Universidad de Oviedo
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DEPARTAMENTO DE INGENIERÍA QUÍMICA Y TECNOLOGÍA DEL MEDIO AMBIENTE

HIDROLISIS TÉRMICA DE BIOMASA CELULAR, SEPARACIÓN Y FERMENTACIÓN DE PRODUCTOS

PROGRAMA DE DOCTORADO EN INGENIERÍA QUÍMICA, AMBIENTAL Y BIOALIMENTARIA

TESIS DOCTORAL

POR

MANUEL GARCÍA SAN MIGUEL

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RESUMEN DEL CONTENIDO DE TESIS DOCTORAL

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2.- Autor	
Nombre: **MANUEL GARCÍA SAN MIGUEL**	DNI/Pasaporte/NIE:
Programa de Doctorado: **PROGRAMA DE DOCTORADO EN INGENIERÍA QUÍMICA, AMBIENTAL Y BIOALIMENTARIA**	
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RESUMEN (en español)

Los lodos de depuradora son un residuo generado diariamente en grandes cantidades y que necesitan una gestión adecuada debido a sus potenciales peligros para la salud y el medioambiente. Aunque actualmente existen métodos para su gestión, éstos se centran en la estabilización, minimización y aprovechamiento energético mediante la producción de biogás, pero ignoran su gran potencial como recurso renovable. El lodo está compuesto por microorganismos, y, por ende, por biomoléculas, como proteínas, carbohidratos o ácidos húmicos. Todas ellas tienen un alto valor comercial debido a sus posibles aplicaciones.

Para poder purificar estas biomoléculas es necesario acometer en primer lugar la rotura del lodo. Aunque existen multitud de técnicas de diferente naturaleza para ello, los tratamientos hidrotérmicos son una excelente alternativa. Estas técnicas se basan en la aplicación de altas temperaturas y presiones que permiten conseguir dos objetivos al mismo tiempo: la estabilización y minimización del lodo y la solubilización de sus componentes. Los tratamientos hidrotérmicos pueden llevarse a cabo en atmósferas inertes, denominándose hidrólisis térmica y produciendo reacciones de hidrólisis que llevan a la rotura de las células y la liberación de los componentes intracelulares; o en atmósferas oxidantes, denominándose oxidación húmeda y presentando reacciones hidrolíticas y de oxidación, que transforman los compuestos solubilizados en moléculas con grupos carboxilo, carbonilo, éter, hidroxilo, etc. Aunque la bibliografía proporciona información sobre los cambios que sufre el lodo, hasta ahora obviaba el potencial de estos tratamientos para generar hidrolizados que permitan recuperar las biomoléculas de interés. Por ello, esta tesis pretende explorar la posibilidad de recuperar las biomoléculas de interés presentes en los efluentes de la oxidación húmeda y de la hidrólisis térmica de lodos de depuradora.

Debido al porcentaje del lodo que suponen los ácidos húmicos, su interferencia con algunos procesos de precipitación de y su baja biodegradabilidad, se estudió de forma específica su comportamiento durante los procesos de oxidación húmeda, buscando así ahondar en el conocimiento sobre el efecto de variables de operación como la temperatura, la presión o el pH de la disolución en la evolución de distintos parámetros de los ácidos húmicos y los productos que se generan a partir de ellos.

Dada la versatilidad de los tratamientos hidrotérmicos para tratar biomasas acuosas, la extensión de su aplicación a otras materias primas podría abrir la puerta a la revalorización de residuos, en términos de recuperación de biomoléculas que hasta ahora no se había explorado. Por ello, en esta tesis se evalúa también la factibilidad de la aplicación de la hidrólisis térmica y de la oxidación húmeda a levaduras de cervecería, con el fin de caracterizar estos procesos y evaluar la posibilidad de recuperar proteínas a partir de los hidrolizados obtenidos mediante varias técnicas sencillas como son la precipitación mediada por pH, la precipitación salina y la cromatografía por afinidad con metales inmovilizados.

En base a la composición bioquímica del lodo, los hidrolizados obtenidos se presentan como medios ricos en nutrientes, y que por tanto pueden ser utilizados como medios para fermentaciones de interés. Una revisión bibliográfica muestra que este enfoque se aplica para

obtener productos de interés como pueden ser los ácidos grasos de cadena corta o los polihidroxialcanoatos mediante diferentes métodos de hidrólisis del lodo, aunque con una clara predominancia de la hidrólisis biológica. Este método es sencillo y económico, pero mediante otros métodos de hidrólisis se podrían obtener hidrolizados aptos para sustentar fermentaciones que acaben en la producción de metabolitos de alto valor, como enzimas.
En este sentido, esta tesis explora además la posible aplicación de los hidrolizados de hidrólisis térmica y de oxidación húmeda como medio de fermentación para *Bacillus licheniformis* CECT 20, un microorganismo capaz de producir enzimas de interés como proteasas y lacasas. Además de subsistir y desarrollarse en los hidrolizados producidos tanto en presencia como en ausencia de oxígeno, *Bacillus licheniformis* fue capaz de producir proteasas, especialmente utilizando el medio procedente de hidrólisis térmica; y lacasas si se cultivaba en el hidrolizado obtenido tras la oxidación húmeda.

RESUMEN (en Inglés)

Sewage sludge is a daily generated waste in huge quantities and requires a proper management owing to its potential hazards for both public health and environment. Currently, there are some methods to this end, but they are mainly focused on sludge stabilisation, minimization and energy recovery via biogas generation, thus ignoring their potential as renewable resource. Sewage sludge is composed by microorganisms and, hence, by biomolecules such as proteins, carbohydrates or humic acids. All of them show a high added value attending to their potential applications.
In order to purify these biomolecules, firstly it is necessary to carry out the sludge breakage. Although it exists several techniques with different basis, hydrothermal treatments appear to be an excellent alternative. Hydrothermal treatments are based on the application of high temperatures and pressures to achieve two objectives at the same time: the sludge stabilisation and minimization and its component solubilization. Hydrothermal treatments can be carried out under inert or oxidant atmospheres, providing different results. For the former, the treatment is called thermal hydrolysis and it is characterised by the presence of hydrolytic reactions that provoke cell lysis and intracellular components release. For the latter, the treatment is called wet oxidation and, besides hydrolytic reactions, oxidation reactions also occur on the solubilised components, turning them into molecules with new functional groups, such as carboxyl, carbonyl, ether, hydroxyl, etc. Although bibliography has dealt with the changes suffered by the sludge during the hydrothermal treatments so far, it was obviated the potential of these treatments to provide hydrolysates that allow the recovery of interesting biomolecules. Therefore, this doctoral thesis aims to explore the feasibility of recovering the biomolecules present in the effluents of wet oxidation and thermal hydrolysis of sewage sludge.
Owing to the percentage of sewage sludge that humic acids represent, the interferences they show with some protein precipitation methods and their low biodegradability, their behaviour during wet oxidation processes was studied. In this way, the knowledge about the effects of operation parameters such as temperature, pressure or pH was augmented, in terms of evolution of different parameters and the products generated.
Given the versatility of the hydrothermal treatments for treating aqueous biomasses, extending their application to other feedstocks could open the door to the revalorization of other wastes in terms of recovering biomolecules that have not been explored yet. Therefore, in this doctoral thesis, the feasibility of the implementation of the thermal hydrolysis and the wet oxidation to brewer's yeast is assessed, in order to characterise the processes and evaluate the potential recovery of proteins from hydrolysates. This recovery was assayed by the study of several techniques such as pH driven precipitation, saline precipitation and immobilized metal affinity chromatography.
Attending to the biochemical composition of the sludge, the hydrolysates appear as media rich in nutrients that can be used as substrate for fermentations with industrial interest. A bibliography review showed that this approach is already applied to obtain interesting products such as short chain fatty acids or polyhydroxyalkanoates by different hydrolysis and fermentation methods, with a clear predominance of biological procedures. These are easy and cheap methods, although other strategies could provide hydrolysates suitable to be used for fermentations to produce high added value metabolites, for instance, enzymes.
In this sense, this doctoral thesis assessed the uses of hydrolysates from thermal hydrolysis and wet oxidation as fermentation media for *Bacillus licheniformis* CECT 20. This microorganism is capable of producing interesting enzymes, such as proteases and laccases.

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Besides from surviving and developing in the hydrolysates obtained either in absence or in presence of oxygen, *Bacillus licheniformis* was able to produce proteases, especially when the hydrolysate from thermal hydrolysis was employed as substrate, or laccases, if it was grown in the hydrolysate obtained by sludge wet oxidation.

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

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RESUMEN

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ABSTRACT

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1.1. PREÁMBULO

La generación de residuos es inherente a cualquier actividad humana, ya sea cotidiana o industrial. De entre todos los residuos generados, el agua residual pasa normalmente desapercibida. El agua que se usa día a día en hogares e industrias es captada y, tras su uso, se transforma en agua residual, que presenta contaminantes que hacen que devolverla al medioambiente en ese estado sea potencialmente peligroso para éste. Es por ello que, previo a la devolución, el agua residual debe ser tratada para reducir los contaminantes presentes, generando así el menor impacto posible para el medio receptor.

1.2. LODOS. GENERACIÓN Y PROBLEMÁTICA

El proceso de tratamiento del agua residual para poder descargarla en el medio natural en condiciones seguras es complejo. Actualmente, una de las técnicas más extendidas es el proceso de lodos activos. Dicho proceso consiste, de forma muy resumida, en la eliminación de materia orgánica por comunidades de microorganismos en un biorreactor que es alimentado con el agua residual. Los microorganismos se alimentan de dicha materia orgánica consumiendo oxígeno en el proceso y eliminándola así del agua. Sin embargo, como consecuencia, los microorganismos crecen y se multiplican, generando los lodos activos. Estos lodos están compuestos por los microorganismos, principalmente procariotas, aunque también hongos, algas, protozoos y rotíferos (Ramalho, 1996).

Para dar una idea de los ingentes volúmenes de lodos generados, se ha observado que se pueden generar entre 3,1 y 8,2 litros por habitante y día, dependiendo de varios factores (Andreoli et al., 2007). En un país como España, con 47 millones de habitantes, se podrían producir 265.550.000 litros al día (tomando como base de cálculo la media de la producción de lodos mencionada anteriormente). Esta cantidad de lodos debe ser gestionada adecuadamente, ya que son un material potencialmente peligroso debido a la presencia de metales pesados y patógenos (Cao et al., 2021).

Los principales métodos de gestión de los lodos se centran en la estabilización y eliminación de los mismos. Tradicionalmente, los lodos se han utilizado como fertilizantes en agricultura, se han llevado a vertederos o se han incinerado con fines energéticos. Aunque estos usos están extendidos, la presencia de los mencionados

contaminantes y patógenos, las emisiones de gases y el alto coste, en el caso de la incineración, desaconsejan estas aplicaciones (Raheem et al., 2018). Otro de los métodos más extendidos se basa en el aprovechamiento del lodo para producir biogás: la digestión anaerobia. Mediante este proceso, el lodo es digerido hasta formar metano. Dicha digestión consta de cuatro etapas: hidrólisis, acidogénesis, acetogénesis y metanogénesis. Debido al potencial energético del metano, se han hecho grandes esfuerzos para optimizar la digestión anaerobia, como se puede ver en la literatura (Kor-Bicakci and Eskicioglu, 2019; Xu et al., 2020). Sin embargo, estos métodos presentan varias desventajas, como condiciones de operación estrictas y poco flexibles, tiempos largos o bajo rendimiento económico.

