samples was spectrophotometrically measured at 600 nm. The biodegradability index (BI) was calculated as the ratio of soluble $sBOD_5$ over soluble sCOD.

The concentration of reducing sugars was determined by the Miller method, using dinitrosalicylic acid (DNS), according to the methodology described by Díaz et al. [34]. Glucose was used as standard. The absorbance was measured at 540 nm using a UV/vis spectrophotometer (Thermo Scientific, Heλios γ).

Total phenolic content was measured using by the Folin–Ciocalteu's phenol method following the procedures of Moussi et al. [35]. In this procedure, 400 μL of sample were mixed with 3 mL of Folin-Ciocalteu reagents (previously diluted 1:10 with distilled water). This mixture was maintained at 22ºC for 5 min. After that, 3 mL of sodium bicarbonate ($NaHCO_3$ 0.6 g/L) were added, and the sample was again incubated at 22ºC for 90 min. After incubation, the absorbance was measured at 725 nm against a blank. Gallic acid was used as standard.

The change in the colour of the KBL was indicated by the colour number (CN), which is defined according to Equation (1), where spectral absorbance coefficients (SAC) are defined as the ratio of the values of the absorbances (Abs) measured at 436, 525 and 620 nm over the cell thickness (x) [36].

$$CN=\frac{SAC_{436}^2+SAC_{525}^2+SAC_{620}^2}{SAC_{436}+SAC_{525}+SAC_{620}} \qquad \text{Eq (1)}$$

For the analysis of Lac, LiP and MnP enzymatic activities the methodology described by Lisboa et al. [37] was followed. For Lac activity the reaction mixture was: 0.8 mL of ABTS (0.03 % v/v), 0.1 mL sodium acetate buffer (0.1M, pH 5.0) and 0.1 mL of sample. The oxidation of ABTS was measured at 420 nm with a molar extinction coefficient (ε) of 3.6×10^4 $M^{-1}cm^{-1}$. The LiP activity was assessed by mixing 1 mL of sodium tartrate buffer solution (125 mM, pH 3.0), 500 μL of veratryl alcohol (10 mM), 500 μL of hydrogen peroxide (2 mM) and 500 μL of sample. The production of veratraldehyde was measured at 310 nm ($\varepsilon = 9.3 \times 10^3$ $M^{-1}cm^{-1}$). The measurement of MnP activity was carried out using the Phenol Red' method by mixing 500 μL of sample, 100 μL of phenol red (0.01% p/v), 100 μL of sodium lactate (250 mM), 200 μL of bovine albumin (0.5% w/v), 50 μL of sulphate manganese (2 mM), 50 μL of

hydrogen peroxide (2 mM) and 1.0 mL of sodium succinate buffer (20 mM, pH 4.0). All the reactions were performed at 30ºC for 5 minutes and were stopped by adding 40 µL of 2 N NaOH. One unit of enzyme (U) was defined as the release of 1 µmol product formed per min under the assay conditions.

Finally, total solids (TS) were analysed by oven-drying (105ºC) a determined volume of sample to constant weight and the value of pH was measured by means of a pH-meter.

3. RESULTS AND DISCUSSION

3.1. KBL biodegradation by *P. chrysosporium* with pH control

Diluted KBL was biotreated by *P. chrysosporium* at pH 4 (**P4**) and pH 6 (**P6**). As can be seen in Table 4.18, the characteristics of the raw material were different according to the initial pH, especially for sCOD, $sBOD_5$ and colour, due to the precipitation of lignin as a consequence of the pH adjustment. This fact was reflected in the increase in dry matter at pH 4. The evolution of soluble COD, soluble reducing sugars, soluble phenolic compounds, colour number (CN), soluble BOD_5 and enzymatic activities (LiP, MnP and Lac) for each experiment and control (CP4 and CP6), is shown in Fig 4.42.

In relation to sCOD degradation (Fig 4.42.A), for the non-inoculated tests, the sCOD concentration of test C-P4 remained almost constant during the treatment, whereas test C-P6 showed a slight reduction, carried out by the endogenous microbiota from KBL. Despite of the high alkalinity of Kraft paper effluents, microorganisms capable to survive in these wastewaters, mainly within *Firmicutes* and *Proteobacteria* phyla have been identified and isolated in previous studies [20]. In both cases, the final sCOD concentration was higher than the KBL before being supplemented with glucose. With respect to experiments inoculated with fungus (P4 and P6), a similar behaviour was observed, very different from controls. In both cases, a fast decrease of sCOD was observed during the first 24 h followed by a progressive degradation until the 4th day of treatment with a final concentration around 2000 mg/L. Afterwards, the sCOD concentration remained almost constant. However, as the initial sCOD was not the same in both cases, due to the lignin precipitation caused for the pH adjustment, the final COD removal achieved in relation with the initial sCOD concentration of the KBL

before glucose addition, was much higher at pH 6 than at pH 4 (65% and 35%, respectively). Results here obtained were higher than the COD removals reported by Wu et al. [38], who achieved COD removals less than 50% after the treatment of diluted black liquor by *P. chrysosporium* and others white-rot fungi after 16 days of treatment at pH 6 and 28ºC.

Regarding soluble reducing sugars evolution easily used by microorganisms (Fig. 4.42.B), the addition of glucose increased the initial values from around 400 mg/L to 3800 mg/L. For the control test at pH 4, these values remained almost constant during the biological treatment, probably due to the protection of the acid pH, which avoid that glucose is consumed by environmental bacteria. At pH 6 a rapid decrease of reducing sugars was observed during the first 24 hours, which corresponds with the reduction observed in sCOD. So, it can be concluded that the removal of sCOD observed in CP-6 was due to the consumption of the added glucose. It is important to keep in mind that the samples used in this work were not sterilized, so the endogenous microbiota from KBL could consume reducing carbohydrates. However, it is important to point out that even at pH 6 remained without being consumed around 2700 mg/L of reducing carbohydrates, probably due to the presence of inhibitory compounds, such as phenolics, which are in a concentration around 600 mg/L.

For the experiments inoculated with the fungus (P4 and P6), significant removals of reducing sugars were achieved compared with the initial one, reaching final values of 73% and 64% for P6 and P4, respectively. In both cases the elimination of reducing carbohydrates occurred mainly during the first 24 hours (see Fig, 4.42.B), with apparent consumption of 2.34 ± 0.02 mg/Lmin and 1.72 ± 0.07 mg /Lmin, at pH 6 and pH 4, respectively. It is necessary to take into account that the real consumption rate is higher because reducing sugars were producing at the same time as they are consumed thanks to the fungal enzymes that broke complex carbohydrates. In this work, it was observed that regardless of the pH adjustment, the highest enzymatic activity was reached at around 96 h (see Fig. 4.42.C), when most of the reducing carbohydrates had been consumed. According to results previously commented, the greatest activities were obtained at pH 6 with 625 ± 11 U/L and 497 ± 8 U/L for MnP and LiP, respectively. These enzymes are responsible for delignification, breaking down the lignin into by-products that the fungus can use as source of nutrients [31]. In addition, the removal of

COD and colour has been mainly related with the release of these enzymes by white-rot fungi [39]. Lac activity was lower than 5 ± 0.6 U/L for both values.

Accordingly to the consumption of reducing sugars, a reduction of $sBOD_5$ concentration was obtained in all cases, reaching 172 ± 8 mg/L for test P4 and 196 ± 7 mg/L for P6 (Fig. 4.42.D). However, due to COD removal, the use of *P. chrysosporium* gave final biodegradability index slightly higher than in the initial KBL, 0.07 and 0.09 for P4 and P6, respectively. Regarding control test, a certain reduction of $sBOD_5$ values was obtained because of glucose consumption by endogenous microbiota. However, these values were higher than the initial $sBOD_5$ of KBL, according to the fact that endogenous microorganisms could not degrade even the glucose added.

The evolution of phenolics concentration is shown in Fig 4.42.E. The removal of phenolic compounds is crucial because of its grave toxic effects on humans, animals and natural environment [40]. Again, the highest removals were reached for the experiment carried out at pH 6, where *P. chrysosporium* could degrade 56% of phenolics after 8 days of treatment, with final concentration below 300 m/L. At pH 4, 44% of phenolics were removed just in 3 days. For both control tests the degradation was lower than 4%.

Although the dephenolization process is mainly related to the activity of Lac, this study probes that other ligninolytic enzymes, such LiP and MnP, also contributes to the degradation of these compounds. Results here obtained agree with other studies on the degradation of phenolics by white-rot fungi, i.e., Sampedro et al. [41] reported removals around 43% using free mycelia of *Phlebia* sp. to treat an olive mill effluent.

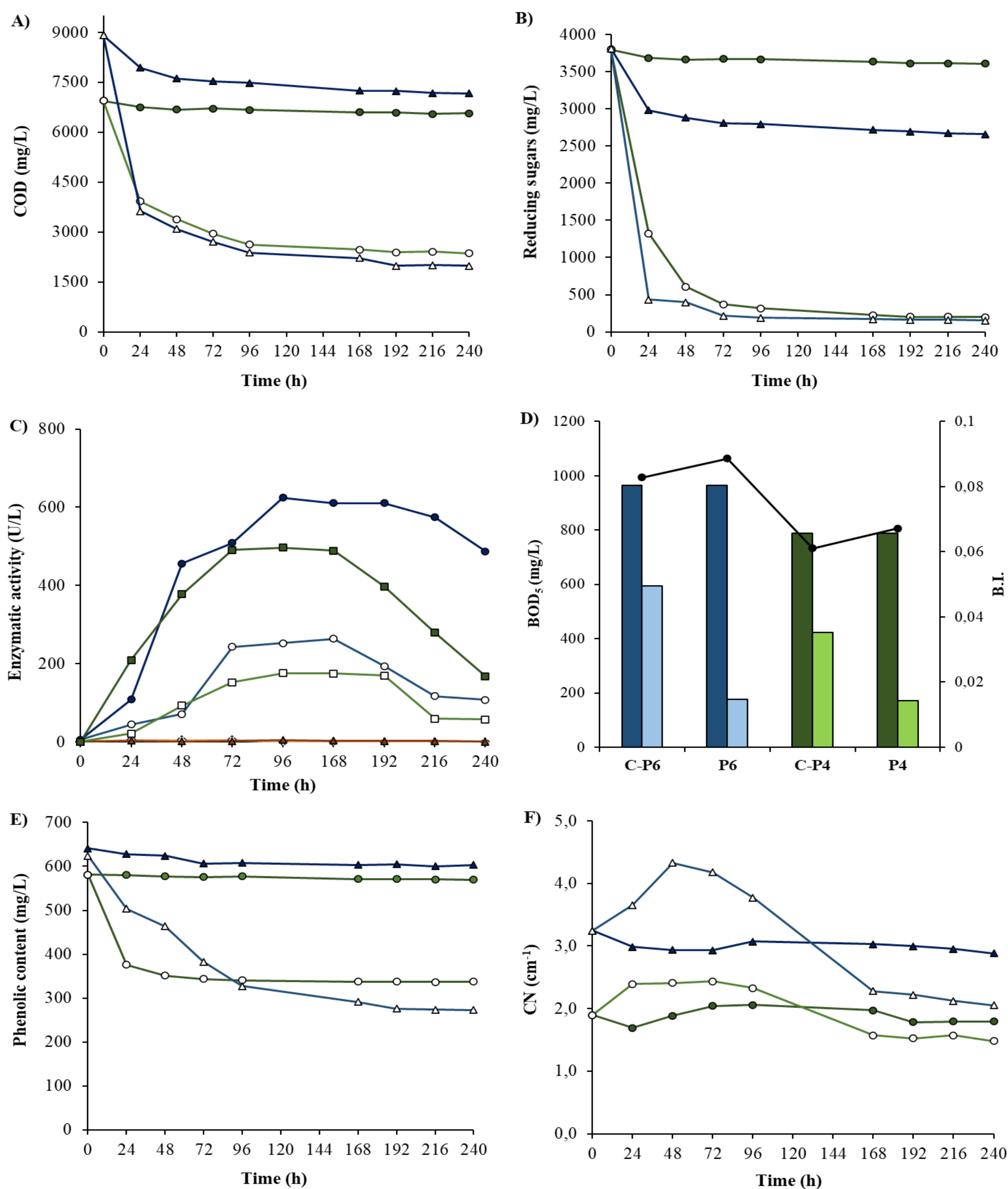


Figura 4. 42. *Evolution of different parameters during the treatment of KBL with P. chrysosporium and pH control.* ***A)*** *Soluble COD* ***B)*** *Reducing sugars* ***C)*** *Enzymatic activities* ***D)*** *BOD_5 and biodegradability index (BI)* ***E)*** *Phenolic compounds.* ***F)*** *Colour number (CN). The standard deviation (SD) of the experimental data were in all cases less than 11% of mean value. In A, B, E and F, empty markers show the inoculated tests, P6 (▲) and P4 (●) and filled markers show the non-inoculated tests, C-P6 (▲) and C-P4 (●). In C, filled markers and empty markers show data of P6 and P4, respectively. MnP (●, ○), LiP (■, □) and Lac (▲, △). In D, initial BOD_5 is shown in dark columns, final BOD_5 in light columns and final BI in circle markers.*

The colour removal achieved by the treatment with *P. chrysosporium*, was lower than 30% for both pH values. It should be noted that for both the pH values here assayed the colour of the sample was higher than the initial one during the first 96 hours of treatment, which coincided with the drop of sCOD and the maximum enzymatic activity. Junnarkar et al. [42] observed the production of a dark brown colour during the treatment of wheat straw by *P. chrysosporium*. This colour was related with secondary products formed during lignocellulosic material biodegradation. This fact might explain the behaviour here obtained, since *P. chrysosporium* could generate coloured by-products from lignin degradation.