Además, en lo referente a la economía, los métodos tradicionales de gestión de lodos pueden suponer entre un 20% y un 60% del coste total de operación de la planta depuradora, por lo que sería de interés encontrar otros métodos que permitan mejorar la rentabilidad de las depuradoras (Andreoli et al., 2007).

Teniendo en cuenta estas desventajas y la alta cantidad de lodo disponible, se continúa la búsqueda de alternativas para la gestión del lodo, que además permitan mejorar el aspecto económico. En este sentido, se ha sometido a los lodos a procesos de pirólisis, que permiten obtener bioaceites y *biochars*; gasificación para obtener productos como *syngas* y biocombustibles; así como procesos de recuperación de fósforo en forma de estruvita (Gao et al., 2020; Sena y Hicks, 2018).

1.3. NUEVAS PERSPECTIVAS EN LA GESTIÓN DE LODOS

Aunque las alternativas previamente descritas pueden parecer prometedoras, la composición bioquímica del lodo indica que subestiman su potencial. Profundizando en este aspecto, el lodo está compuesto mayoritariamente por biomoléculas como proteínas (61%) y carbohidratos (11%), pero también por ácidos húmicos (20%) (Chen et al., 2007; Frølund et al., 1996; Suárez-Iglesias et al., 2017). Purificadas, estas moléculas presentan un alto valor añadido, ya que tienen múltiples usos. Por ejemplo, las proteínas pueden emplearse en la fabricación de resinas, bioplásticos, adhesivos, recubrimientos o fertilizantes (Adamczyk et al., 2010; Jones et al., 2015; Skeist, 1990; Somanathan et al., 1992; Tian et al., 2018). Los ácidos húmicos presentan propiedades surfactantes y son excelentes fertilizantes (Salati et al., 2011) y los carbohidratos sirven como fuente de carbono para fermentaciones (Tekin et al., 2014).

Visto el potencial de las biomoléculas que componen el lodo, su recuperación a partir de éste es un paso esencial para generar valor a partir un residuo que, actualmente, no tiene demasiadas opciones de revalorización. Además, la sociedad está cada vez más concienciada en la sustitución de fuentes no renovables de recursos por otras renovables, por lo que la posibilidad de obtener moléculas de interés de un residuo renovable, barato y abundante encaja a la perfección con la perspectiva de economía circular que tanta fuerza está cobrando hoy en día.

1.4. MÉTODOS DE HIDRÓLISIS DE LODOS

Sin embargo, la recuperación de las biomoléculas no es una operación sencilla, ya que forman parte del lodo, bien de los microorganismos o bien de los flóculos. Por ello, el primer paso debe ser la rotura y solubilización de estas estructuras. Existe un amplio abanico de métodos para este fin que han sido profundamente revisados. Dada la importancia de los métodos de rotura para comprender el resto de la tesis, se hará una breve descripción.

Los métodos físicos se basan en la generación de fuerzas de corte que rompen las células y flóculos, liberando así sus contenidos. Dichas fuerzas se pueden obtener de diversas fuentes, como homogeneizadores, molinos de bolas, ultrasonidos o por altas presiones (Le et al., 2015; Shehadul Islam et al., 2017).

Los métodos biológicos son aquellos que emplean microorganismos para conseguir la disrupción de las estructuras del lodo. Normalmente son los propios microorganismos del lodo los que llevan a cabo este proceso mediante diversas actividades enzimáticas (Chatterjee y Mazumder, 2019).

Los métodos químicos se basan en reacciones químicas que acaban en la rotura de membranas y flóculos. Dichas reacciones se pueden generar mediante la adición de distintos reactivos, como pueden ser ozono, CaO, CaO_2, $Ca(OH)_2$, NaOH, ácido nitroso libre, amonio libre, ácido etilendiaminotetraacético (EDTA), persulfatos o peroxidisulfatos entre otros muchos (Appels et al., 2008; Luo et al., 2020, 2019; Yuan et al., 2019).

La rotura del lodo también se puede conseguir mediante métodos bioquímicos, es decir, mediante la adición de enzimas adecuadas, como proteasas y celulasas, entre otras. Con un cóctel enzimático adecuado, podría incluso lograrse la disrupción de la membrana celular (Gonzalez et al., 2018; Massanet-Nicolau et al., 2008).

1.5. MÉTODOS HIDROTÉRMICOS. DESCRIPCIÓN Y VENTAJAS

Otro agente que puede ser empleado para lograr la rotura de las membranas es el calor. Los métodos térmicos normalmente emplean temperaturas inferiores a la de ebullición del agua, aunque también se pueden emplear temperaturas que superen dicho punto de ebullición, lo que hace necesario incrementar la presión del sistema. En este caso, los métodos se denominan hidrotérmicos. Estas altas presiones (normalmente por encima de 20 bar) y altas temperaturas (por encima de 150 °C) provocan la rotura de las mencionadas estructuras liberando los componentes, pero también son capaces de romper estos componentes en sus unidades básicas, es decir, son capaces de romper polímeros en oligómeros o monómeros. Así, estos tratamientos se denominan de hidrólisis térmica (HT) (Hii et al., 2014).

Pero las posibilidades de los tratamientos hidrotérmicos son amplias. Una modificación común consiste en la adición de agentes oxidantes, como puede ser el O_2. En este caso, el tratamiento pasa a denominarse oxidación húmeda (OH). La naturaleza hidrolítica de la oxidación húmeda es similar a la de la hidrólisis térmica, sin embargo, la presencia de oxidantes sirve como promotor de reacciones de oxidación. Esto es importante por dos motivos. En primer lugar, las reacciones de oxidación son más agresivas, por lo que la solubilización será previsiblemente mayor que en la hidrólisis térmica (Urrea et al., 2018). En segundo lugar, las moléculas que se vayan solubilizando serán oxidadas, por lo que pasarán a tener grupos funcionales tipo carboxilo, carbonilo, éter, hidroxilo, etc. Es, por tanto, una fuente de moléculas ácidas (Suárez-Iglesias et al., 2017). En un proceso ideal, la oxidación húmeda sería capaz de convertir la materia orgánica en CO_2 y agua (Hii et al., 2014).

De este modo, los tratamientos hidrotérmicos suponen una excelente alternativa para la gestión de los lodos por varios motivos:

- Permiten tratar el lodo sin operaciones previas de deshidratación. De hecho, se necesita agua en el sistema.
- Permiten la minimización del lodo. Al solubilizar sus componentes, se consigue reducir su contenido de sólidos suspendidos.
- Permiten la higienización del lodo debido a las altas temperaturas.

Aunque inicialmente se pudiera pensar que son tratamientos costosos debido a las altas temperaturas necesarias y la adición de gases para conseguir la presión deseada en el sistema, se debe tener presente que, especialmente en la oxidación húmeda, se generan

radicales libres. Estas especies son altamente reactivas y provocan reacciones exotérmicas que hacen que los requerimientos de calor del sistema no sean tan elevados. De hecho, el proceso de oxidación húmeda puede llegar a ser autotérmico (Bhargava et al., 2006).

Teniendo todo lo anterior en cuenta, los tratamientos hidrotérmicos se presentan como una alternativa para el tratamiento de lodos con un alto potencial, ya que permiten la minimización e higienización del lodo al mismo tiempo que ofrecen la posibilidad de recuperar sus componentes, al ser éstos solubilizados durante el proceso.

1.6. RECUPERACIÓN DE BIOMOLÉCULAS DE HIDROLIZADOS

Una vez se cuenta con los componentes del lodo solubilizados, la recuperación es factible. Las biomoléculas que más interés suscitan son las proteínas, ya que son las que mayores concentraciones presentan en el lodo y, como ya se mencionó, cuentan con una amplia variedad de aplicaciones. La recuperación de proteínas del lodo se ha intentado empleando diferentes técnicas tanto de hidrólisis como de separación. Una descripción detallada de éstas se puede consultar en Xiao y Zhou (2020). Cabe destacar que en algunos estudios concretos, como es el caso de Hwang et al., (2008), la recuperación de proteínas del lodo hidrolizado puede llegar a ser completa; sin embargo, no tuvieron en cuenta los ácidos húmicos, moléculas que también forman parte del lodo y que interfieren en los métodos de medida.

En cualquier caso, la recuperación de moléculas concretas es aún un campo que necesita avances que permitan aprovechar todo el potencial del lodo como fuente renovable de biomoléculas.

1.7. USO DIRECTO DE LOS HIDROLIZADOS

El lodo hidrolizado puede ser aprovechado también como medio de fermentación. De hecho, éste ya es un campo en funcionamiento, pues, sin ir más lejos, la digestión anaerobia utiliza un hidrolizado biológico de lodo como sustrato para obtener biogás. También es posible la obtención de biohidrógeno mediante procesos similares. Estos dos campos han sido ampliamente estudiados, y en el caso de la digestión anaerobia, explotados a nivel industrial. Por ello, estos productos no serán estudiados en detalle en esta tesis ya que, además, existen amplias revisiones sobre el tema (Bozkurt y Apul, 2020; Elalami et al., 2019; Fu et al., 2021; Kor-Bicakci y Eskicioglu, 2019).

Pero no sólo el aprovechamiento energético de los hidrolizados de lodo es posible. Por ejemplo, si se profundiza en los mecanismos de la digestión anaerobia, es posible obtener ácidos grasos de cadena corta (AGCC), como ácido acético, propiónico, butírico o valérico. Éstos son productos de gran interés comercial y con varias aplicaciones, como materias primas de industrias químicas o sustratos para la producción de biológica de electricidad, entre otras (Aghapour Aktij et al., 2020; Bhatia and Yang, 2017; Bhatt et al., 2020; Lee et al., 2014; Patel et al., 2020). Una de estas aplicaciones es la síntesis de polihidroxialcanoatos (PHA), biopolímeros sintetizados por microorganismos como fuente de energía en condiciones de hambruna a partir del consumo de AGCC (Satoh et al., 1999). Los PHA son similares a ésteres de lípidos, y presentan propiedades parecidas a las del polipropileno, por lo que son excelentes alternativas a los plásticos procedentes de derivados del petróleo (Bernard, 2014; Reddy et al., 2003). La actual conciencia social en la reducción del uso de materiales no renovables justifica el gran interés de la optimización de la producción de PHA a partir de lodos.

Los hidrolizados ricos en AGCC también pueden ser utilizados como sustrato en la síntesis de lípidos, que posteriormente pueden ser transformados en biocombustibles (J. Liu et al., 2019).

La hidrólisis biológica, como ya se ha visto, no es el único método susceptible de ser empleado para obtener hidrolizados de lodo. Sin embargo, permite obtener hidrolizados con unas características adecuadas para la producción de AGCC y PHA. Otros métodos, como los tratamientos hidrotérmicos producen hidrolizados en los que las biomoléculas, como las proteínas, están solubilizadas sin sufrir grandes cambios en su naturaleza química. Es por ello que, empleando microorganismos adecuados y utilizando el hidrolizado como sustrato, se pueden obtener diferentes metabolitos de interés, como enzimas. Por ejemplo, se ha explorado la producción de proteasas a partir de hidrolizados de lodo (Drouin et al., 2008). Sin embargo, aún es una rama poco explorada, pero que presenta un gran potencial, pues la variedad de productos que podrían obtenerse es muy extensa, y sería prácticamente ilimitada si se emplea de forma conjunta con técnicas de ingeniería genética.

De este modo, la implementación de hidrólisis térmica u oxidación húmeda como métodos de hidrólisis podría servir para introducir las plantas de tratamiento de aguas residuales de lleno en la economía circular, bien a través de la obtención de las biomoléculas ya presentes en el lodo o por el empleo de éste como sustrato para

fermentaciones de interés. Todo ello está en la línea de los objetivos marcados por la Unión Europea en su nuevo Plan de Acción para la Economía Circular (Comisión Europea).

1.8. EXTRAPOLACIÓN A OTRAS BIOMASAS

Visto el potencial de los lodos y sus hidrolizados como fuente renovable de biomoléculas, parece lógico buscar otros sustratos donde sea factible emplear técnicas similares para obtener productos de interés. Obviamente, deben tratarse de biomasas constituidas por microorganismos. Aunque existe una amplia variedad de este tipo de biomasas, hay un sector en concreto que está experimentando un gran auge en los últimos tiempos, el sector cervecero. Para la producción de cerveza se emplean levaduras, que, salvando las diferencias, podrían asemejarse a los lodos activos: ambos son sustratos baratos y abundantes, constituidos por microorganismos unicelulares y con un alto contenido en proteínas.

Aunque las levaduras de cerveza tienen actualmente otros usos, basta con ver las cantidades producidas para darse cuenta de que pueden aplicarse otras alternativas de gestión. En Europa se produjeron, sólo en 2018, 39.000 millones de litros de cerveza (Eurostat). Asumiendo que por m^3 se producen entre 1,7 y 2,3 kg de levadura (Ferreira et al., 2010), se cuenta con una producción anual de entre 66.300 y 89.700 toneladas de levadura.

Teniendo en cuenta que es un sustrato rico en proteínas, se podrían aplicar los tratamientos mencionados para recuperarlas de los hidrolizados. No es un campo explorado, aunque sí se ha comprobado que la hidrólisis térmica es una tecnología capaz de solubilizar las proteínas presentes en las levaduras (Lamoolphak et al., 2006). Por ello, la extrapolación a levaduras de todo lo mencionado para lodos es una línea de investigación de gran interés.

2. OBJETIVOS

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El objetivo principal de esta tesis es el estudio de posibles vías de revalorización de hidrolizados de biomasas celulares obtenidos mediante tratamientos hidrotérmicos para la producción de compuestos no energéticos con interés industrial. Para ello, se deben alcanzar los siguientes sub-objetivos:

- Caracterizar los procesos y productos de OH e HT de lodos de depuradora.
- Caracterizar los procesos y productos de OH e HT levadura de cerveza.
- Evaluar distintos métodos de separación de los biopolímeros a partir de lodo hidrolizado por vía térmica.
- Evaluar distintos métodos de separación de los biopolímeros a partir de levadura hidrolizada por vía térmica.
- Caracterizar los procesos y productos de OH de ácidos húmicos.
- Analizar el potencial del hidrolizado de lodo como medio de fermentación.

3. MATERIALES Y MÉTODOS

3. MATERIALES Y MÉTODOS

3.1. LODO

El lodo usado en todos los experimentos fue lodo secundario espesado por flotación, procedente de la planta de tratamiento de aguas residuales de Baíña (Mieres, Asturias). Hasta su uso, el lodo se almacenó a 4 ºC. Las características de este lodo se muestran en la tabla 1.

Tabla 1. *Parámetros iniciales (media ± desviación típica) del lodo usado en los experimentos. * indica concentraciones en disolución.*

Parámetro	***Valor***
pH	6,4 ± 0,1
SST (g/l)	39,4 ± 4,1
SSV (g/l)	29,6 ± 1,4
DQOt (g O_2/L)	35,7 ± 3,3
DQOs (g O_2/L)	2,61 ± 0,63
TOC* (mg/L)	734,7 ± 68,7
Proteínas* (mg/L)	170,4 ± 73,5
Ác. húmicos* (mg/L)	433,5 ± 108,7
Carbohidratos* (mg/L)	129,5 ± 52,9
Ác. urónicos* (mg/L)	15,7 ± 0,9
ADN* (mg/L)	20,9 ± 1,3

3.2. LEVADURA

La otra materia prima empleada, levaduras de la especie *Saccharomyces pastorianus*, procedieron de la fábrica de Cervezas Caleya (La Felguera, Asturias). Estas levaduras se recogieron del fondo de un tanque cilindro-cónico tras el proceso de fermentación. Es importante remarcar que en dicho proceso no se emplearon lúpulos ni otros agentes aromáticos.

Estas levaduras mostraron altas concentraciones de SSV (150 g/L) y, además, contenían elevados contenidos de carbohidratos residuales procedentes de la producción de cerveza. Por ello, se realizaron 3 lavados con agua destilada, llegando a concentraciones de SSV de aproximadamente 30 g/L. Al igual que en el caso de los lodos, las levaduras lavadas se almacenaron a 4 °C hasta su uso. Las características de las levaduras lavadas se recogen en la tabla 2.

Tabla 2. *Parámetros iniciales (media ± desviación típica) de las levaduras usadas en los experimentos. * indica concentraciones en disolución.*

Parámetro	***Valor***
pH	5,6 ± 0,5
TSS (g/L)	26 ± 6
VSS (g/L)	26 ± 6
SVI (mL/g)	18 ± 3
DQOt (g O_2/L)	41,6 ± 6,6
DQOs (g O_2/L)	5,5 ± 1,9
TOC* (mg/L)	1,9 ± 0,5
Proteínas* (mg/L)	1,7 ± 1,1
Carbohidratos* (mg/L)	0,6 ± 0,5

3.3. TRATAMIENTOS HIDROTÉRMICOS

Los tratamientos hidrotérmicos se llevaron a cabo en un reactor PARR 4520 series de 1 litro de capacidad. Este reactor está equipado con un agitador de hélice con 6 palas que proporciona 500 rpm. El gas (oxígeno puro para OH o nitrógeno puro para HT) se suministra al reactor tras pasar por un humidificador de 2 litros de capacidad, a fin de evitar las pérdidas de volumen en el reactor causadas por la evaporación. Por seguridad, tanto el reactor como el humidificador se llenaron sólo hasta el 70% de su capacidad. En la figura 1 se muestra un esquema de la configuración del reactor.

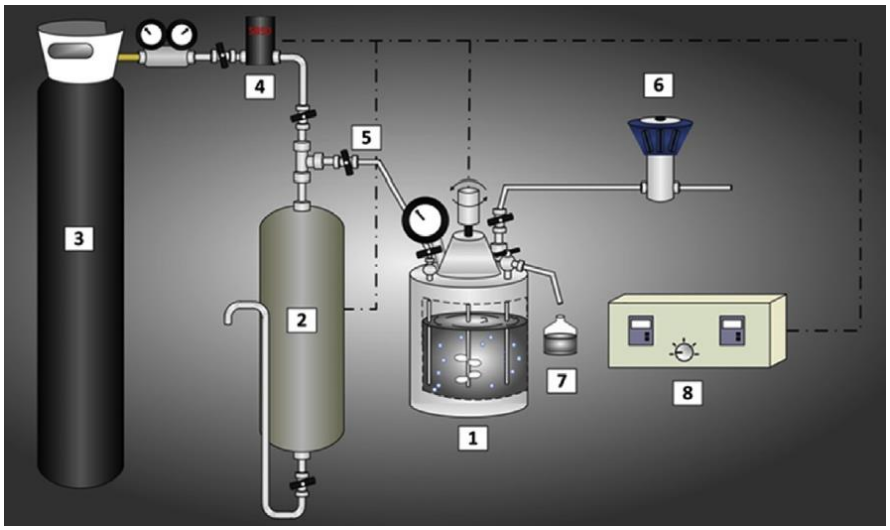

Figura 1. *Configuración del reactor empleado para los tratamientos hidrotérmicos. En la imagen, 1: reactor, 2: humidificador, 3: botella de O_2 o N_2, 4: controlador de flujo, 5: válvula de control, 6: válvula de control de la presión, 7: puerto de toma de muestras, 8: controlador PID. Extraído de Urrea et al., (2016).*

En cuanto a los parámetros operacionales, la temperatura del reactor y del humidificador se ajustaron mediante un controlador PID a los valores deseados. Todos estos valores se encontraron siempre dentro del rango típico de los empleados habitualmente en tratamientos hidrotérmicos y pueden ser consultados en el apartado de resultados correspondiente (Hii et al., 2014).

3.4. CULTIVOS

Esta técnica fue empleada para el ensayo de las posibilidades de los hidrolizados obtenidos en el apartado anterior (3.3.) como medio de fermentación para obtener productos de interés. Dada la presencia de fuentes de carbono como proteínas y, en menor medida, carbohidratos, es lógico pensar que un microorganismo proteolítico sea capaz de crecer en el hidrolizado. Por ello, de entre todos los microorganismos proteolíticos, se buscó uno de baja peligrosidad (nivel 1), de requerimientos nutricionales sencillos y que, además, fuese capaz de crecer en un medio con ácidos húmicos. Con este fin, *Bacillus licheniformis* CECT 20 fue seleccionado, ya que es capaz de producir una amplia variedad de enzimas, como proteasas, pudiendo alimentarse así de las proteínas presentes en el hidrolizado; o lacasas, siendo capaz de degradar los ácidos húmicos. Estas enzimas, además, tienen aplicaciones industriales, que serán vistas más adelante (apartado 4.5).

Para el cultivo de *B. licheniformis*, los hidrolizados se obtuvieron tras 80 minutos HT u OH a 160 °C y 40 bar. Dichos hidrolizados se centrifugaron y se pasaron a botes estériles. Aquí es dónde se presenta una importante ventaja de los tratamientos hidrotérmicos para obtener medios de cultivo, pues los hidrolizados son estériles, debido a las agresivas condiciones del interior del reactor. Una vez centrifugados, se ajustó el pH al óptimo del microorganismo (7,2), utilizando NaOH 1M ó HCl 1M. Tras ello, se tomaron 5 mL de un cultivo de *B. licheniformis* en fase exponencial (realizado en medio *Nutrient Broth* (NB), a pH 7,2; 37 °C; 150 rpm y entre 12 y 24 h) y se añadieron a 95 mL de hidrolizado de HT u OH. Las condiciones de cultivo en los hidrolizados fueron las mismas que en medio NB: 37 °C, 150 rpm. Se tomaron muestras diariamente, durante varios días, para analizar la densidad óptica (DO), proteínas solubles, carbohidratos solubles, ácidos húmicos solubles, actividad proteasa global y actividad lacasa. Los métodos de medida se detallan en el apartado siguiente (3.6).

También se realizaron cultivos en las mismas condiciones, pero diluyendo los hidrolizados con agua destilada convenientemente esterilizada. Con ello, se pretendió

comprobar el posible efecto tóxico de algunos compuestos que se pueden generar durante los tratamientos hidrotérmicos, especialmente en el caso de OH (Martín et al., 2007).

Finalmente, y puesto que los tratamientos hidrotérmicos producen reacciones de naturaleza hidrolítica entre otras, se preparó un medio sintético con los tres principales biopolímeros presentes en el hidrolizado. Para las proteínas se empleó albúmina sérica bovina (BSA, Sigma), para los ácidos húmicos se empleó ácido húmico puro (Sigma) y para los carbohidratos, almidón o glucosa (ambos de Sigma). Estas moléculas se añadieron en cantidades que permitieron obtener concentraciones similares a las encontradas en los hidrolizados: 5 g/L de BSA, 2.5 g/L de ácidos húmicos, 2 g/L de carbohidratos. El empleo de almidón o glucosa permitió evaluar el efecto de una fuente de carbono compleja frente a otra más fácilmente asimilable.