3.2. KBL biodegradation by *A. uvarum* with pH control

The industrial application of *A. uvarum* has hardly been studied. Therefore, its use for the treatment of KBL effluents has not been previously reported. This fungus was selected based on the good results reported by Salgado et al. [43] for the treatment of olive mill and winery wastewaters, in terms of colour and phenolic compounds removal. *A. uvarum* belongs to the group of black Aspergilli and has the capacity to produce secalonic acid, asteric acid, geodyn, erdine and dihydrogeodyine [44].

The evolution of sCOD for the KBL treatment by *A. uvarum* is shown in Fig. 4.43.A. In this case, the degradation of sCOD was progressive during the treatment with the inoculated fungus, whereas in the control tests sCOD remained almost constant at pH 4 and decreased slightly at pH 6. As occurred with *P. chrysosporium*, final sCOD was similar at both pH values. However, as the initial sCOD was higher at pH 6, the percentage of sCOD removal was higher at this pH. So, *A. uvarum* accomplished 61% and 43% of sCOD degradation at pH 6 and pH 4, respectively. Liu et al. [45] reported similar results with a maximum COD removal of 60% at pH 6.0 when an alkaline pulping effluent was treated by *Aspergillus niger*.

Regarding the evolution of reducing sugars concentration (Fig 4.43.B), again an abrupt decrease was observed during the first 72 h with degradation rates of 1.49 ± 0.11 mg/Lmin and 2.02 ± 0.08 mg/Lmin for U4 and U6, respectively. Compared to *P. chrysosporium*, the consumption of reducing sugars attained after 10 days was slightly higher, without significant differences between U4 and U6 (78% and 75%, respectively).

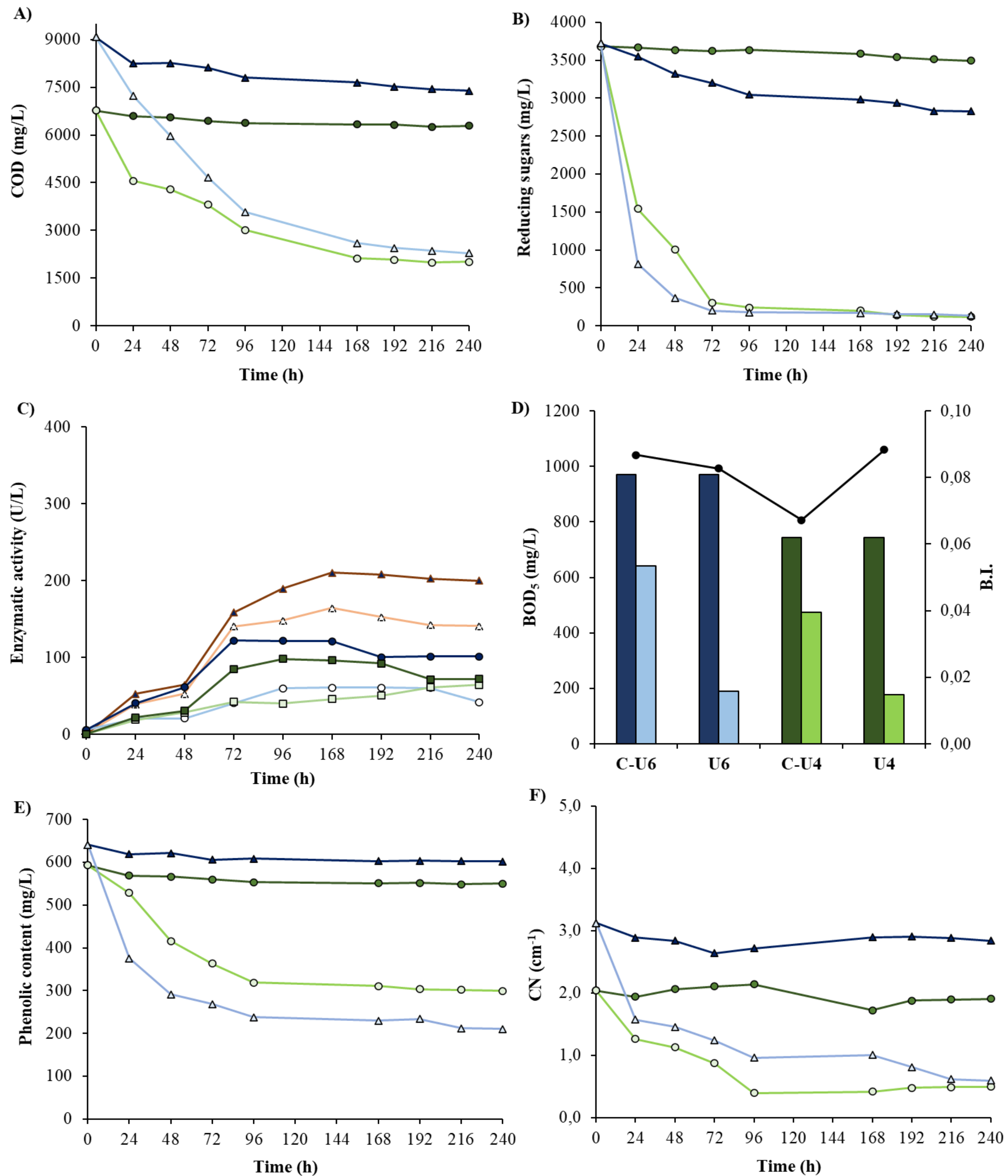


Figura 4. 43. *Evolution of different parameters during the treatment of KBL with A. uvarum and pH control.* ***A)*** *Soluble COD* ***B)*** *Reducing sugars* ***C)*** *Enzymatic activities* ***D)*** *BOD_5 and biodegradability index (BI)* ***E)*** *Phenolic compounds.* ***F)*** *Colour number (CN). The standard deviation (SD) of the experimental data were in all cases less than 12% of mean value. In A, B, E and F, empty markers show the inoculated tests, U6 (△) and U4 (○) and filled markers show the non-inoculated tests, C-U6 (▲) and C-U4 (●). In C, filled markers and empty markers show data of U6 and U4, respectively. MnP (●, ○), LiP (■, □) and Lac (▲, △). In D, initial BOD_5 is shown in dark columns, final BOD_5 in light columns and final BI in circle markers.*

Compared to *P. chrysosporium*, the fungus *A. uvuarum* showed a lower capacity to synthesize LiP and MnP, with maximum activities achieved at pH 6 of 98 ± 9 U/L and 122 ± 6 U/L, respectively (see Fig. 4.43.C). On the contrary, enzymatic activity of Lac was much higher with *A. uvarum*, reaching values of 210 ± 7 U/L and 164 ± 12 U/L for U6 and U4, after 168 h of treatment. Salgado et al. [43], reported the ability of *A. uvarum* to synthesize other enzymes with proteolytic, lipolytic, and tannase activities, which together with the commented MnP, LiP and Lac, allowed the high efficacy of sCOD degradation.

Regarding BOD_5 concentration (Fig. 4.43.D), the behaviour was similar to that obtained with *P. chrysosporium*. As soon as enzymes broke recalcitrant organic matter into compounds more biodegradable, they were consumed by the microorganisms. Final BI were also very low with values of 0.08 and 0.09 for U6 and U4, respectively. The main different observed between the inoculation of *P. chrysosporium* or *A. uvarum* was observed with respect to the degradation of phenolic compounds, *A. uvarum* allowed to obtain higher removals for these kinds of pollutants, especially at pH 6 where 67% of the phenolics were degraded (see Fig. 4.43.E). At both pH values, the degradation process of phenolics was progressive until 96 h, and from this time on, concentration remained almost constant. The higher dephenolization observed is likely to be related with the higher Lac activity achieved with *A. uvarum*.

For both pH values, the use of *A. uvarum* gave better results in decolourization than those achieved with *P. chrysosporium* (Fig. 4.43.F). The use of *A. uvarum*, gave a removal of 76% and 81% for U4 and U6 after 10 days, respectively. In both cases (U4 and U6), the reduction in the CN was in parallel with the reduction in phenolics concentration, with the only exception of the slight decolourization observed after 168 h of treatment at pH 6. This confirms the fact that the main responsible for the dark colour of KBL are phenolic compounds. Gulzar et al. [46] reported decolourization efficacies of 78% in synthetic textile effluent after 72 hours of treatment by *Aspergillus niger*, whereas only 52% was achieved for real wastewater, results quite lower than that here achieved.

3.3 Biodegradation of KBL with and without solids, and without pH control by *A. uvarum*.

As results obtained in terms of sCOD degradation were similar for both microorganisms assayed, and phenolics degradation and consequently decolourization, was better with *A. uvarum*, this last fungus was selected for the next experiments. The qualities of the final effluents obtained at controlled pH 4 and 6 were quite similar, except for the concentration in phenolics that was lower at pH 6. Taking these results into account and in order to know if the control of pH during the treatment was necessary, the treatment of KBL with *A. uvarum* adjusting the initial pH at 6 and with free pH evolution during the treatment was carried out. Diluted KBL with solids (US) and without solids (UL) were used for these experiments.

The pH profiles are shown in Fig. 4.44.A, as can be observed, a reduction of pH took place in all cases. The control tests (CS and CL) suffered a reduction of just one unit, reaching final values around 5.0 in both cases. On the contrary, in both inoculated test (US and UL), the pH value showed a fast drop during the first 96 h, reaching values below 2. After this time, pH continued to decrease progressively until reaching pH values around 1.0 after 10 days of treatment. The genus *Aspergillus* has been widely studied for its ability to produce organic acids such as citric, gluconic, or fumaric acid, using lignocellulosic materials as substrates [47]. The organics acids may be responsible for the acidification observed in this work.

In Fig 4.44.B, the change of the concentration of soluble reducing carbohydrates is shown, with a behaviour very similar to that obtained with pH controlled at pH 6. It must be noted that, even for pH values below, sugars consumption was observed. Similar final BOD_5 values were observed for US and UL, with a BI of 0.08, as in U6 test (Fig. 4.44.D).

With respect to the enzymatic activity, the values obtained without pH control was much lower than those obtained in the pH-controlled test. As can be seen in Fig. 4.44.C, the enzymatic activities were slightly higher for the US test, with maximum values of 47 ± 6 U/L for MnP and 31 ± 4 U/L LiP after 72 h, and 53 ± 5 U/L for Lac after 24 h. Then, the enzymatic activity drastically decreased, especially for Lac. This abrupt reduction in Lac activity happened when pH was less than 3.0.