3.5. MÉTODOS DE SEPARACIÓN

Las proteínas son las moléculas que más interés generan debido a las aplicaciones ya mencionadas en el apartado 1.3. de la Introducción. Por ello, los métodos de separación se enfocaron a la precipitación de las proteínas. Se buscaron métodos sencillos y fácilmente escalables.

3.5.1. Precipitación con acetona

Una de las técnicas aplicadas fue la precipitación con acetona a -20 ºC (Sigma), a razón de tres volúmenes de acetona por volumen de muestra. Se dejó precipitar durante dos horas a -20 ºC y se centrifugó a 4 ºC, 10000 rpm durante 10 minutos (Jiang et al., 2004).

3.5.2. Precipitación con ácido tricloroacético

El ácido tricloroacético (TCA) es un agente precipitante. Se usó una concentración del 20% de TCA en la muestra, dejándolo precipitar a 4 ºC durante 30 minutos. Posteriormente se centrifugó a 4 ºC y 10000 rpm, durante 10 minutos (Oliveira et al., 1999).

3.5.3. Precipitación con sulfato amónico

El sulfato amónico ($(NH_4)_2SO_4$) es comúnmente usado para precipitar proteínas. Este método se basa en el conocido fenómeno *salting out*, es decir, la adición de un compuesto (generalmente una sal) que compite por el agua con el resto de solutos. La concentración usada para lodos fue cercana a la saturación, de 0,6 g/mL, siendo 0,74 g/mL el punto de saturación (Haynes, 2014).

Esta técnica también se aplicó para los hidrolizados de levaduras, si bien se ensayaron diferentes concentraciones de $(NH_4)_2SO_4$.

3.5.4. Precipitación por pH

La precipitación por pH se basa en que, cuando la disolución tiene un pH igual al punto isoeléctrico de una proteína, esta pierde su estructura y precipita (Galanakis, 2012). Por ello, tanto para lodo como para levadura, se probaron diferentes pH ácidos, comprendidos entre 2 y 5.5, así como un pH alcalino.

3.5.5. Cromatografía de afinidad con metales inmovilizados

La cromatografía de afinidad con metales inmovilizados (IMAC) se basa en la afinidad de ciertos metales como el hierro o el cobre por los enlaces peptídicos de las proteínas. Dichos enlaces se unen al catión metálico, previamente unido a una resina, reteniendo a la proteína sobre ellos. Esta técnica es de gran interés, pues la resina no presenta elevados costes y se puede reutilizar, ya que la proteína puede ser fácilmente eluída con soluciones de NaCl.

La técnica IMAC se aplicó sobre los hidrolizados de lodos y levaduras. En el caso de los lodos, se encontraron varias dificultades que serán descritas en el apartado 4.1.

La resina empleada fue la Lewatit – TP 207 (Sigma). Dicha resina está funcionalizada con ácido iminodiacético, que presenta gran afinidad por cationes metálicos. La resina se sumergió en una disolución 1M de $CuCl_2$ durante 4 horas, ya que el Cu^{2+} es el catión con mayor afinidad por el enlace peptídico (Ueda et al., 2003). Posteriormente se lavó con agua destilada. Los experimentos se realizaron en *batch*, con diferentes relaciones líquido-sólido (L/S) para obtener las isotermas de adsorción que caracterizan el proceso. El líquido obtenido tras la adsorción se evaluó en términos de concentración de biopolímeros solubles.

3.5.6. Recuperación y selectividad de biopolímeros

Los diferentes métodos descritos están enfocados a la precipitación de proteínas, sin embargo, también es posible la precipitación de ácidos húmicos debido a la similitud en grupos funcionales y tamaño que pueden presentar con las proteínas, así como de carbohidratos, ya que éstos pueden formar parte de las proteínas, como es el caso de las glucoproteínas. Por ello, para evaluar la eficiencia de la separación se tuvo en cuenta el porcentaje precipitado de cada biopolímero (ecuación 1), pero también un factor de selectividad α (ecuación 2), calculado en base a la precipitación relativa de cada biomolécula.

$$\%_{precipitatado} = \left(1 - \frac{concentración\ en\ el\ sobrenadante\ tras\ la\ precipitación}{concentración\ en\ el\ sobrenadante\ \textit{antes de}\ la\ precipitación}\right) x\ 100 \text{ (ec. 1)}$$

$$\alpha_{i/j} = \frac{\left(\frac{(C_i)_{antes\ de\ la\ precipitación} - (C_i)_{sobrenadante\ tras\ la\ precipitación}}{(C_j)_{antes\ de\ la\ precipitación} - (C_j)_{sobrenadante\ tras\ la\ precipitación}}\right)}{\frac{(C_i)_{sobrenadante\ tras\ la\ precipitación}}{(C_j)_{sobrenadante\ tras\ la\ precipitación}}} \text{ (ec. 2)}$$

La resolución de la ecuación 2 puede devolver tres tipos de soluciones:

- **$\alpha \approx 1$**. Indica que no hay buena separación, es decir, que las proporciones de *i* y *j* en sobrenadante y precipitado son similares.
- **$\alpha > 1$**. Si α es mucho mayor que 1, hay buena separación del componente *i*, es decir, habrá precipitado mucho más *i* que *j*.
- **$\alpha < 1$**. Si α es mucho menor que 1, indica que *j* ha precipitado más que *i*, por lo que la separación es buena.

3.6. MÉTODOS ANALÍTICOS

3.6.1. Medidas de parámetros típicos

Las medidas de pH, DQO, SST y SSV, IVL se realizaron siguiendo *Standard Methods* (APHA, 1998). Dichas medidas, exceptuando la DQOS, se realizaron sobre el hidrolizado total, mientras que el resto de parámetros se midieron sobre el hidrolizado soluble, es decir, el sobrenadante recuperado tras una centrifugación a 10000 g durante 10 minutos.

Teniendo esto en cuenta, el TOC se midió utilizando un equipo Shimadzu TOC-VCSH (Japón).

La determinación de proteínas y ácidos húmicos se realizó siguiendo el método Lowry modificado, utilizando BSA y ácido húmico puro como patrones (Frølund et al., 1996). Éste se basa en la reacción de los grupos fenólicos de las proteínas con el ácido fosfomolibdotúngstico, que se reduce pasando de color verdoso a azul en presencia de estos grupos aromáticos. Dado que las proteínas tienen una estructura tridimensional en la que los grupos aromáticos suelen estar en la zona interior (hidrófoba), es necesario desplegarlas, para lo que se usa una solución que aporta cobre. El cobre se unirá a los enlaces peptídicos (también a otros grupos amino) desnaturalizando la proteína y dejando expuestos los grupos aromáticos de sus aminoácidos, que luego darán la coloración que se va a cuantificar (*absorbancia total*). Sin embargo, también hay grupos aromáticos de

otras moléculas capaces de reducir el ácido fosfomolibdotúngstico, como es el caso de los ácidos húmicos (como se puede ver en la figura 2).

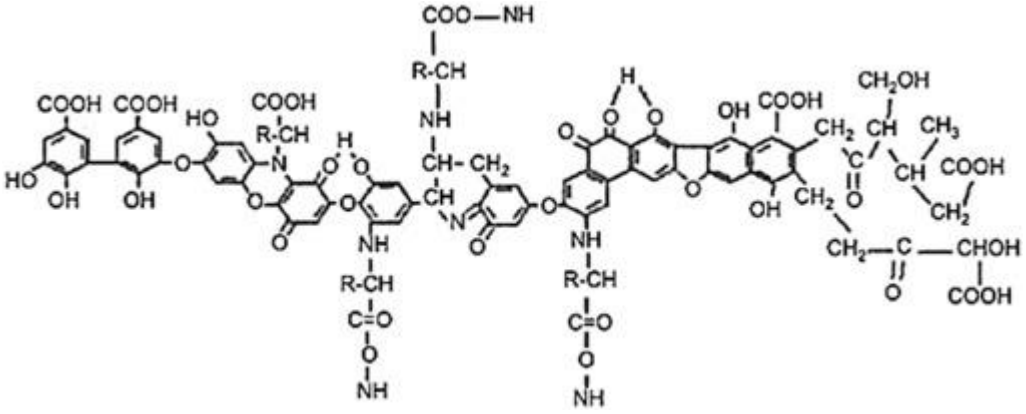

Figura 2. *Estructura típica de un ácido húmico. Extraída de de Melo et al., (2016).*

Debido a ello, el método se modifica y se tiene en cuenta la presencia de estas moléculas. Para ello, se hace una segunda medida, pero sin añadir cobre, de modo que sólo intervendrían los grupos aromáticos de los ácidos húmicos en la generación del color (*absorbancia ciega*). Con estos datos se aplican las siguientes ecuaciones empíricas (ecuación 3 y ecuación 4) para obtener la absorbancia correspondiente a proteínas y la correspondiente a ácidos húmicos:

$$\textbf{\textit{Abs. Proteína}} = 1{,}25 \cdot (\textit{Abs. Total} - \textit{Abs. Ciega}) \quad \text{(ec. 3)}$$

$$\textbf{\textit{Abs. Ác. húmico (ciega)}} = \textit{Abs. ciega} - 0{,}2 \cdot \textit{Abs. Proteína} \quad \text{(ec. 4)}$$

Con la curva de calibrado obtenida empleando BSA y ácido húmico, se puede obtener la concentración de cada molécula.

El método del fenol-sulfúrico fue el empleado para determinar los carbohidratos totales, en base a un patrón de glucosa (Sigma) (DuBois et al., 1956). Este método se basa en la transformación de los azúcares en furfurales en un medio ácido. Éstos son capaces de intercambiar protones con el fenol generando compuestos coloreados, de un tono anaranjado.

El ADN se midió utilizando el método propuesto por Burton, (1956), utilizando ADN de timo de ternera como patrón. Este método se basa en la coloración que proporciona la reacción entre la difenilamina y el β-hidroxilevulinaldehído formado a partir de la desoxirribosa del ADN en medio ácido. Puesto que el ADN es una molécula intracelular, sirve como excelente indicador de la rotura celular, puesto que al romperse el ADN pasa al medio líquido. Es decir, a mayor concentración de ADN en el hidrolizado, mayor rotura de las células.

En cuanto a los ácidos urónicos, su concentración fue determinada de acuerdo al trabajo de Blumenkrantz y Asboe-Hansen, (1973), que indica que estas moléculas se hidrolizan en medios ácidos fuertes, en los que también se deshidratan formando derivados furfurilos, que son capaces de reaccionar con el metadifenilo, generando un complejo rosado.

Otro parámetro medido en el sobrenadante fue el número de color (NC), si bien éste se obtiene a partir de los datos de absorbancias a 436 nm, 525 nm y 620 nm, aplicando la ecuación 5 (Tizaoui et al., 2007):

$$NC = \frac{CAE_{436}^2 + CAE_{525}^2 + CAE_{620}^2}{CAE_{436} + CAE_{525} + CAE_{620}} \qquad \text{(ec. 5)}$$

En esta ecuación, CAE (coeficiente de absorción espectral) indica la absorbancia registrada a cada una de las longitudes de onda anteriormente indicadas.

A partir de los datos obtenidos para DQOS y TOC, se calculó el estado medio de oxidación del carbono (AOSC, por sus siglas en inglés: *average oxidation state of carbon*). Dicho cálculo se realizó por medio de la ecuación 6 (Vogel et al., 2000):

$$AOSC = 4 - 1.5 \times \frac{TOC}{DQOS} \qquad \text{(ec. 6)}$$

Este parámetro puede tomar valores entre –4 y +4, si el carbono está totalmente reducido o si está totalmente oxidado, respectivamente. En la sección de resultados aparecerá el término MOC, que proviene del inglés *Mean Oxidation number of Carbon*, y es el equivalente al AOSC, pero para una molécula concreta.

3.6.2. Medidas de parámetros específicos para el cultivo de B. licheniformis

En el caso de los parámetros medidos en los cultivos de *B. licheniformis* realizados sobre los hidrolizados de lodos, las medidas de proteínas, ácidos húmicos, carbohidratos y actividad proteasa se realizaron sobre el sobrenadante obtenido tras centrifugar a 13200 rpm durante 5 minutos. El pellet obtenido se usó para evaluar la densidad óptica (DO), resuspendiéndolo en 1 mL de NaCl 0,7%, y diluyendo en caso de que fuese necesario. En el caso de la actividad lacasa, algunos estudios indican que es intracelular (Sharma et al., 2007), por lo que para su determinación se sometió 1 mL del cultivo a un tratamiento con ultrasonidos en tres pulsos de 10 segundos espaciados por 15 segundos, para evitar sobrecalentamiento de la muestra y la desnaturalización de la enzima.

La medida de la actividad proteasa se realizó en base al método de la azocaseína (Ginther, 1979; Romero et al., 2001). En este método se define una unidad de actividad proteasa como la cantidad de enzima necesaria para incrementar la absorbancia a 420 nm

en 0,1 unidades. Para determinar la actividad lacasa, se utilizó el método del ABTS (Niku-Paavola et al., 1990). En este caso, una unidad de actividad lacasa se establece como la cantidad de enzima que oxida 1 µmol de ABTS por minuto.

4. RESULTADOS

4. RESULTADOS

En este apartado se recogen los resultados obtenidos a lo largo de la tesis, tanto aquellos que han sido publicados como artículos científicos, como los que han servido para generar el conocimiento necesario sobre el tema y poder plantear líneas de experimentación acordes con lo observado.

Con el objetivo de mostrar la estructura de los resultados, se incluye la siguiente figura:

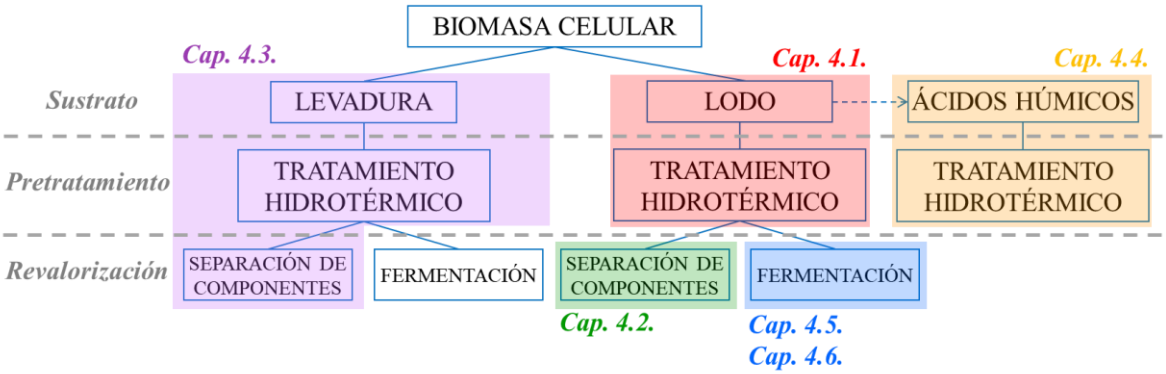

Figura 3. *Esquema de la estructura del capítulo de resultados.*

Como se puede ver en la figura 3, en primer lugar, en el capítulo 4.1. se realizará una descripción del trabajo realizado sobre tratamientos hidrotérmicos de lodos de depuradora, a "altas" y "bajas" temperaturas, comentando los principales hallazgos. También se hará en este apartado un inciso sobre otro trabajo relacionado con la separación de proteínas, si bien los resultados no fueron los esperados, por lo que se incluye en este apartado a título informativo, mostrando los principales motivos que impidieron lograr una buena separación.

En el capítulo 4.2., se incluyen los resultados relativos a la separación de componentes de hidrolizados térmicos de lodos obtenidos a altas temperaturas, empleando diversas técnicas de separación.

El capítulo 4.3. recoge los resultados de los tratamientos hidrotérmicos de levaduras de cervecería, en términos de caracterización del proceso en presencia o ausencia de una atmósfera oxidante, su modelizado cinético y el efecto de ésta en las etapas de recuperación de proteínas.

En el capítulo 4.4., se mostrarán los resultados de la OH de ácidos húmicos, un tema de interés para el trabajo con tratamientos hidrotérmicos y lodos de depuradora, ya que los ácidos húmicos son una parte importante del lodo y, como se pudo comprobar,

interfieren con las etapas de separación, por lo que conocer los cambios que sufren durante la oxidación húmeda proporcionará información de gran interés para futuros trabajos en esta línea.

El aprovechamiento global de los hidrolizados térmicos de lodo es otra estrategia factible en la revalorización de lodos de depuradora, como se mostrará en el capítulo 4.5. Éste recoge los resultados del uso de los hidrolizados obtenidos tras HT y OH como medio de fermentación de *Bacillus licheniformis* y la posibilidad de obtener productos de alto valor como son proteasas y lacasas.

En línea con la estrategia de emplear el lodo como sustrato para fermentaciones de interés, el capítulo 4.6. consiste en una revisión bibliográfica sobre el uso de lodos como medio de fermentación para la obtención de productos no energéticos, mostrando las principales estrategias de fermentación y los productos que se pueden obtener a partir de éstas.

4.1. INVESTIGACIÓN ADICIONAL

4.1.1. Tratamientos hidrotérmicos de lodos a altas temperaturas

Durante las primeras etapas del desarrollo de la presente tesis, los resultados obtenidos del trabajo de caracterización de los lodos de depuradora fueron suficientes para redactar un artículo científico donde se compara el efecto de la presencia del oxígeno durante el tratamiento hidrotérmico en las evoluciones de los diferentes parámetros característicos del lodo y los productos generados. Dicho artículo (Urrea et al., 2018) ya forma parte de otra tesis doctoral realizada en el grupo de investigación y se añade en el anexo I:

"*Sludge hydrothermal treatments. Oxidising atmosphere effects on biopolymers and physical properties*"

La información obtenida durante el desarrollo del artículo resultó muy útil en términos de caracterización y comprensión de los procesos que suceden durante los tratamientos hidrotérmicos. Así, permitió determinar el tiempo óptimo de tratamiento térmico del lodo, en base a la agresividad del mismo y el daño que podría causar a los productos de interés antes de las etapas de recuperación de biopolímeros.

4.1.2. Tratamientos hidrotérmicos de lodos a bajas temperaturas

Como se menciona en el apartado inmediatamente anterior, la información obtenida indicaba un posible daño en las proteínas durante los tratamientos hidrotérmicos a alta temperatura. Por ello, y a fin de comprobar el papel de la temperatura en el proceso, se

llevaron a cabo una serie de experimentos a “bajas temperaturas”. Las condiciones experimentales en estos casos fueron tratamientos de OH e HT a temperaturas entre 60 y 120 °C. Estos experimentos se caracterizaron del mismo modo que los realizados en las condiciones indicadas en los apartados 3.3. y 3.6.

En lo relativo a la caracterización del tratamiento hidrotérmico, como se esperaba, la solubilización fue baja, pues los SSV sólo se redujeron entre un 20% y un 30% para 60 °C y 120 °C, respectivamente; mientras que, como se verá para temperaturas más elevadas, las reducciones llegan a ser del 70% en el caso de la OH a 160 °C. La solubilización de la DQO tampoco fue muy marcada en comparación con la obtenida a altas temperaturas, siendo la DQOS un 32% de la total a baja temperatura mientras que, a 160 °C, llegó a ser un 60%. Un comportamiento similar se observó en la solubilización de los biopolímeros, obteniendo solubilizaciones mucho menores a temperaturas más bajas.

También pudo comprobarse el efecto de la temperatura sobre las reacciones de oxidación típicas de la OH. A temperaturas bajas no se observaron diferencias significativas entre los datos obtenidos para HT y OH, indicando que las reacciones de oxidación no fueron relevantes en la evolución de los parámetros. Sin embargo, en los resultados obtenidos en los tratamientos a altas temperaturas, las diferencias fueron evidentes. Sirva como ejemplo la disminución de SSV en OH, un 70%, frente a la de HT, un 50%. Una consecuencia de ello es la ausencia del perfil característico de las concentraciones de los productos solubles en OH: incremento inicial, fase de estabilización y destrucción por oxidación.

Sin embargo, aunque las solubilizaciones fueron mucho menores, las recuperaciones de proteínas fueron más selectivas tanto para los hidrolizados obtenidos tras la HT como para los hidrolizados obtenidos tras la OH, especialmente empleando el método del *salting out* con $(NH_4)_2SO_4$. Estos resultados se asocian a un menor daño a las proteínas, que no pierden su estructura ni sufren cambios en sus grupos funcionales. Sin embargo, se requeriría un trabajo completo para evaluar si la mayor pureza de los productos obtenidos compensa la marcada disminución en la solubilización.

Todo este trabajo está recogido en una comunicación tipo póster acompañada de una breve presentación oral en las XXXV Jornadas Nacionales de Ingeniería Química, celebradas en Salamanca en Junio del 2018 (ver anexo II):

"*Recuperación de proteínas de lodos mediante tratamientos hidrotérmicos a bajas temperaturas*" (XXXV JORNADAS NACIONALES DE INGENIERÍA QUÍMICA. LIBRO DE ABSTRACTS, 2018).

4.1.3. Separaciones de proteínas del hidrolizado de lodo por IMAC

En muchas ocasiones, los resultados no acompañan al trabajo realizado. Dado el interés en recuperar las proteínas de los hidrolizados de lodo, se propusieron varias técnicas que perseguían este fin. Entre ellas, la técnica IMAC, ya explicada en el apartado 3.5.5., que ya había sido utilizada en el grupo previamente con excelentes resultados (Arévalo et al., 2000).

Sin embargo, su aplicación en el lodo presentó diversos problemas, que analizados detenidamente y en base a la bibliografía existente, indican que la IMAC no es una técnica adecuada para la recuperación de proteínas de hidrolizados hidrotérmicos de lodos de depuradora.

4.1.3.1. Resultados

A continuación, se detallan los estudios realizados para llegar a esta conclusión, así como los diferentes problemas encontrados.

En primer lugar, es necesario recordar que la resina empleada estaba dopada con ácido iminodiacético (IDA), sobre el que se adsorbió cobre II. Esto se debe a la alta afinidad que presentan el cobre y las proteínas, propiciada por la coordinación del cobre con los átomos de nitrógeno presentes en los enlaces peptídicos a pH básicos. También son susceptibles de reacción otros átomos de nitrógeno, como los presentes en los grupos funcionales de los aminoácidos básicos (arginina, etc). Esto, en una solución de proteína pura, llevaría a la unión de la proteína al cobre, quedando así retenida en la resina. En pasos posteriores, podría separarse utilizando una disolución concentrada de NaCl, obteniendo así la proteína purificada.