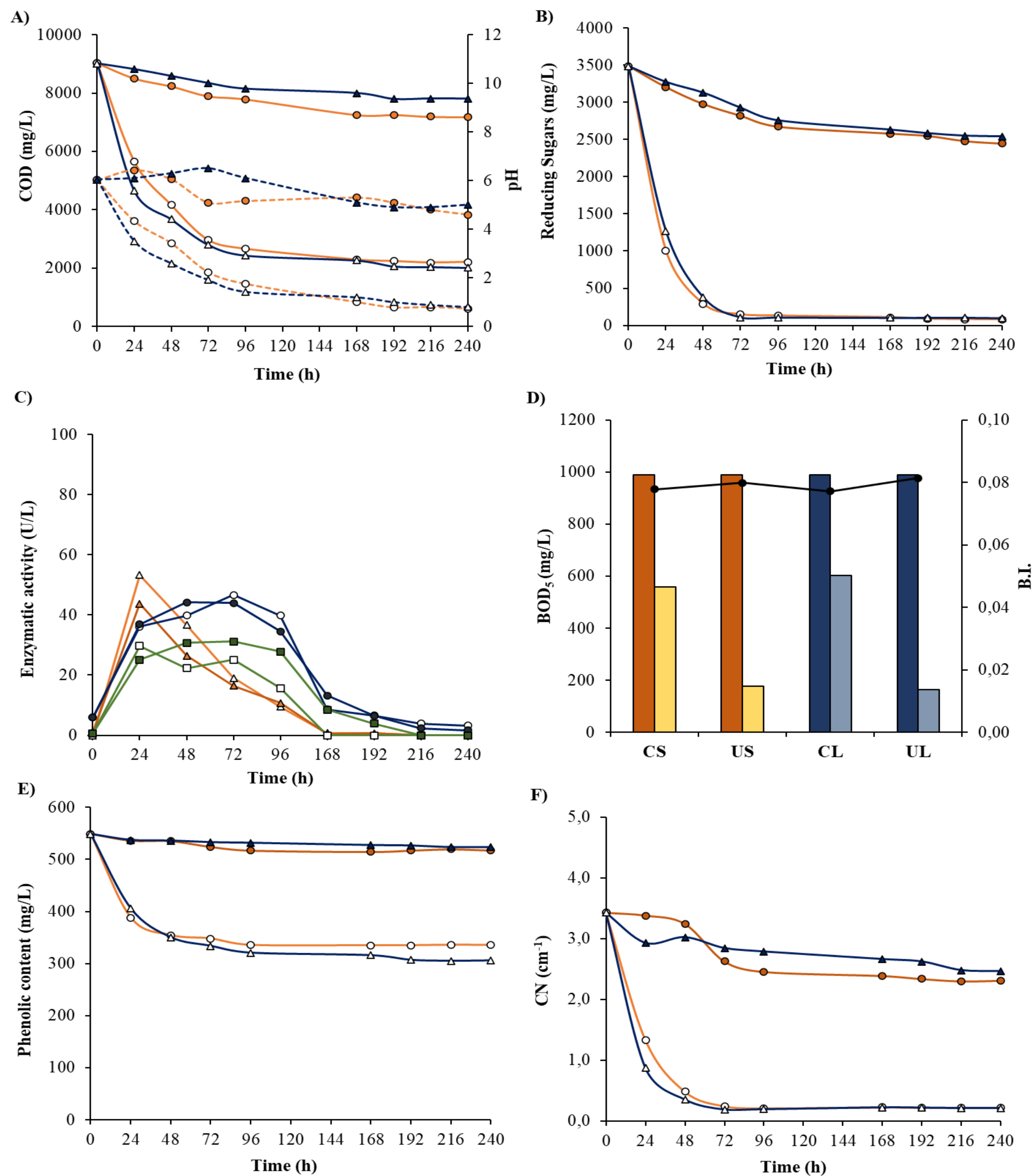


Figura 4. 44. *Evolution of different parameters during the treatment of KBL with and without solids, and without pH control with A. uvarum.* ***A)*** *Soluble COD (solid lines) and pH (dashed lines)* ***B)*** *Reducing sugars* ***C)*** *Enzymatic activities* ***D)*** *BOD_5 and biodegradability index (BI)* ***E)*** *Phenolic compounds.* ***F)*** *Colour number (CN). The standard deviation (SD) of the experimental data were in all cases less than 12% of mean value. In A, B, E and F, empty markers show the inoculated tests, UL (△) and US (○) and filled markers show the non-inoculated tests, CL (▲) and CS (●). In C, filled markers and empty markers show data of U6 and U4, respectively. MnP (●, ○), LiP (■, □) and Lac (▲, △). In D, initial BOD_5 is shown in dark columns, final BOD_5 in light columns and final BI in circle markers.*

Dhakar et al., (2015) who studied the effect of temperature and pH on the production of ligninolytic enzymes (MnP, LiP and Lac) by *A. niger*, reported that the minimum production of Lac (0.8 U/L) corresponded to a pH 3.5, while at pH 7.5 the measured activity was 8.9 U/L. In addition, an increment in MnP and LiP production was described when pH increased considering pH 5.5 and pH 9.5 as optimum for MnP (1737 U/L) and LiP (215 U/L) production, respectively. In this work, the drop of pH suffered in UL and US, explains the low fungal enzymatic activities with respect to tests carried out at controlled.

Surprisingly, despite the pH drop and the low enzymatic activities, the sCOD degradation observed (Fig. 4.44.A) in US and UL was very similar to that obtained with the controlled pH (U6). Removals over 60% were achieved in presence or absence of solids, indicating that the enzymatic activities achieved in the first three days are enough to break most part of complex carbohydrates.

Some differences were observed for phenolics removal compared to tests carried out with pH control, where the enzymatic activity, especially Lac, was higher. In this case, efficiencies around 40% were obtained for US and UL after 4 days of treatment (see Fig.4.44.E), whereas with pH controlled at pH 6 a percentage of 67% was achieved. As stated before, the low pH significantly affected the enzymatic production by fungus and therefore the dephenolization process.

Finally, the changes in CN can be seen in Fig 4.44.F. In control samples (CS and CL) a colour removal close to 30% took place, whereas the inoculation of the fungus allowed to obtain a colour removal of 94%. In this case decolourization was due to phenolics degradation, but also by the acidification occurred during the treatment. In fact, acid precipitation is the most widely used technique to extract lignin. Normally strong acids such as sulfuric or hydrochloric are used to precipitate Kraft lignin obtaining a the treated effluent with lower COD and CN [48]. In this work CN decreased until 72 h, indicating that a pH around 2 is enough to achieve almost a total decolourization. To sum up, similar results were obtained with and without solids and, with and without pH control (with the exception of phenolics concentration). So, a KBL treatment with *A. uvarum* without previous filtration and without pH control, which reduces operational costs, seems to be a good option to considerably reduce COD, phenolics and colour.

4. CONCLUSIONS

The white-rot fungus *P. chrysosporium* and the ascomycete fungus *A. uvarum* were tested for the treatment of non-sterilized KBL effluent. Whereas very low biodegradations were observed in non-inoculated control test, both fungi were effective for the treatment of KBL, with higher efficiencies for the experiments carried out at pH 6, especially for phenolics degradations. In these controlled-pH tests, the fungus *P. chrysosporium* allowed to obtain removal efficiencies of 65%, 50% and 37% for COD, phenolic compounds, and colour, respectively, whereas 61%, 67%, and 81% were obtained when *A. uvarum* was used. The higher efficiencies obtained with *A. uvarum* for phenolics degradation and decolourization are related with the capacity of this fungus to produce Lac with enzymatic activities of around 200 U/L. Regarding the test carried out with and without solids by *A. uvarum*, no significant differences were observed, so it is not necessary to remove the solids before the biological treatment. Additionally, when pH was initially adjusted to 6, allowing it evolved freely during the treatment, a fast drop of pH occurred (below 3 after 3 day) and sCOD, colour and phenolics removals of 65%, 94% and 44%, were obtained respectively. MnP, LiP and Lac enzymatic activities were notably lower due to the acidification, which was reflected in the lower phenolics degradation obtained in the test without pH control. Nevertheless, pH did not affect sCOD degradation and even allowed a higher decolourization thanks to lignin precipitation. Results obtained in this work probed the interest of using these fungi, especially *A. uvarum*, to treat KBL effluent in an economic and eco-friendly way.

ACKNOWLEDGEMENTS

Authors acknowledge the financial support from the Spanish Ministry of Economy and Competitiveness (MINECO) through Project CTM2015-63864-R. The authors particularly thank to ENCE Navia (Spain) for their support in the sample collection and to AMG group and Micoteca of University of Minho for their useful advice and material support. Ana Isabel Díaz also acknowledges an FPI grant (BES-2016-077963) from Spanish Ministry of Science, Innovation and Universities (MICINN).

REFERENCES

[1] M.A. Hubbe, J.R. Metts, D. Hermosilla, M.A. Blanco, L. Yerushalmi, F. Haghighat, P. Lindholm-Lehto, Z. Khodaparast, M. Kamali, A. Elliott, Wastewater treatment and reclamation: A review of pulp and paper industry practices and opportunities, BioResources. 11 (2016) 7953–8091. https://doi.org/10.15376/biores.11.3.hubbe.

[2] R. Toczyłowska-Mamińska, Limits and perspectives of pulp and paper industry wastewater treatment – A review, Renew. Sustain. Energy Rev. 78 (2017) 764–772. https://doi.org/10.1016/j.rser.2017.05.021.

[3] D. Pokhrel, T. Viraraghavan, Treatment of pulp and paper mill wastewater - A review, Sci. Total Environ. 333 (2004) 37–58. https://doi.org/10.1016/j.scitotenv.2004.05.017.

[4] L. Pola, S. Collado, P. Oulego, P. Calvo, M. Díaz, Characterisation of the wet oxidation of black liquor for its integration in Kraft paper mills, Chem. Eng. J. 405 (2021) 126610. https://doi.org/10.1016/j.cej.2020.126610.

[5] E. Nikolskaya, P. Janhunen, M. Haapalainen, Y. Hiltunen, Solids Content of Black Liquor Measured by Online Time-Domain NMR, Appl. Sci. 9 (2019) 2169. https://doi.org/10.3390/app9102169.

[6] G. Adhikari, K.G. Bhattacharyya, Impact of pulp and paper mill effluents and solid wastes on soil mineralogical and physicochemical properties, Environ. Monit. Assess. 187 (2015). https://doi.org/10.1007/s10661-015-4330-z.

[7] E. Mateos-Espejel, L. Savulescu, F. Maréchal, J. Paris, Unified methodology for thermal energy efficiency improvement: Application to Kraft process, Chem. Eng. Sci. 66 (2011) 135–151. https://doi.org/10.1016/j.ces.2010.09.032.

[8] M. Kamali, Z. Khodaparast, Review on recent developments on pulp and paper mill wastewater treatment, Ecotoxicol. Environ. Saf. 114 (2015) 326–342. https://doi.org/10.1016/j.ecoenv.2014.05.005.

[9] T. Kinnarinen, M. Golmaei, E. Jernström, A. Häkkinen, Separation, treatment

and utilization of inorganic residues of chemical pulp mills, J. Clean. Prod. 133 (2016) 953–964. https://doi.org/10.1016/j.jclepro.2016.06.024.

[10] Z. Al-Kaabi, R. Pradhan, N. Thevathasan, A. Gordon, Y.W. Chiang, P. Arku, A. Dutta, Ash removal from various spent liquors by oxidation process for bio-carbon production, J. Environ. Chem. Eng. 8 (2020) 103520. https://doi.org/10.1016/j.jece.2019.103520.

[11] E.C.L. Coimbra, A.H. Mounteer, A.L.V. do Carmo, M.J.F. Michielsen, L.A. Tótola, J.P.F. Guerino, J.G.A.N. Gonçalves, P.R. da Silva, Electrocoagulation of kraft pulp bleaching filtrates to improve biotreatability, Process Saf. Environ. Prot. 147 (2021) 346–355. https://doi.org/10.1016/j.psep.2020.09.039.

[12] J.P. Ribeiro, C.C. Marques, I. Portugal, M.I. Nunes, AOX removal from pulp and paper wastewater by Fenton and photo-Fenton processes: A real case-study, in: Energy Reports, Elsevier Ltd, 2020: pp. 770–775. https://doi.org/10.1016/j.egyr.2019.09.068.

[13] L. Bijan, M. Mohseni, Using ozone to reduce recalcitrant compounds and to enhance biodegradability of pulp and paper effluents, Water Sci. Technol. 50 (2004) 173–182. https://doi.org/10.2166/wst.2004.0190.

[14] M. Efgan Kibar, S. Veli, A. Arslan, S. Ketizmen, A. Nilgün Akin, Catalytic Wet Air Oxidation of Pulp and Paper Industry Wastewater 1, J. Water Chem. Technol. 41 (2019) 36–43. https://doi.org/10.3103/S1063455X19010065.

[15] S.S. Hassan, G.A. Williams, A.K. Jaiswal, Emerging technologies for the pretreatment of lignocellulosic biomass, Bioresour. Technol. 262 (2018) 310–318. https://doi.org/10.1016/j.biortech.2018.04.099.

[16] S. Madan, P. Sachan, U. Singh, A review on bioremediation of pulp and paper mill effluent – An alternative to conventional remedial technologies, J. Appl. Nat. Sci. 10 (2018) 367–374. https://doi.org/10.31018/jans.v10i1.1632.

[17] A. Abhishek, A. Dwivedi, N. Tandan, U. Kumar, Comparative bacterial degradation and detoxification of model and kraft lignin from pulp paper wastewater and its metabolites, Appl. Water Sci. 7 (2017) 757–767.

https://doi.org/10.1007/s13201-015-0288-9.

[18] H. Vashi, O.T. Iorhemen, J.H. Tay, Degradation of industrial tannin and lignin from pulp mill effluent by aerobic granular sludge technology, J. Water Process Eng. 26 (2018) 38–45. https://doi.org/10.1016/j.jwpe.2018.09.002.

[19] A. Brink, C.M. Sheridan, K.G. Harding, A kinetic study of a mesophilic aerobic moving bed biofilm reactor (MBBR) treating paper and pulp mill effluents: The impact of phenols on biodegradation rates, J. Water Process Eng. 19 (2017) 35–41. https://doi.org/10.1016/j.jwpe.2017.07.003.

[20] R. Hooda, N.K. Bhardwaj, P. Singh, Screening and Identification of Ligninolytic Bacteria for the Treatment of Pulp and Paper Mill Effluent, Water, Air, Soil Pollut. 226 (2015) 305. https://doi.org/10.1007/s11270-015-2535-y.

[21] D.M. Brown, J. Pawlak, A.M. Grunden, Bacterial valorization of pulp and paper industry process streams and waste, Appl. Microbiol. Biotechnol. 105 (2021) 1345–1363. https://doi.org/10.1007/s00253-021-11107-2.

[22] S. Collado, P. Oulego, O. Suárez-Iglesias, M. Díaz, Leachates and natural organic matter. A review of their biotreatment using fungi, Waste Manag. 96 (2019) 108–120. https://doi.org/10.1016/J.WASMAN.2019.07.018.

[23] E.J. Espinosa-Ortiz, E.R. Rene, K. Pakshirajan, E.D. Van Hullebusch, P.N.L.L. Lens, E.D. van Hullebusch, P.N.L.L. Lens, E.D. Van Hullebusch, P.N.L.L. Lens, Fungal pelleted reactors in wastewater treatment: Applications and perspectives, Elsevier, 2016. https://doi.org/10.1016/j.cej.2015.07.068.

[24] S. Rodríguez-Couto, Industrial and environmental applications of white-rot fungi, Mycosphere. 8 (2017) 456–466. https://doi.org/10.5943/mycosphere/8/3/7.

[25] S. Collado, P. Oulego, O. Suárez-Iglesias, M. Díaz, Biodegradation of dissolved humic substances by fungi, Appl. Microbiol. Biotechnol. 102 (2018) 3497–3511. https://doi.org/10.1007/s00253-018-8851-6.

[26] M.B. Asif, F.I. Hai, L. Singh, W.E. Price, L.D. Nghiem, Degradation of Pharmaceuticals and Personal Care Products by White-Rot Fungi—a Critical

Review, Curr. Pollut. Reports. 3 (2017) 88–103. https://doi.org/10.1007/s40726-017-0049-5.

[27] S.K. Sen, S.S.S. Raut, P. Bandyopadhyay, S.S.S. Raut, S. Kumar, S.S.S. Raut, P. Bandyopadhyay, Fungal decolouration and degradation of azo dyes : A review, Fungal Biol. Rev. 30 (2016) 112–133. https://doi.org/10.1016/j.fbr.2016.06.003.

[28] X. Font, G. Caminal, X. Gabarrell, S. Romero, M.T. Vicent, Black liquor detoxification by laccase of *Trametes versicolor* pellets, J. Chem. Technol. Biotechnol. 78 (2003) 548–554. https://doi.org/10.1002/jctb.834.

[29] S. Costa, D.G. Dedola, S. Pellizzari, R. Blo, I. Rugiero, P. Pedrini, E. Tamburini, Lignin Biodegradation in Pulp-and-Paper Mill Wastewater by Selected White Rot Fungi, Water. 9 (2017) 935. https://doi.org/10.3390/w9120935.

[30] N. Sharma, V.C. Gupta, Batch Biodegradation of Phenol of Paper and Pulp Effluent by *Aspergillus Niger*, Int. J. Chem. Eng. Appl. (2012) 182–186. https://doi.org/10.7763/ijcea.2012.v3.183.

[31] P. Sharma, S.K. Rath, Potential applications of fungi in the remediation of toxic effluents from pulp and paper industries, in: Fungi Bio-Prospects Sustain. Agric. Environ. Nano-Technology, Elsevier, 2021: pp. 193–211. https://doi.org/10.1016/B978-0-12-821925-6.00010-1.

[32] A.I. Díaz, A. Laca, M. Díaz, Fungal treatment of an effluent from sewage sludge digestion to remove recalcitrant organic matter, Biochem. Eng. J. 172 (2021) 108056. https://doi.org/10.1016/j.bej.2021.108056.

[33] J. Saetang, S. Babel, Effect of glucose on enzyme activity and color removal by *Trametes versicolor* for high strength landfill leachate, Water Sci. Technol. 62 (2010) 2519–2526. https://doi.org/10.2166/wst.2010.552.

[34] A.I. Díaz, A. Laca, A. Laca, M. Díaz, Treatment of supermarket vegetable wastes to be used as alternative substrates in bioprocesses, Waste Manag. 67 (2017) 59–66. https://doi.org/10.1016/j.wasman.2017.05.018.

[35] K. Moussi, B. Nayak, L.B. Perkins, F. Dahmoune, K. Madani, M. Chibane,

HPLC-DAD profile of phenolic compounds and antioxidant activity of leaves extract of *Rhamnus alaternus L.*, Ind. Crops Prod. 74 (2015) 858–866. https://doi.org/10.1016/j.indcrop.2015.06.015.

[36] A.I. Díaz, P. Oulego, J.M. González, A. Laca, M. Díaz, Physico-chemical pre-treatments of anaerobic digestion liquor for aerobic treatment, J. Environ. Manage. 274 (2020) 111189. https://doi.org/https://doi.org/10.1016/j.jenvman.2020.111189.

[37] D.S. Lisboa, C. Santos, R.N. Barbosa, O. Magalhães, L.M. Paiva, K.A. Moreira, N. Lima, C.M. Souza-Motta, Requalification of a *Brazilian Trichoderma* collection and screening of its capability to decolourise real textile effluent, Int. J. Environ. Res. Public Health. 14 (2017) 1–16. https://doi.org/10.3390/ijerph14040373.

[38] J. Wu, Y.Z. Xiao, H.Q. Yu, Degradation of lignin in pulp mill wastewaters by white-rot fungi on biofilm, Bioresour. Technol. 96 (2005) 1357–1363. https://doi.org/10.1016/j.biortech.2004.11.019.

[39] M. Islam, A. Wai, G. Hausner, Q. Yuan, Effect of lignocellulosic enzymes on the treatment of mature landfill leachate, J. Environ. Manage. 233 (2019) 400–409. https://doi.org/10.1016/J.JENVMAN.2018.12.045.

[40] A.F. Ariste, R.A. Batista-García, V.K. Vaidyanathan, N. Raman, V.K. Vaithyanathan, J.L. Folch-Mallol, S.A. Jackson, A.D.W. Dobson, H. Cabana, Mycoremediation of phenols and polycyclic aromatic hydrocarbons from a biorefinery wastewater and concomitant production of lignin modifying enzymes, J. Clean. Prod. 253 (2020) 119810. https://doi.org/10.1016/j.jclepro.2019.119810.

[41] I. Sampedro, S. Marinari, A. D'Annibale, S. Grego, J.A. Ocampo, I. García-Romera, Organic matter evolution and partial detoxification in two-phase olive mill waste colonized by white-rot fungi, Int. Biodeterior. Biodegrad. 60 (2007) 116–125. https://doi.org/10.1016/j.ibiod.2007.02.001.

[42] N. Junnarkar, N. Pandhi, N. Raiyani, N. Bhatt, R. Raiyani, Production of LiP by Phanerochaete chrysosporium MTCC 787 Through Solid State Fermentation of

Wheat Straw and Assessing its Activity Against Reactive Black B, Int. J. Adv. Res. J. 4 (2016) 812–819. http://www.journalijar.com/uploads/275_IJAR-8575.pdf.

[43] J.M. Salgado, L. Abrunhosa, A. Venâncio, J.M. Domínguez, I. Belo, Combined bioremediation and enzyme production by *Aspergillus* sp. in olive mill and winery wastewaters, Int. Biodeterior. Biodegrad. 110 (2016) 16–23. https://doi.org/10.1016/j.ibiod.2015.12.011.

[44] G. Perrone, J. Varga, A. Susca, J.C. Frisvad, G. Stea, S. Kocsubé, B. Tóth, Z. Kozakiewicz, R.A. Samson, J. Nos Varga, A. Susca, J.C. Frisvad, G. Stea, S. Ndor Kocsubé, B. Ta Tó, Z. Kozakiewicz, R.A. Samson, *Aspergillus uvarum* sp. nov., an uniseriate black Aspergillus species isolated from grapes in Europe, Int. J. Syst. Evol. Microbiol. 58 (2008) 1032–1039. https://doi.org/10.1099/ijs.0.65463-0.

[45] T. Liu, H. Hu, Z. He, Y. Ni, Treatment of poplar alkaline peroxide mechanical pulping (APMP) effluent with *Aspergillus niger*, Bioresour. Technol. 102 (2011) 7361–7365. https://doi.org/10.1016/j.biortech.2011.04.043.

[46] T. Gulzar, T. Huma, F. Jalal, S. Iqbal, S. Abrar, S. Kiran, S. Nosheen, W. Hussain, M. Rafique, Bioremediation of Synthetic and Industrial Effluents by *Aspergillus niger* Isolated from Contaminated Soil Following a Sequential Strategy, Molecules. 22 (2017) 2244. https://doi.org/10.3390/molecules22122244.

[47] K. Dhakar, R. Kooliyottil, A. Joshi, A. Pandey, Simultaneous production of ligninolytic enzymes by a temperature and pH tolerant strain of *Aspergillus niger* under different cultural conditions, Indian J. Biotechnol. 14 (2015) 81–86. http://nopr.niscair.res.in/handle/123456789/31478.

[48] L. Pola, S. Collado, P. Oulego, M. Díaz, Production of carboxylic acids from the non-lignin residue of black liquor by hydrothermal treatments, Bioresour. Technol. 284 (2019) 105–114. https://doi.org/10.1016/j.biortech.2019.03.066.

Capítulo 5.

CONCLUSIONES

CONCLUSIONES

- Las técnicas fisicoquímicas (oxidación húmeda, hidrólisis térmica, ozonización y ultrasonidos) y biológicas (tratamientos con hongos) ensayadas en esta tesis doctoral han resultado adecuadas para el tratamiento de residuos con compuestos fenólicos complejos, consiguiendo reducir el contenido en materia orgánica y/o aumentar la biodegradabilidad del efluente final.

- El análisis metagenómico de los efluentes resultantes de la centrifugación y decantación del digestato procedente de la biometanización de lodos de depuradora indicó una mayor predisposición de los microorganismos del filo *Proteobacteria* hacia la fase sólida y del filo *Firmicutes* hacia la fase líquida. La alta presencia de bacterias hidrolíticas en esta última fracción indica que la recirculación de parte de este licor al reactor anaerobio puede resultar una opción ventajosa.

- La clasificación taxonómica realizada en el proceso biológico de tratamiento de lixiviados indicó que no existían diferencias significativas entre las corrientes analizadas procedentes de los reactores de nitrificación y desnitrificación. Así, la alta recirculación logró mantener una composición microbiológica homogénea entre los reactores óxicos y anóxicos, tanto a nivel de filo como de clases.

- Todos los métodos fisicoquímicos evaluados para el tratamiento del licor de biometanización lograron aumentar significativamente la biodegradabilidad del efluente. Además, los métodos de oxidación avanzada (ozonización y oxidación húmeda) fueron óptimos para la degradación de la materia orgánica, mientras que la hidrólisis térmica y el tratamiento con ultrasonidos sólo fueron efectivas en términos de solubilización. Las mejores eficacias en lo que respecta a la eliminación de materia orgánica fueron obtenidas mediante el tratamiento por oxidación húmeda a 200ºC y 60 bar durante 8 h, consiguiéndose reducciones en la DQO y el color del 71% y 91%, respectivamente.