Sin embargo, los hidrolizados de lodos son una mezcla heterogénea que consta de proteínas, ácidos húmicos, carbohidratos y otros biopolímeros en menores concentraciones. Dada la alta concentración de proteínas presentes en los hidrolizados, del orden de 7 g/l para los hidrolizados de 160 °C y 40 bar y de 3 g/l para los de 60 °C y 10 bar; es lógico pensar que el IMAC es un buen método para purificar dichas proteínas.

El problema surgió con los ácidos húmicos. Los ácidos húmicos son agrupaciones de anillos aromáticos con gran cantidad de sustituyentes alifáticos. Atendiendo a su origen, son moléculas procedentes de la degradación de biomoléculas, principalmente

proteínas, vitaminas... Este hecho hace que compartan con estas moléculas muchos grupos funcionales similares, y, por tanto, propiedades similares. Además, su tamaño es también similar al de las proteínas. El factor que interesa en relación a la aplicación de la IMAC a hidrolizados es el hecho de que proteínas y ácidos húmicos posean grupos funcionales, y, por ende, cargas similares, puesto que también serán susceptibles de coordinarse con el cobre de la resina. Este fenómeno pudo ser observado en los experimentos.

Así, al añadir el hidrolizado de lodo a la resina preparada, se observó un cambio inmediato en el color de éste, desde el marrón anaranjado inicial hacia el verde azulado, indicando así que el cobre fue eluído de la resina.

Se intentó evitar esto tamponando el hidrolizado a pH 7, ya que la forma de liberar los cationes de cobre de la matriz de la resina es a pH ácidos. En este caso, sin embargo, la liberación del cobre también fue inmediata y la coloración aún más intensa. Se cree que esto es debido a la interacción de los ácidos húmicos con el cobre, ya que hay autores que indican que la capacidad de los ácidos húmicos de retener cobre es mayor a pH cercanos a 6 (Vidali et al., 2010).

Ya que en algunos experimentos se vio que la liberación del cobre no se apreciaba hasta pasados unos 5 minutos, se llevaron a cabo una serie de experimentos cortos, de 2 ó 3 minutos. A pesar de no observar un cambio de coloración significativo, los datos obtenidos reflejaron que ya se había liberado algo de cobre al medio, pues se volvieron a obtener porcentajes negativos para los ácidos húmicos, es decir, que en la disolución habría más ácidos húmicos que en el hidrolizado de partida, lo cual es imposible (recordar que el cobre es una interferencia en la determinación de ácido húmicos; ver apartado 3.6.1.).

4.1.3.2. Discusión

Tratando de buscar una explicación al hecho de que el cobre unido a la resina se liberase al medio, se realizó una revisión bibliográfica.

Se encontró que la unión de cobre a ácidos húmicos va aumentando a medida que lo hace el pH. Esto explica que en los hidrolizados tamponados a pH 7 la coloración azulada fuese mayor (Vidali et al., 2010). Este artículo indica además la mayor capacidad de retención de cobre por ácidos húmicos fotooxidados, que serán similares a los que podríamos obtener en el reactor. La combinación de los dos hechos (pH y oxidación) es,

presumiblemente, la causa de no poder aplicar IMAC a la separación de una mezcla de proteínas y ácidos húmicos que ha sido hidrolizada y/o oxidada.

Más investigadores indicaron que, a menor tamaño de ácido húmico, mayor es la unión a cationes metálicos (por ejemplo, Cu^{2+}), ya que la proporción de grupos fenólicos y carboxílicos es mayor (de Melo et al., 2016). En lo relativo a los experimentos realizados, al hacer la OH de los ácidos húmicos presentes en el lodo, se reduce su tamaño y aumentan los grupos carboxílicos y, por tanto, su interacción con el cobre.

En cuanto al hecho de separar proteínas de ácidos húmicos, independientemente del método utilizado, es una tarea compleja y muy difícil, pues hay autores que indican que ambas moléculas son capaces de formar agregados heterogéneos, debido a su naturaleza anfifílica (Tan et al., 2008).

Por otra parte, la liberación de cobre desde la resina hacia el hidrolizado afecta también al método de medida. El método de Lowry modificado, descrito previamente en el apartado 3.6.1. se ve interferido por la presencia de cobre en disolución, que provoca una sobreestimación en la absorbancia ciega (ver ec. 4), lo que lleva a obtener medidas no válidas tanto para proteínas como para ácidos húmicos.

Con el fin de subsanar los errores que se obtienen con el método de Lowry modificado, se buscaron otros métodos de medida. Sin embargo, la coloración típica de los ácidos húmicos altera los resultados del método de Bradford (Bradford, 1976), y su absorbancia a 280 nm impide la determinación de la concentración de proteínas a esta longitud de onda, que es otra de las técnicas más habituales de medida de proteína (Stoscheck, 1990).

4.2. TRATAMIENTOS HIDROTÉRMICOS DE LODOS. RECUPERACIÓN DE PROTEÍNAS

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Estado: Publicada
Índice de impacto: 5,448
Índice de impacto de los últimos 5 años: 5,997

En el apartado 4.1.1. se habló sobre el trabajo previo que condujo a la publicación de un artículo en el que se comparan los efectos de una atmósfera oxidante frente a los de una no oxidante durante los tratamientos hidrotérmicos de lodos. Dicho artículo permitió obtener información sobre la solubilización de los distintos biopolímeros. En base a esta información, es posible determinar un tiempo óptimo de tratamiento para obtener la mayor cantidad posible de proteína soluble, la molécula de mayor interés.

Una vez determinado este tiempo, es necesario afrontar la separación y recuperación de proteínas del hidrolizado, ya que forman parte de una mezcla heterogénea en la que también están presentes otras biomoléculas. Con este fin, se aplicaron las técnicas de separación descritas en el apartado 3.5., evaluando la cantidad de proteína precipitada y las selectividades alcanzadas con cada separación.

En resumen, el objetivo del presente capítulo es estudiar la revalorización de lodos mediante la recuperación de las proteínas solubles presentes en los hidrolizados obtenidos tras la aplicación de tratamientos hidrotérmicos.

Protein recovery from solubilized sludge by hydrothermal treatments

*José L. Urrea[a], Manuel García[a], Sergio Collado[a], Paula Oulego[a], Mario Díaz[a]**

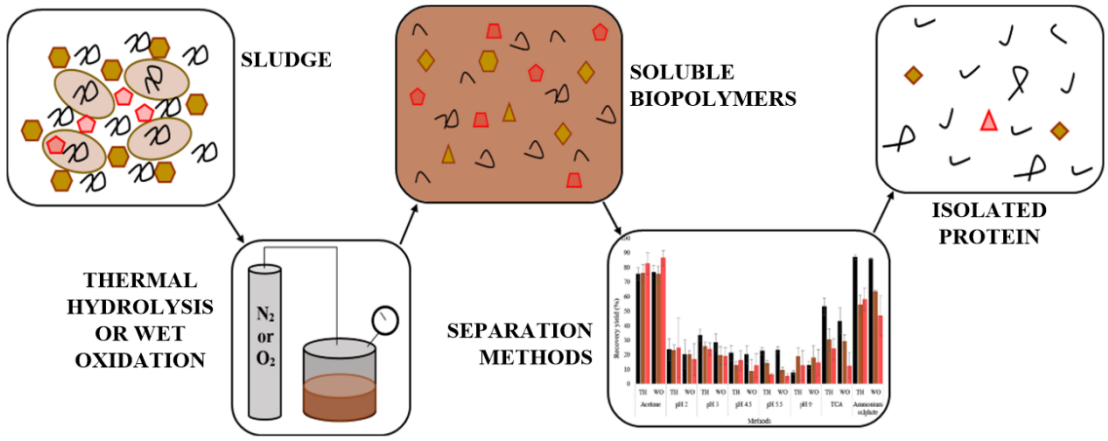
[a] Department of Chemical and Environmental Engineering. University of Oviedo.

C/ Julián Clavería s/n. E-33071 Oviedo, Spain. Tel.:34985103439; Fax: 34985103434

* Mario Díaz. Department of Chemical and Environmental Engineering. University of Oviedo.

C/ Julián Clavería s/n. E-33071 Oviedo, Spain. Tel.: +34 985 10 34 39; Fax: +34 985 10 34 34 mariodiaz@uniovi.es

GRAPHICAL ABSTRACT



HIGHLIGHTS

WO and TH improve sludge management by recovering valuable compounds.

Optimal treatment time was determined around 87 minutes of experiment for WO and TH.

Products from thermal treatments were characterised in terms of composition and molecular size.

WO solubilise proteins up to 0.291 g/gVSSo and TH does it up to 0.272 g/gVSSo for TH.

Protein recovery is possible adding $(NH_4)_2SO_4$, although it showed poor selectivity.

ABSTRACT

New alternatives for sludge management have been developed in recent years, with hydrothermal treatments being one of the most attractive ones. Even though many studies have been made on the application of hydrothermal treatments as pre-treatment or end-line technologies for sludge stabilisation and/or minimization, there is a lack of knowledge about the products generated during the process and its characteristics. This information is a crucial step for the assessment of the recovery of valuable products of the sludge, mainly proteins, humic acids and carbohydrates, which can considerably improve the economic balance of the hydrothermal treatment.

This work assesses, for the first time, the potential of hydrothermally hydrolysed sludge as renewable source for proteins recovery. For this purpose, firstly, the concentrations and properties of the main soluble biopolymers generated during the hydrothermal treatment, either in presence (wet oxidation, WO) or absence (thermal hydrolysis, TH) of oxygen, were measured, determining the reaction time necessary for a maximum solubilisation. Peak concentrations of 7.7 g/l (0.291 g/gVSSo) of proteins for WO and 7.2 g/l (0.272 g/gVSSo) for TH, were achieved at 87 minutes of experiment.

Afterwards, different separation methods, usually applied at industrial scale, were assessed for the separation of protein from the hydrolysed sludge, in terms of protein recovery and selectivity. Ammonium sulphate addition was found to be the best separation method, achieving 87% and 86% of protein recovery for TH and WO samples respectively, and the highest selectivity. Although further studies are required in order to achieve complete protein purification, a new perspective in sludge management is now open, by recovering valuable compounds.

KEYWORDS: activated sludge, thermal hydrolysis, wet oxidation, biopolymers, protein recovery

1. Introduction

Every day, huge amounts of sludge are being generated in municipal wastewater treatment plants (WWTP). For instance, in the EU, more than 10 million tons of dry solids of sewage sludge were produced in 2008 (Comission, 2008). Traditionally, sludge has been majority recycled as fertilizer in agriculture, or has been disposal in landfill. However, sludge landfill disposal is highly regulated due to its hazardous characteristics, such as pathogens presence and toxic compounds that can be generated when the sludge is disposed in landfills. Most countries are focused in the recycling of the sludge, for example, using it to produce energy by incineration or anaerobic digestion (Kacprzak et al., 2017). Either way, the management of this sludge represents a big problem, due to their high organic load and low dewaterability (Neyens and Baeyens, 2003). Sludge dewatering by physical operations such as flotation, thickening, filtration or sedimentation are extended options, but they are too expensive (almost 50% of the total disposal cost) (Neyens et al. 2004) and the required operations are difficult since they depend on the sludge characteristics such as structure, which, in turn, is affected by its composition (Dursun, and Dentel, 2009; Tsang and Vesilind, 1990). As mentioned before, anaerobic digestion processes are the most common solution in the sludge management. These techniques have the methane generation as main advantage, which considerably improves the economic balance of the WWTP, not only by using methane itself, but selling the energy produced. However, anaerobic digestion is a very slow process, requiring well controlled conditions, which supposes its main disadvantage.