- Los métodos de hidrólisis térmica, ácida y enzimática ensayados se mostraron efectivos para el tratamiento de residuos de verduras en cuanto a la obtención de

azúcares reductores. Los mejores resultados se observaron al aplicar un proceso combinado de hidrólisis térmica (135ºC, 5 min) y enzimática (60ºC, 60 min), que permitió hidrolizar tanto los compuestos lignocelulósicos (tomate y pimiento) como el almidón procedente del residuo de patata, lográndose extraer en todos los casos más del 75% de los azúcares potenciales del residuo. Además, los residuos hidrolizados resultaron aptos para ser utilizados directamente como medio de fermentación ya que las concentraciones de inhibidores analizadas fueron muy bajas.

- La degradación de materia orgánica recalcitrante mediante el empleo del hongo *Phanerochaete chrysosporium* ha demostrado ser una técnica de gran versatilidad ya que se ha empleado para el tratamiento de efluentes de composiciones muy diferentes (licor de biometanización, permeado de ultrafiltración, alperujo, purines y licor negro), y en todos los casos se han conseguido eliminaciones significativas de DQO y color (entre el 30% y el 75%, dependiendo del tipo de residuo).

- El empleo de hongos ascomicetos, en concreto de *Aspergillus uvarum*, para el tratamiento de licor negro proporcionó resultados muy prometedores, alcanzando degradaciones del 61%, 67%, y 81% para la DQO, los compuestos fenólicos y el color, respectivamente. Los resultados obtenidos adquieren especial interés al no existir apenas referencias bibliográficas sobre la aplicación biotecnológica de este microorganismo para el tratamiento de residuos.

- Un aspecto clave para optimizar el tratamiento fúngico de residuos ha sido el pH. Así, el control del pH en valores próximos a la neutralidad permitió aumentar la eficacia de degradación de la materia orgánica recalcitrante, gracias al efecto sinérgico del hongo y la microbiota endógena. Por ejemplo, cuando se trató el licor de biometanización a pH 6, se alcanzó un 77% de eliminación de DQO, de la que sólo el 59% de DQO se debía a la acción del hongo.

- La presencia de una fuente de carbono fácilmente biodegradable resultó conveniente para la activación del hongo. De esta manera, en el caso de los

tratamientos de permeado de lixiviado y licor de biometanización, la suplementación con glucosa permitió aumentar significativamente las eliminaciones de DQO. En particular, la adición de 3 g/L de glucosa al licor de biometanización proporcionó degradaciones de DQO superiores al 70%, mientras que sólo un 20% fue eliminado sin suplementación.

CONCLUSIONS

- The physico-chemical (wet air oxidation, thermal hydrolysis, ozonation and ultrasound) and biological (fungal treatments) techniques tested in this PhD thesis have turned out to be suitable for the treatment of wastes containing complex phenolic compounds. For all the waste effluents tested the organic matter content could be reduced and/or the biodegradability increased.

- The metagenomic analysis of the effluents from the centrifugation and decantation of a digestate from a sewage sludge biomethanization showed that microorganisms belonging to the phylum *Proteobacteria* had a greater predisposition towards the solid phase and the phylum *Firmicutes* towards the liquid phase. The high presence of hydrolytic bacteria in this last fraction indicates that recirculating part of this liquor to the anaerobic reactor may be an advantageous option.

- The taxonomic classification carried out throughout the biological treatment of landfill leachates indicated that there were not significant differences between the analysed samples taken from the nitrification and denitrification reactors. Thus, the high recirculation employed was able to maintain a similar microbiological composition, at phylum and class level, between the oxic and anoxic reactors.

- All the physico-chemical methods evaluated for the treatment of a biomethanization liquor significantly increased the effluent biodegradability. Additionally, the advanced oxidation methods (ozonation and wet air oxidation) were optimal for degrading the organic matter, while thermal hydrolysis and ultrasound treatments were only effective in terms of solubilization. The best efficiencies with respect to the organic matter removal were obtained when the wet oxidation treatment was used at 200°C and 60 bar for 8 h, achieving COD and colour removals of 71% and 91%, respectively.

- The assayed thermal, acid and enzymatic hydrolysis were effective for the treatment of vegetable wastes to obtain reducing sugars. The best results were obtained when a process combining thermal (135ºC, 5 min) and enzymatic hydrolysis (60ºC, 60 min) was used. This treatment allowed the hydrolysis of both lignocellulosic compounds (tomato and pepper) and starch from potato wastes, extracting in all cases more than 75% of the potential sugars of the waste. Moreover, the hydrolyzed wastes were suitable to be used directly as fermentation media since the measured concentrations of inhibitors were very low.

- The employment of the fungus *Phanerochaete chrysosporium* to remove the recalcitrant organic matter has proven to be a highly versatile technique. So, it has been used for the treatment of effluents with very different compositions (biomethanization liquor, leachate permeate, olive processing waste, slurry and black liquor) and, in all cases, significant eliminations of COD and colour have been achieved (between 30% and 75%, depending on the type of waste).

- Promising results have been obtained when the ascomycete fungus *Aspergillus uvarum* was used to treat black liquor, achieving degradations of 61%, 67%, and 81% for COD, phenolic compounds and colour, respectively. The results obtained are particularly interesting as there is hardly any bibliographical reference on the biotechnological application of this microorganism for waste treatment.

- A key aspect to optimise the fungal treatment of wastes has been pH. Thus, by controlling the pH at values close to neutrality the degradation efficiency of recalcitrant organic matter increased, thanks to the synergistic effect of the fungus and the endogenous microbiota. For example, when the biomethanization liquor was treated at pH 6, 77% of initial COD was removed and only 59% of removal was due to the action of the fungus.

- The presence of a readily biodegradable carbon source turned out to be advisable for fungal activation. Thus, in the case of the treatments of leachate

permeate and biomethanization liquor, the supplementation with glucose allowed an important increase in the COD removal. In particular, the addition of 3 g/L of glucose to the biomethanization liquor provided COD degradations higher than 70%, whereas only 20% was removed without supplementation.

ANEXOS

ANEXO I: Supplementary material for "Impact of anaerobic digestion and centrifugation/decanting processes in bacterial communities fractions"

Authors: Ana Isabel Díaz[1], Paula Oulego[1], Sergio Collado[1], Adriana Laca[1], J. Manuel González[2] and Mario Díaz[1]*

[1]Department of Chemical and Environmental Engineering. University of Oviedo. Oviedo. Spain

[2]R&D, COGERSA SAU. Gijón, Asturias, Spain E-33697

*Corresponding author's e-mail: mariodiaz@uniovi.es

Phone: +34 985 10 34 39; Fax: +34 985 10 34 34

Table of contents

1- Fig.S1: Taxonomic classification for initial leachate (S1)
2- Fig.S2: Taxonomic classification for digestate (S2)
3- Fig.S3: Taxonomic classification for initial solid phase (S3)
4- Fig.S4: Taxonomic classification for clarified (S4)
5- Fig.S5: Taxonomic classification for decanted product (S5)

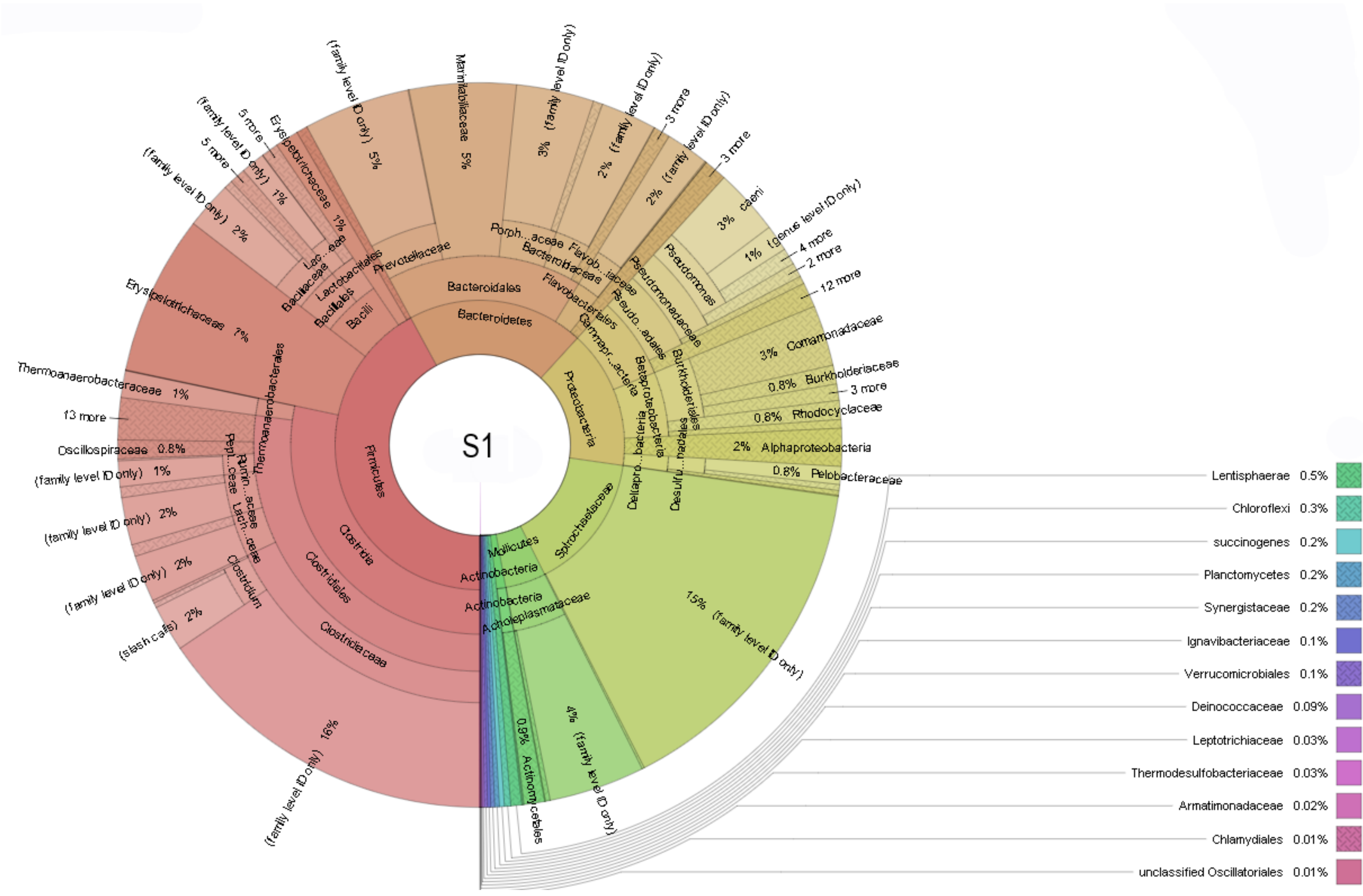


Figure S1: Taxonomic classification for initial leachate (S1)

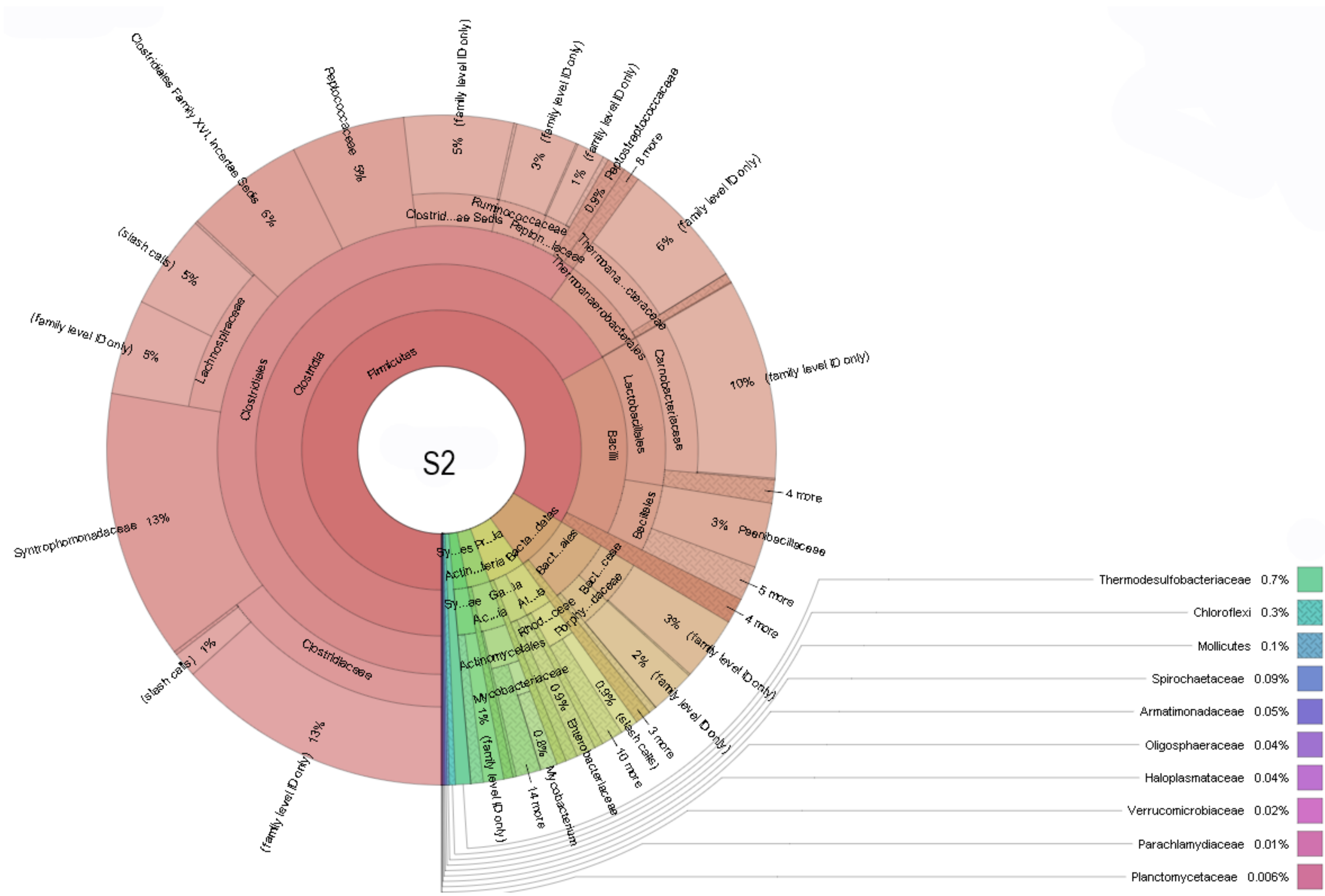


Figure S2: Taxonomic classification for digestate (S2)

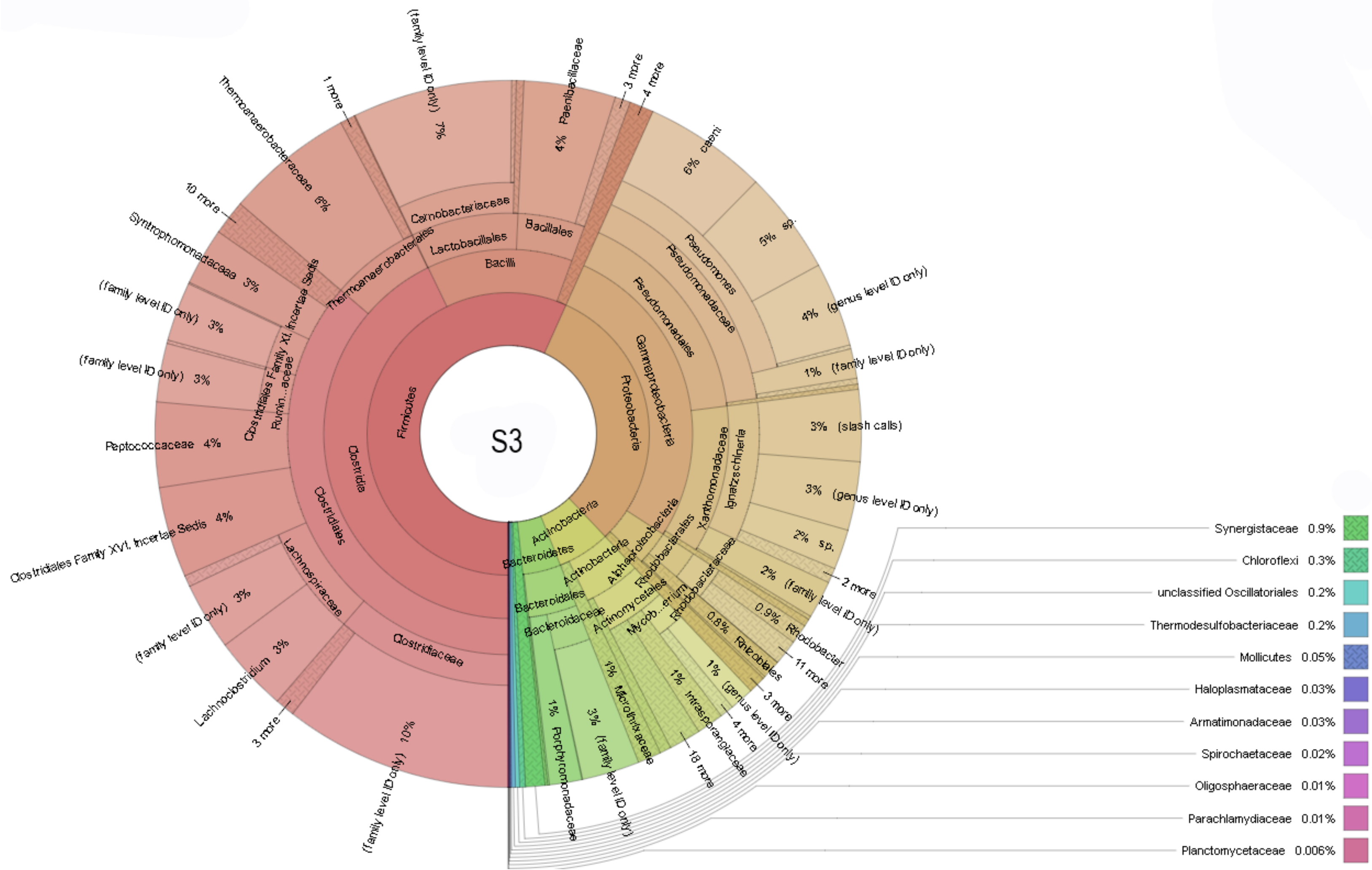


Figure S3: Taxonomic classification for initial solid phase (S3)

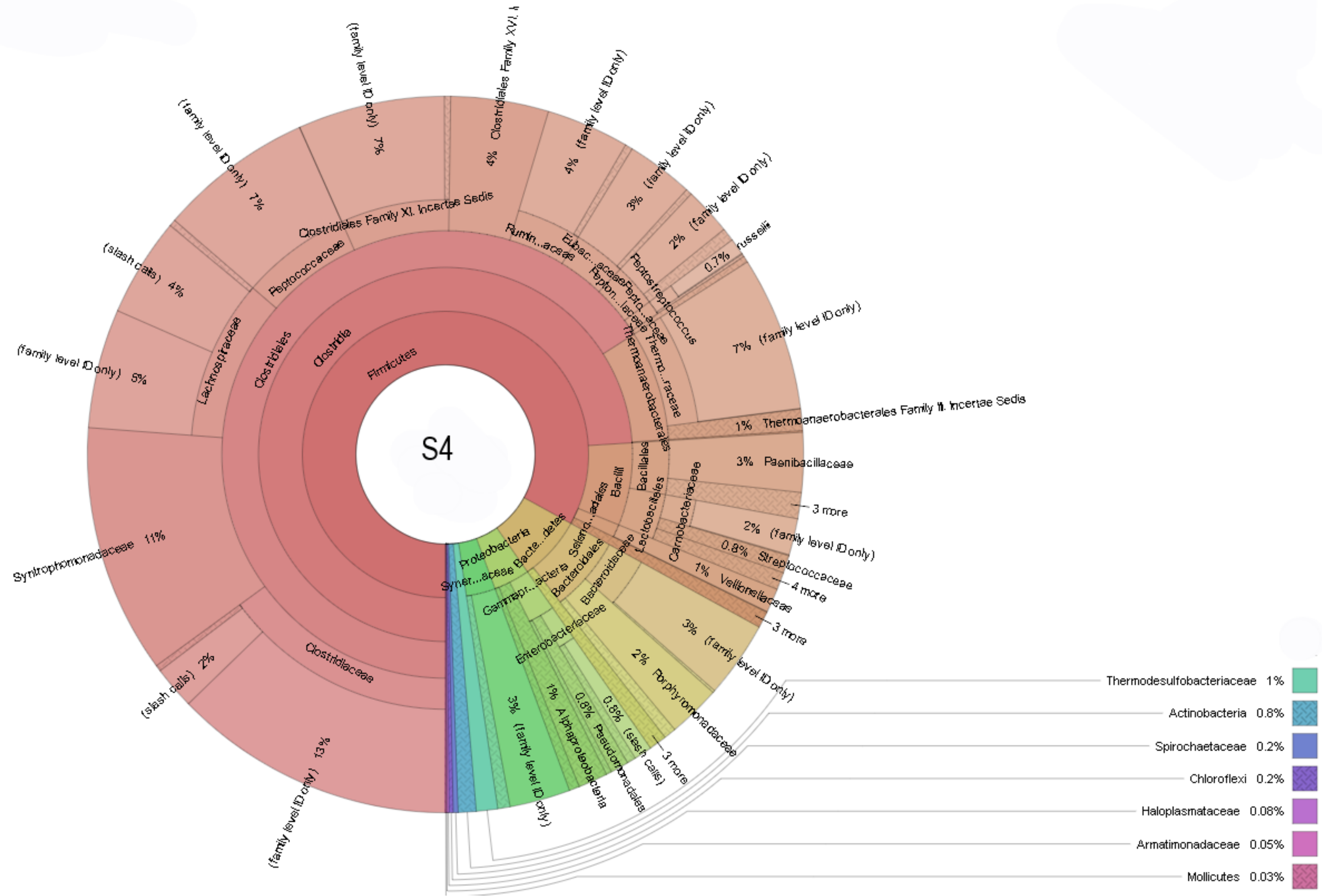


Figure S4: Taxonomic classification for clarified (S4)

Figure S5: Taxonomic classification for decanted product (S5)

ANEXO II: Supplementary material for "Physico-chemical pre-treatments of anaerobic digestion liquor for aerobic treatment"

Authors: Ana Isabel Díaz[1], Paula Oulego[1], Adriana Laca[1]*, José Manuel González[2], and Mario Díaz[1]

[1]Department of Chemical and Environmental Engineering, University of Oviedo, Oviedo, Spain

[2] R&D, COGERSA SAU, Gijón, Asturias, Spain

Correspondence: Dr. A. Laca, Department of Chemical and Environmental Engineering, University of Oviedo, Oviedo, Spain

Email: lacaadriana@uniovi.es

Supplementary material:

- Figure 5.1.S1: Comparison between model and experimental data for ultrasound
- Figure 5.1.S2. Comparison between model and experimental data for ozonation
- Figure 5.1.S3. Comparison between model and experimental data for hydrolysis
- Figure 5.1.S4. Comparison between model and experimental data for WAO

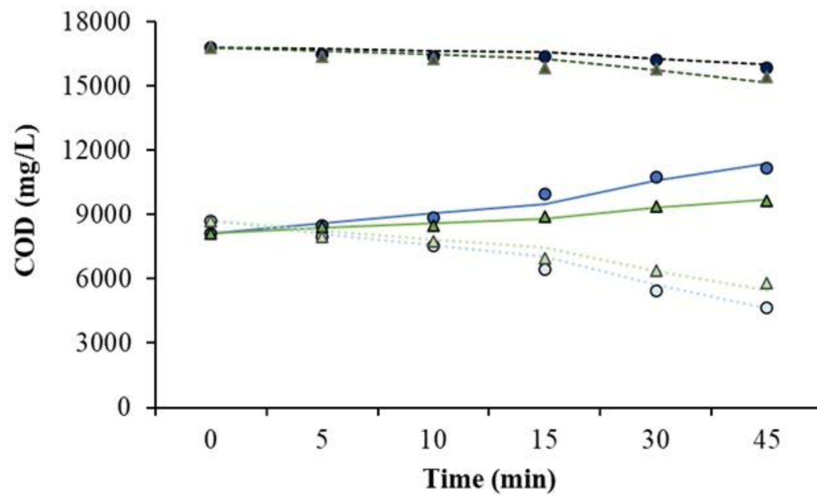


Figure 5.1.S1: Changes of tCOD (dashed lines), sCOD (solid lines) and nsCOD (dotted lines) during ultrasound treatment at 50% of amplitude and 90% of amplitude. The lines shown the theoretical data obtained with the kinetic model proposed and markers shown the experimental data.