Nowadays, new pre-treatment methods are being studied in order to speed up this process (Carrère et al. 2010), highlighting among others the hydrothermal techniques (Abe et al. 2013), either thermal hydrolysis (TH) or wet oxidation (WO). Both require high temperatures, usually over 200 ºC, and high pressures, usually over 60 bar. The difference between them is that an oxidant agent is needed in WO, while it is not required for TH, so the final effluent characteristics and products will differ. WO allows the solubilisation and partial oxidation of the sludge, whereas there is no degradation, only solubilisation in TH, due to the absence of any oxidant. This fact means that TH is a good pre-treatment for anaerobic digestion, improving its methane yield up to 50% (Haug et al. 1978). Either TH or WO seem to be promising methods for sludge management, but they are in the optimization period yet, as can be observed in the existing bibliography.

In fact, most of the studies related to these techniques are focused on the effect of the operation conditions on solubilisation or mineralization degrees and methane yields (Hii et al. 2014; Hii et al. 2013). Nevertheless, there is a lack of knowledge about the mechanisms involved and deeper characterizations of the products obtained than COD or TOC measurements are not available.

For example, it is well known that hydrothermal treatments break cells, thus releasing intracellular compounds, mainly proteins, humic acids and carbohydrates. Nevertheless, the characterization and separation of these biopolymers is a topic scarcely studied (Urrea et al. 2016), even when it could provide valuable information to the development of the hydrothermal treatments by two main reasons. Firstly, a deep knowledge of the biopolymers formed during either the wet oxidation or thermal hydrolysis is key for identifying improvements in the subsequent treatment. Knowing these mechanisms and products is necessary, because they condition the design of the following operations, such as pumping, anaerobic digestion or membrane operations (Judd, 2010). This is due to the effect of the soluble biopolymers, mainly proteins, on the main physicochemical properties of the effluent, such as viscosity, biodegradability, settleability or dewaterability (Hoa et al. 2003; Ruiz-Hernando et al. 2015; Wang et al. 2014, Wang et al. 2009; Zhang et al. 2015). At the same time, information about the separation and purification of these products, as well as reducing the effluent organic load, also brings a different perspective in the sludge management, considering it as a new renewable source for not only energy, but also for resources recovery (Suárez-Iglesias et al. 2017; Tyagi and Lo, 2013). This fact could be the key point in the implementation of hydrothermal technologies for sludge management at full scale, due to its high costs. As previously explained, products obtained by hydrothermal treatments include proteins, humic acids and carbohydrates, which have high commercial value if can be isolated from the hydrolysed sludge stream. For example, predominant biopolymers, proteins, can be used as fertilizers, adhesives or animal feed (Hwang et al. 2008) and their separation considerably reduces the nitrogen content of the effluent. Humic acids, which usually exhibit a low biodegradability, thus reducing the methane yield of a subsequent anaerobic digestion, have already been used as biosurfactants and fertilizers (Salati et al. 2011). Finally, carbohydrates from sludge are a cheap substrate for fermentations (Tekin et al. 2014). Proteins generate more interest, owing to their high

proportion in the sludge, which is a 61% (Chen et al. 2007). Hence, isolation and revaluing of these molecules should be studied firstly.

Taking into account the previous information, the aim of this work was to study and characterise the hydrothermal techniques (TH and WO), either as sludge management process or as revaluing treatments by soluble proteins recovery. In order to carry it out, this includes the analysis of the physical-chemical parameters of the sludge as well as the characterisation of the products generated and their purification by the application of several isolation methods.

2. Material and methods

2.1 Sludge characterisation

The experiments were performed using a secondary sludge thickened by flotation in a municipal WWTP (Asturias, Spain) with the characteristics showed in Table 1.

Table 1. Main parameters of the sludge used in the experiments. (*) indicates soluble concentrations.

Parameter	*Units*	*Value*
pH		6.54
TCOD	mg 0_2/l	37200
SCOD	mg 0_2/l	210
TSS	g/l	31.92
VSS	g/l	26.47
SVI	ml/g	31
Proteins*	mg/l	181
Humic acids*	mg/l	281
Carbohydrates*	mg/l	82
Uronic acids*	mg/l	22.46
DNA*	mg/l	18.1
TOC*	mg C/l	373.20
IC*	mg C/l	126.80

2.2 Analytical methods

Total suspended solids (TSS), volatile suspended solids (VSS), fixed suspended solids (FSS), total and soluble chemical oxygen demand (TCOD and SCOD), sludge

volume index (SVI) and pH measurements were carried out according to Standard Methods (APHA, 1998).

TOC and IC values were measured by means of a TOC analyser (Shimadzu TOC-VCSH, Japan). SCOD concentrations were determined by closed reflux, colorimetric method, using a DR2500 spectrophotometer (Hach Company, USA).

Protein and humic acid concentrations were simultaneously measured using the Lowry modified method (Lowry et al. 1951, Frølund et al. 1996) with BSA (Bovine Serum Albumin) and humic acid as standards, respectively. Carbohydrate concentration was estimated by the Dubois method (Dubois et al. 1956) using D-glucose as standard. DNA concentrations were measured according to Burton (1956) by using calf-thymus DNA as standard. Uronic acid concentration was measured using the method of Blumenkrantz (Blumenkrantz and Hasboe-Hansen, 1973) with glucuronic acid as standard. All these methods are spectrophotometric, and absorbances were measured using a UV/vis spectrophotometer (Thermo Scientific, Helios γ).

Soluble biopolymers molecular sizes were determined by HPLC (Agilent 1200, Agilent Technologies Inc., California, USA), employing a Yarra SEC-2000 (300 x 7.8 mm) column. The column preparation and operational terms were the same as in Urrea et al. (2016), but in the present study, a wavelength of 280 nm was selected. For the sake of having a better understanding of the molecular size evolution, the fingerprint area was divided into four regions. The first three ones were located in the zone of size exclusion, corresponding to low (0–35 kDa or 10.02 min to 11.8 min), medium (35–150 kDa or 7.6 min to 10.02 min) and high (>150 kDa or 5.45 min to 7.6 min) molecular weights. The last one was established in the final area of the column total volume (11.8 min). The peaks with a retention time higher than the one relating to the total column volume have been associated to molecules that interact with the filling material, being commonly observed this phenomenon with either extracellular or intracellular polymeric substances from sludge (Frølund et al. 1996, Urrea et al. 2016). Görner et al. (2003) reported that some of those peaks were retained for longer times due to hydrophobic interactions. Therefore, these facts suggest that the polymers which were effectively separated according to their size, that is to say, those eluted before of 11.8 min, had more hydrophilic characteristics.

In order to have a simpler view of the size distribution of the molecules and its hydrophilic or hydrophobic characteristic, the percentage of area for each size and the hydrophobic zone were calculated in relation to the total area of the analysis.

2.3 Biopolymers solubilisation

Thermal hydrolysis and wet oxidation experiments were carried out using a PARR 4520 (Parr Instrument Company, Illinois, USA) series reactor of one litre capacity, equipped with a one-propeller stirrer and a gas humidifier steel tank of two litres filled with water. The experiment parameters, such as gas flow, humidifier and reactor temperature, were handled by a proportional integral differential controller. The pressure was adjusted through a backpressure controller located at the end of the gas line. For security purposes, both the reactor and the humidifier were filled to 70% of their total capacities. A more detailed description of the experimental setup can be found in Urrea et al. (2016).

Both TH and WO experiments were performed at 160 ºC and 40 bar, in presence of nitrogen or oxygen respectively. This conditions were chosen in order to get a balance between sludge solubilisation and biomolecule recovering, because at higher temperatures the formation of refractory compounds and the destruction of the soluble biopolymers begin to be significant (Neyens and Baeyens, 2003). The gas flow was 1200 ml/min either for nitrogen or oxygen and the stirrer speed was adjusted to 500 rpm.

A total of eight samples were taken from the reactor either for TH or WO experiments. The first one was withdrawn when the temperature of the reactor reached a value of 100 ºC. The next sample was collected for a temperature of 160 ºC and the following were periodically taken until the end of the reaction (120 minutes after reaching reaction conditions).

2.4 Separation procedures

Although there are large numbers of different biomolecules existing in the sludge, only three have enough quantity to generate interest: proteins, humic acids and carbohydrates. Proteins are the most common biomolecule, so we focused the separation procedures on isolating them. For this reason, separation procedures were applied to those samples obtained by either wet oxidation or thermal treatment, after the reaction time which ensured the maximum concentration of these biomolecules (20 minutes after reaching reaction conditions).

Although there are several methods to isolate proteins, the methods here applied were selected according to its simplicity and/or low cost. Taking this into account, the following four methods, several of them used at industrial scale, were tested.

2.4.1 pH adjustment

The pH value was adjusted to five different values: 2, 3, 4.5, 5.5 and 9, by adding 2M H_2SO_4 or 1M NaOH. This values agree with those found in bibliography (Pervaiz and Sain, 2011), being pH=2 and pH=9 added by us, because pH=2 is the isoelectric point of humic acids, and pH=9 is a good indicator of alkaline conditions, where no precipitation of any biopolymer is expected. Once the pH value was reached, it was kept in stirring for 15 minutes at 350 rpm and then centrifuged at 10000 rpm during 10 minutes, at 4 ºC.

2.4.2 Acetone precipitation

Acetone (Sigma-Aldrich, 34850 M) at -20 ºC was added at a 3:1 ratio to the sample, being stored in a cooler at -18 ºC for two hours to achieve precipitation. Afterwards, it was centrifuged at 10000 rpm during 10 minutes, at 4 ºC (modified from Jiang et al. 2014).

2.4.3 Trichloroacetic acid precipitation

Trichloroacetic acid (TCA) was found to be a good precipitation agent (Oliveira et al. 1999). It was added in order to obtain a 20% TCA (Sigma-Aldrich, T6399) in the sample, keeping it in ice for 30 minutes to precipitate the proteins. The mixture was centrifuged at 10000 rpm during 10 minutes, at 4 ºC.

2.4.4 Ammonium sulphate precipitation

This method is based in the *salting out* phenomenon, so a near saturation concentration of ammonium sulphate, 0.6 g/ml (saturation point is 0.764 g/ml (Haynes, 2014)), was required. Once added, the sample was centrifuged at 10000 rpm during 10 minutes, at 4 ºC.

All precipitation tests were performed in triplicate, so the results showed in the figures are mean values.

Biopolymers concentrations after the precipitation methods were only measured in the supernatant, so the amount precipitated results from the supernatant concentrations before and after the separation procedure being applied.