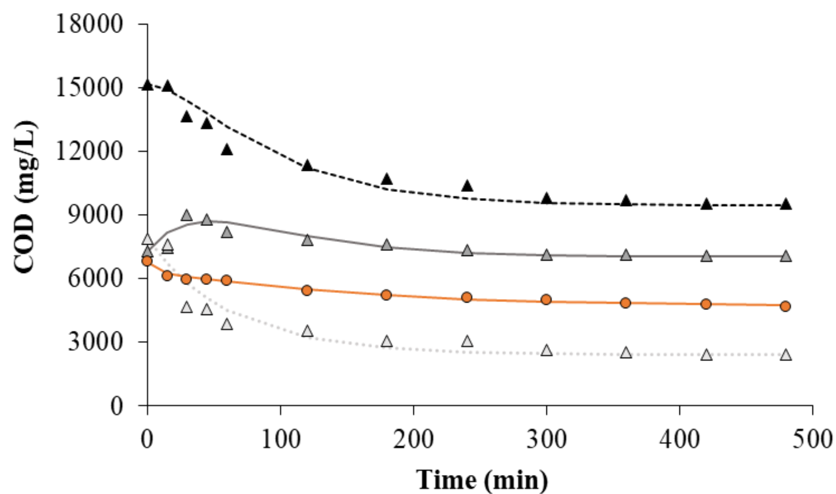


Figure 5.1.S2: Changes of tCOD (dashed lines), sCOD (solid lines) and nsCOD (dotted lines) during ozonation treatment. The experiment A corresponds to the sample with solids and the experiment B (orange colour) corresponds to the sample without solids. The lines shown the theoretical data obtained with the kinetic model proposed and markers shown the experimental data.

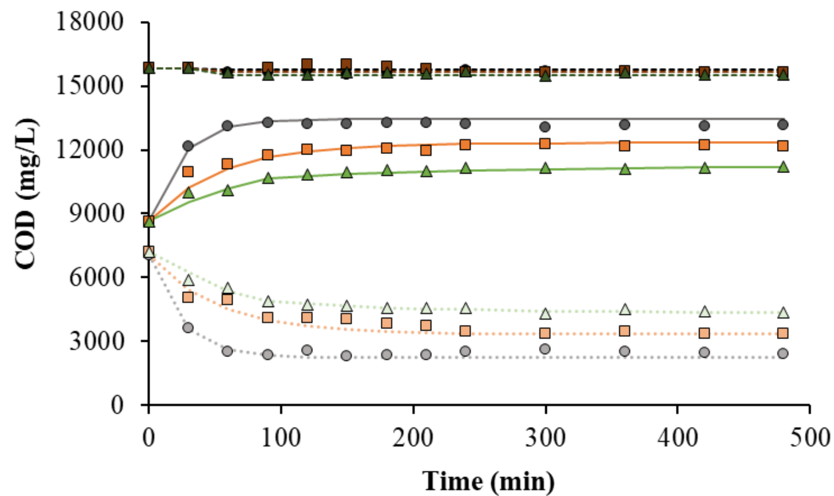


Figure 5.1.S3: Changes of tCOD (dashed lines), sCOD (solid lines) and nsCOD (dotted lines) during hydrolysis treatment at 160 ºC, at 180 ºC and at 200 ºC. The lines shown the theoretical data obtained with the kinetic model proposed and markers shown the experimental data.

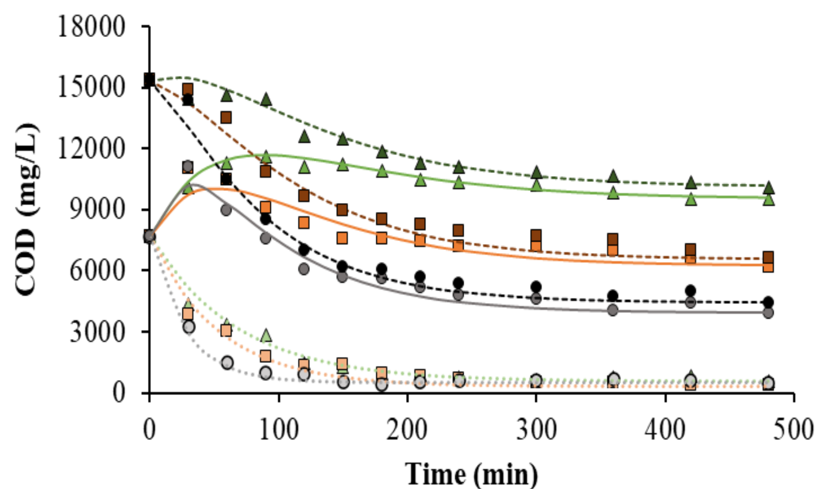


Figure 5.1.S4: Changes of tCOD (dashed lines), sCOD (solid lines) and nsCOD (dotted lines) during wet air oxidation treatment at 160 ºC, at 180 ºC and at 200 ºC. The lines shown the theoretical data obtained with the kinetic model proposed and markers shown the experimental data.

ANEXO III. Supplementary material for "Metagenomic analysis of bacterial communities from a nitrification-denitrification treatment of landfill leachates".

Authors: Ana Isabel Díaz[1], Paula Oulego[1], Adriana Laca[1]*, José Manuel González[2], and Mario Díaz[1]

[1]Department of Chemical and Environmental Engineering, University of Oviedo, Oviedo, Spain

[2] R&D, COGERSA SAU, Gijón, Asturias, Spain

Correspondence: Dr. A. Laca, Department of Chemical and Environmental Engineering, University of Oviedo, Oviedo, Spain

Email: lacaadriana@uniovi.es

Supplementary material:

- Figure 5.2.S1: Taxonomic classification for raw leachate (S1)
- Figure 5.2.S2. Taxonomic classification for denitrification reactor output (S2)
- Figure 5.2.S3. Taxonomic classification for nitrification reactor output (S3)
- Figure 5.2.S4. Taxonomic classification for nitrification reactor output (S4)
- Figure 5.2.S5. Taxonomic classification for ultrafiltration sludge (S5)

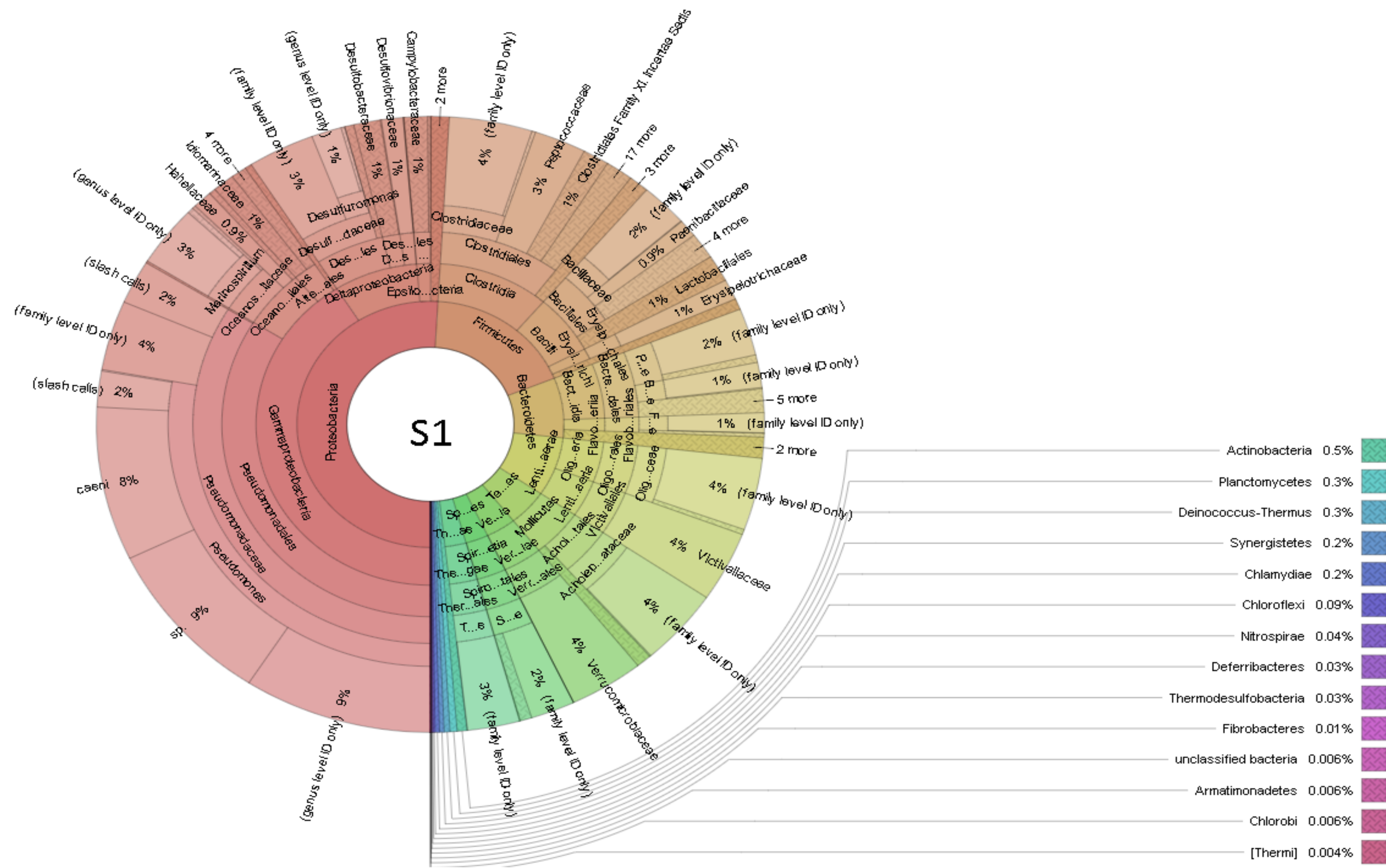


Figure S1: Taxonomic classification for raw leachate (S1)

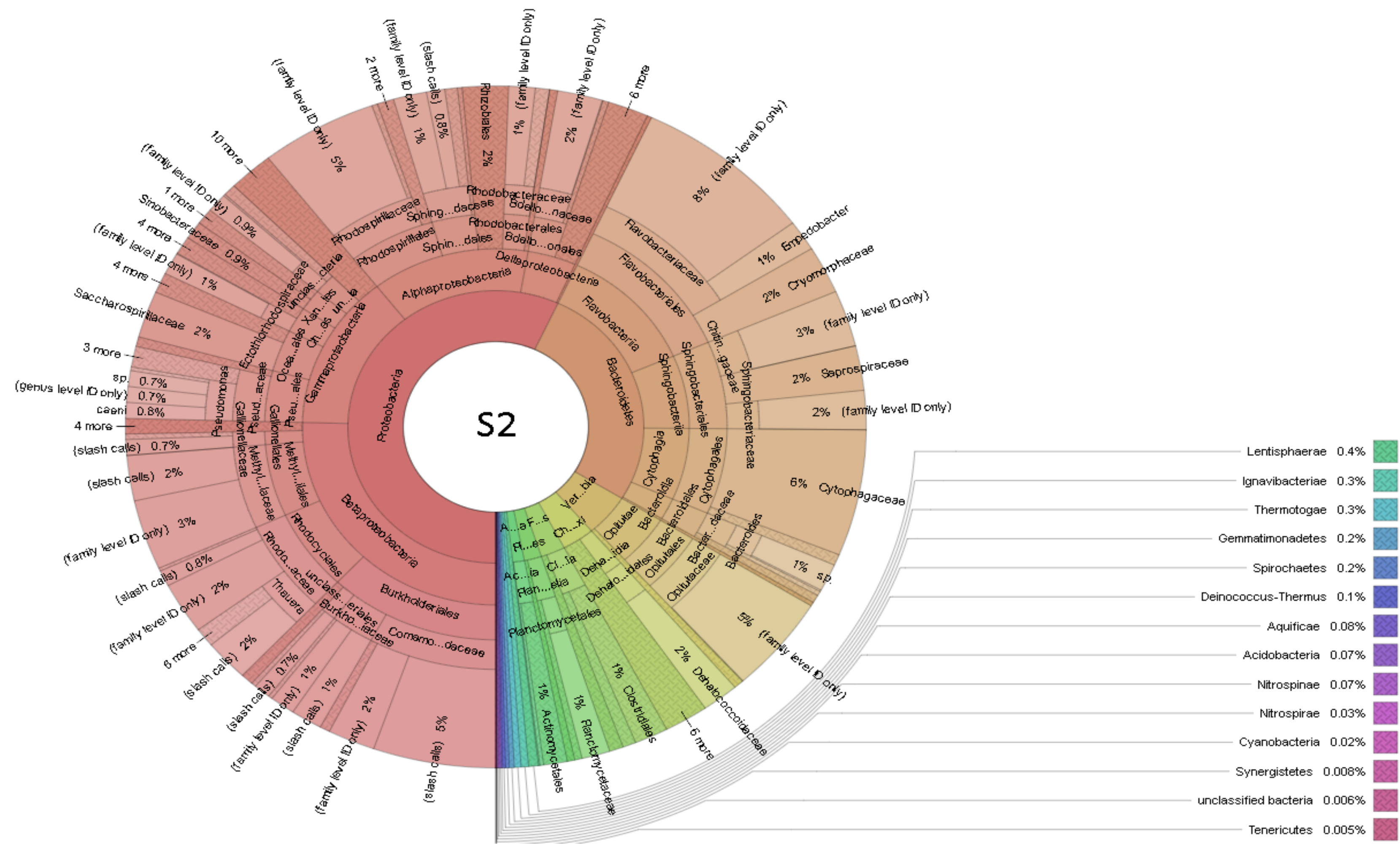


Figure S2. Taxonomic classification for denitrification reactor output (S2)

Figure S3. Taxonomic classification for nitrification reactor output (S3)

Figure S4. Taxonomic classification for nitrification reactor output (S4)

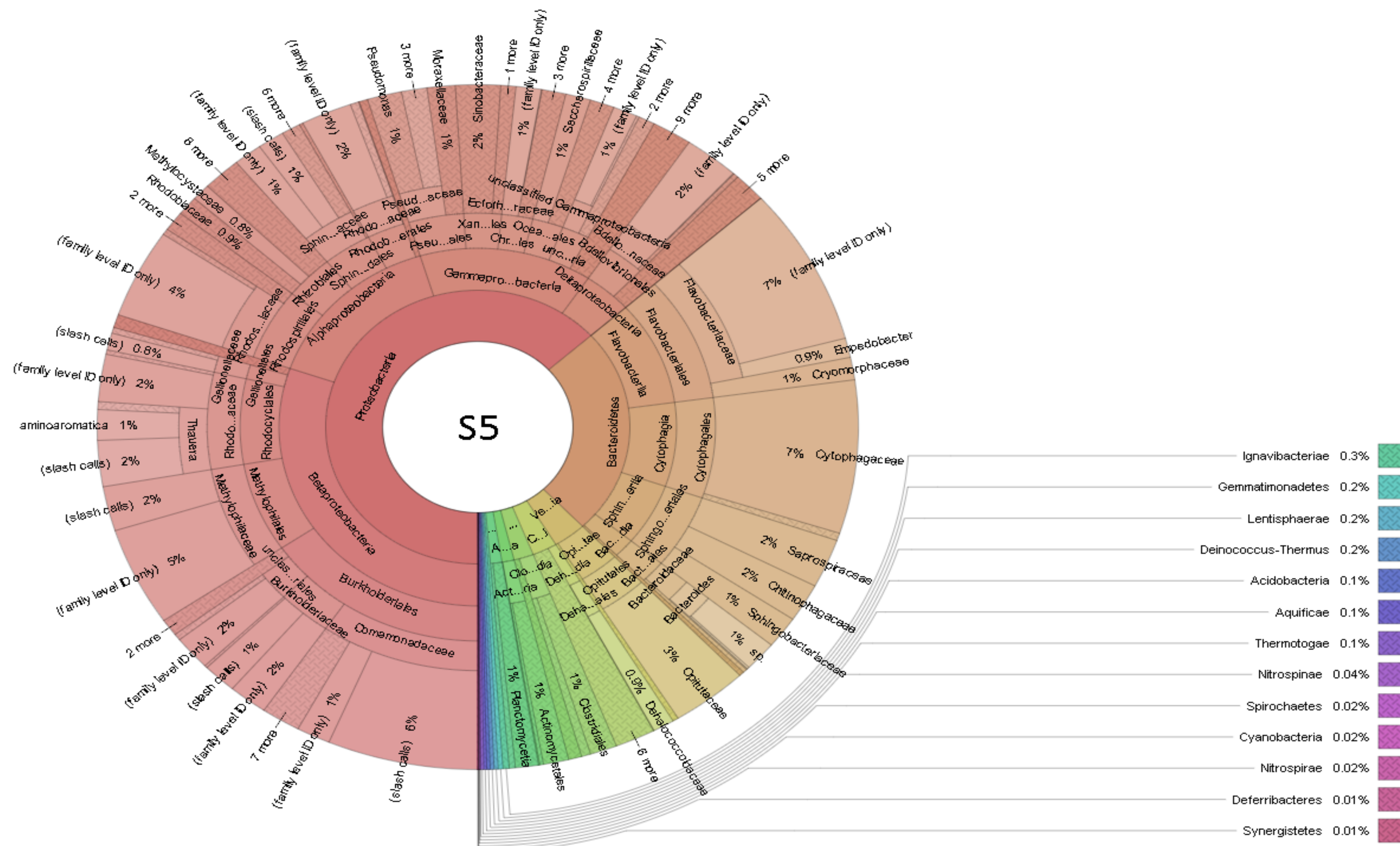


Figure S5. Taxonomic classification for ultrafiltration sludge (S5).

DIFUSIÓN DE LA TESIS DOCTORAL

PUBLICACIÓNES

1. **“Treatment of supermarket vegetable wastes to be used as alternative substrates in bioprocesses”**. (2017). Autores: Ana Isabel Díaz, Amanda Laca, Adriana Laca y Mario Díaz. Revista: Waste Management, Vol. 67, Pages 59 – 66. (Q1, 4.723)

2. **“Impact of anaerobic digestion and centrifugation/decanting processes in bacterial communities fractions”**. (2018). Autores: Ana Isabel Díaz, Paula Oulego, Sergio Collado, Jose Manuel González, Adriana Laca y Mario Díaz. Revista: Journal of Bioscience and Bioengineering, Vol. 126 (6), Pages 742-749 (Q2, 2.032)

3. **“Metagenomic Analysis of Bacterial Communities from a Nitrification–Denitrification Treatment of Landfill Leachates”**. (2019). Autores: Ana Isabel Díaz, Paula Oulego, Adriana Laca, Jose Manuel González y Mario Díaz. Revista: Clean – Soil, Air, Water; Vol. 47 (11), 1900156 (Q2, 1.603).

4. **“Physico-chemical pre-treatments of anaerobic digestion liquor for aerobic treatment”**. (2020). Autores: Ana Isabel Díaz, Paula Oulego, Jose Manuel González, Adriana Laca y Mario Díaz. Revista: Journal of Environmental Management, Vol. 274, 111189 (Q1, 6.789).

5. **“Fungal treatment of an effluent from sewage sludge digestion to remove recalcitrant organic matter”**. (2021). Autores: Ana Isabel Díaz, Adriana Laca y Mario Díaz. Revista: Biochemical Engineering Journal, Vol 172, 108056. (Q2, 3.978).

6. **“Biodegradation of olive mill effluent by white-rot fungi”.** (2021). Autores: Ana Isabel Díaz, Marta Ibañez, Adriana Laca, Mario Díaz. Revista: Applied Science, Vol. 11, 9930. (Q2, 2.679)

PARTICIPACIÓN EN CONGRESOS Y SEMINARIOS:

1- **Congreso:** I Congreso Nacional de Jóvenes Investigadores de Ciencia, Ingeniería y Tecnología de los Alimentos.
Título: *"Hidrólisis de residuos de la venta de hortalizas para producir bioetanol"*. **Autores:** Ana Isabel Díaz, Adriana Laca y Mario Díaz. **Tipo de participación:** Oral y Póster **Lugar:** Escuela Técnica Superior de Ingenierías Agrarias de Palencia. **Fecha:** Noviembre, 2015

2- **Congreso:** Congreso Iberoamericano de Biotecnología (BIO.IBEROAMERICA) **Título:** *"Hydrolysis of vegetable market wastes to be used as substrates for fermentative production of value-added products"* **Autores:** Ana Isabel Diaz, Amanda Laca, Adriana Laca y Mario Diaz. **Tipo de participación:** Póster **Lugar:** Salamanca **Fecha:** Junio, 2016

3- **Congreso:** 5th European Conference on Environmental Applications of Advanced Oxidation Processes (EAAOP5).
Título: *"AOPs as pretreatment techniques for anaerobic digestion liquor"* **Autores:** Paula Oulego, Ana Isabel Díaz, Sergio Collado, Jose Manuel González, Adriana Laca y Mario Díaz **Tipo de participación:** Póster **Lugar:** Praga **Fecha:** Junio, 2017

4- **Congreso:** VI Jornadas Internacionales de Doctorado por la Universidad de Oviedo
Título: *"Changes in sewage sludge microbial communities through anaerobic digestion and separation processes"*. **Autores:** Ana Isabel Díaz, Adriana Laca y Mario Díaz. **Tipo de participación:** Póster **Lugar:** Mieres **Fecha:** Julio, 2017

5- **Seminario Técnico:** VI Asamblea General de Mesa Española de Tratamiento de Aguas. Seminario Técnico "Lodos: Producción y Aprovechamiento".
Título: *"Changes in sewage sludge microbial communities through anaerobic digestion and separation processes"*. **Autores:** Ana Isabel Díaz, Adriana Laca y Mario Díaz. **Tipo de participación:** Oral. **Lugar:** Oviedo **Fecha:** Julio, 2017

6- **Congreso:** 10th World Congress of Chemical Engineering (WCCE10).
Título: "Microbial community in leachate nitrification-denitrification processes". **Autores:** Ana Isabel Díaz, Adriana Laca, Jose Manuel González, Paula Oulego, Sergio Collado y Mario Díaz. **Tipo de participación:** Oral **Lugar:** Barcelona **Fecha:** Octubre, 2017

7- **Congreso:** II Congreso Nacional de Jóvenes Investigadores en Ciencia, Ingeniería y Tecnología de los alimentos (JICITA)
Título: "Obtención de bioetanol a partir de hidrolizados de frutas". **Autores:** Celia Hernández, Ana Isabel Díaz, Amanda Laca, Adriana Laca y Mario Díaz. **Tipo de participación:** Oral y Póster **Lugar:** León **Fecha:** Octubre, 2017

8- **Congreso:** VI Jornadas Doctorales y I Jornadas de Divulgación científica del grupo G-9 universidades
Título: "Impact of anaerobic digestion and separation processes in bacterial communities fractions". **Autores:** Ana Isabel Díaz, Adriana Laca, Mario Díaz. **Tipo de participación:** Oral y Póster **Lugar:** Santander **Fecha:** Mayo, 2018

9- **Congreso:** XIII Congreso Español de Tratamiento de Aguas
Título: "Tratamiento de efluentes industriales complejos mediante el empleo de hongos". **Autores:** Ana Isabel Díaz, Adriana Laca y Mario Díaz. **Tipo de participación:** Póster **Lugar:** León **Fecha:** Junio, 2018

10- **Congreso:** 2nd International Research Conference on Sustainable Energy, Engineering, Materials and Environment.
Título: "Fungal treatment for the hydrolysis of complex carbohydrates from fruit wastes". **Autores:** Laura Losada, Amanda Laca, Ana Isabel Díaz, Adriana Laca y Mario Díaz. **Tipo de participación:** Póster **Lugar:** Mieres **Fecha:** Julio, 2018

11- **Congreso:** VII Jornadas Internacionales de Doctorado por la Universidad de Oviedo
Título: "Tratamiento de aguas industriales complejas mediante el empleo del hongo de pudrición blanca *Phanerochaete chrysosporium*". **Autores:** Ana Isabel Díaz, Adriana Laca y Mario Díaz. **Tipo de participación:** Oral y Póster **Lugar:** Mieres **Fecha:** Julio, 2018

12- **Congreso:** III Congreso Español de Sociología de la Alimentación
Título: "Bioeconomía en el sector alimentario: planteamiento y casos prácticos"
Autores: Ana Isabel Díaz, Amanda Laca, Paula Oulego, Adriana Laca, Manuel Rendueles, Mario Díaz. **Tipo de participación:** Oral **Lugar:** Gijón **Fecha:** Septiembre, 2018

13- **Congreso:** Congreso Nacional de Biotecnología
Título: "Biological treatment of anaerobic digestion liquor by white-rot fungi"
Autores: Ana Isabel Díaz, Adriana Laca y Mario Díaz. **Tipo de participación:** Poster **Lugar:** Vigo **Fecha:** Junio, 2019

14- **Congreso:** Congress of Microbiology and Biotechnology (MICROBIOTEC)
Título: "Fungal treatment of Kraft Black Liquor using *Phanerochaete chrysosporium* and *Aspergillus uvarum*"
Autores: Ana Isabel Díaz, Adriana Laca, Nelson Lima y Mario Díaz. **Tipo de participación:** Poster **Lugar:** Lisboa (Online) **Fecha:** Noviembre, 2021