2.4.5 Recovery and selectivity

The recovery of the biopolymers, as percentage precipitated for each precipitation procedure and treatment, was expressed according to equation (1).

$$\%_{precipitated} = \left(1 - \frac{supernatant\ concentration\ after\ precicipitation}{supernatant\ concentration\ before\ precipitation}\right) x100 \qquad (1)$$

Due to the fact that there are many different molecules present in the hydrolysed sludge, it is a high likelihood that these molecules precipitate together with the proteins when applying the precipitation methods. Thus, the percentage precipitated for each method do not show how well the method isolated the proteins against humic acids and carbohydrates. In order to quantify the selective precipitation of each method, the next selectivity factors were defined from equations 2 and 3 as follows:

$$\alpha_{p/h.a.} = \frac{\left(\frac{(C_p)_{before\ precipitation} - (C_p)_{supernatant\ after\ precipitation}}{(C_{h.a.})_{before\ precipitation} - (C_{h.a.})_{supernatant\ after\ precipitation}}\right)}{\frac{(C_p)_{supernatant\ after\ precipitation}}{(C_{h.a.})_{supernatant\ after\ precipitation}}} \qquad (2)$$

$$\alpha_{p/c} = \frac{\left(\frac{(C_p)_{before\ precipitation} - (C_p)_{supernatant\ after\ precipitation}}{(C_c)_{before\ precipitation} - (C_c)_{supernatant\ after\ precipitation}}\right)}{\frac{(C_p)_{supernatant\ after\ precipitation}}{(C_c)_{supernatant\ after\ precipitation}}} \qquad (3)$$

where α is the selectivity factor for proteins against humic acids ($\alpha_{p/h.a.}$) or carbohydrates ($\alpha_{p/c}$), c_p is protein concentration, $c_{h.a.}$ refers to humic acid concentration and c_c is carbohydrate concentration. When applying equations 2 and 3 in order to calculate α, three possible results could be obtained: $\alpha = 1$, $\alpha > 1$ and $\alpha < 1$. In the first case, there is no separation, because the relation between concentrations of the studied molecules are the same in the supernatant after applying the separation method and in the precipitate, measured as the difference between initial concentration and the concentration in the supernatant after applying the separation method. This means that precipitation has not been selective. When $\alpha > 1$, there is a good separation, since proteins have precipitated in a higher proportion than the other molecules; obviously, the higher the selectivity factor the better the separation was. An $\alpha < 1$ also indicates a good separation, but, in this case, proteins will remain in the liquid, due to a higher precipitation of the other component. In conclusion, the best separation methods are those with α values much higher than 1 or much lower than 1.

3. Results and discussion

3.1 Sludge disintegration and solubilisation yield of biopolymers

The effect of wet oxidation and thermal hydrolysis on TCOD, SCOD and VSS from sludge (Figure 1) has profusely been studied in the bibliography (Baroutian et al. 2015; Urrea et al. 2015, 2014), so these results will be here briefly commented. As expected, both treatments were effective in producing the solubilisation of sludge. However, wet oxidation showed a better yield, achieving higher rates and final degrees of sludge disintegration than thermal hydrolysis, particularly from minute 45 to 87 of treatment. In fact, the VSS concentration decreased by 57% after 77 minutes of wet oxidation, being this value 35% for thermal hydrolysis for the same time. These differences of solubilisation are due to the formation and attack of hydroxyls radicals during wet oxidation, which favoured a higher level of sludge disintegration. These radicals are also responsible for the partial oxidation of the solubilized matter, thus having the dual effect of increasing the soluble COD by a more effective disintegration of the sludge but, at the same time, also decreasing it due to oxidation reactions in the liquid phase. These oxidation reactions in liquid phase explain why TCOD is reduced at the end of treatment to 62% of its initial value, whereas this parameter remained constant for thermal hydrolysis.

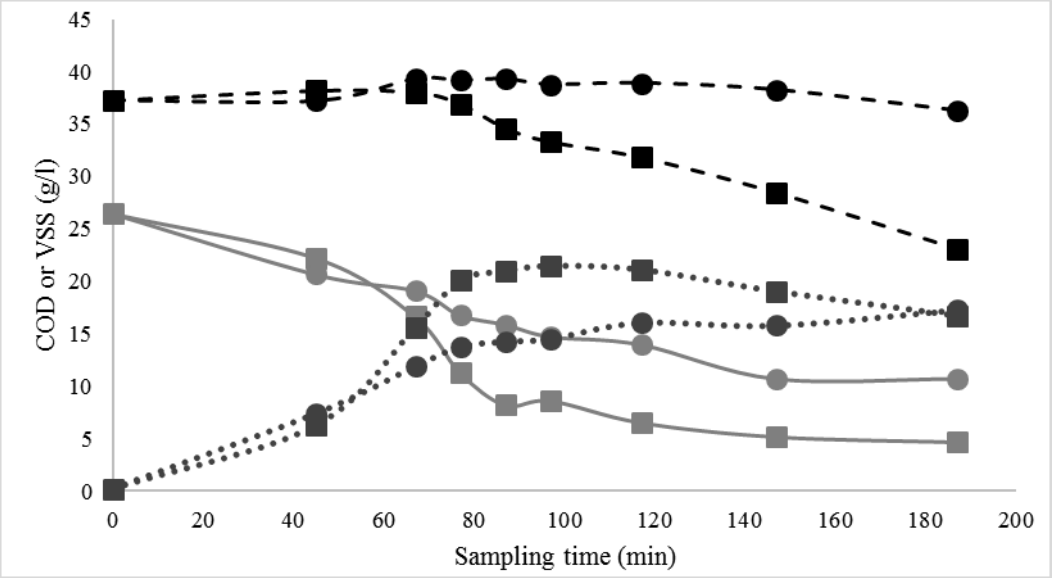


Figure 1. Comparison between TH (●) and WO (■) effects on TCOD (— — —), SCOD (· · ·) and VSS (——). In all cases, 160 ºC and 40 bar.

Nevertheless, the solubilisation yields for specific biopolymers caused by thermal treatments is an issue not studied in depth. Figures 2 a, b, c, d and e show the evolution

of the five main biopolymer families detected (carbohydrates, proteins, humic acids, nucleic and uronic acids) during a thermal treatment at 160 ºC and 40 bar either in absence or presence of an oxidant atmosphere. For both treatments, results reveal a high degree of biopolymers solubilisation, mainly proteins, followed by humic acids and carbohydrates. In fact, proteins supposed around the 50% of the total soluble biopolymers. Again, the employment of an oxidant atmosphere involves a faster and more intense solubilisation of biopolymers, achieving a maximum soluble protein concentration of 7.7 g/l (that is, 0.291 g protein/gVSSo) after 87 minutes of reaction. For the same reaction time, the soluble proteins concentration for thermal hydrolysis was 7.2 g/l (0.272 g protein/gVSSo), thus being a 7% lower.

In a similar way, the peak concentrations in wet oxidation for humic acids and carbohydrates were 4.3 g/l (0.164 g/gVSSo) and 3.7 g/l (0.141 g/gVSSo), and 3.8 g/l (0.146 g/gVSSo) and 2.8 g/l (0.106 g/gVSSo) for thermal hydrolysis. As expected, oxidation reactions provoke a reduction in all soluble biopolymers concentrations during the second half of the reaction, when solubilisation rate decay due to the depletion of organic solids. This effect is particularly strong for soluble proteins, whose concentration is almost halved during the last 100 minutes of reaction. Nevertheless, thermal hydrolysis, where oxidation reactions are negligible, involves a progressive increase in the concentrations of all polymers, except for proteins, whose concentration initially increases, achieving a maximum in 87th minute and then decreasing. This final reduction in the soluble proteins content, although less intense than for wet oxidation, is significant, with an 18% reduction during the last 100 minutes, probably due to the hydrolysis of the peptide bonds. In the same way as protein concentration, DNA concentration decreases in the second half of the TH experiment. This decrease is probably due to the hydrolysis reactions that occur in the reactor, which lead to DNA polymer breakage and degradation. Therefore, before recovering proteins from the treated medium, it is essential to determine the optimum reaction time in which their concentration will be maximum. This concentration was found at 87 minutes of treatment either for TH or WO.

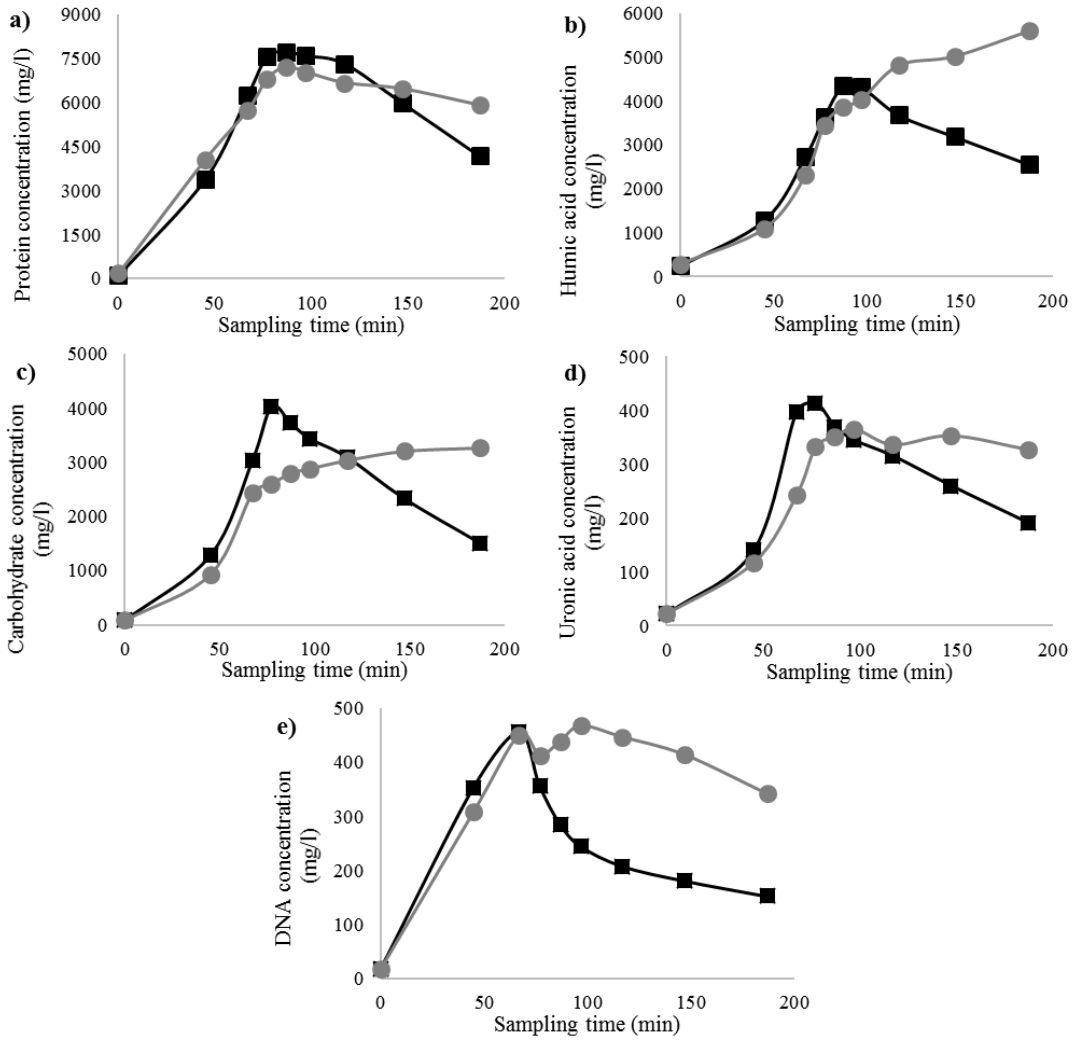


Figure 2. Comparison of the concentration of the five biopolymers detected during TH (●) and WO (■). In all cases, 160 ºC and 40 bar.

3.2 Size distribution of solubilised biopolymers (fingerprints)

In the previous section, the effects of either wet oxidation or thermal hydrolysis on the total amount of soluble biopolymers have been discussed in detail. However, it is also necessary to determine the effects on their size during the corresponding treatment. Figure 3a and 3b show the fingerprints evolution, obtained by size exclusion chromatography, of the supernatants obtained along the experiment of thermal hydrolysis or wet oxidation, respectively. To the best of our knowledge, there are not previous studies comparing the fingerprints obtained by wet oxidation and thermal hydrolysis.

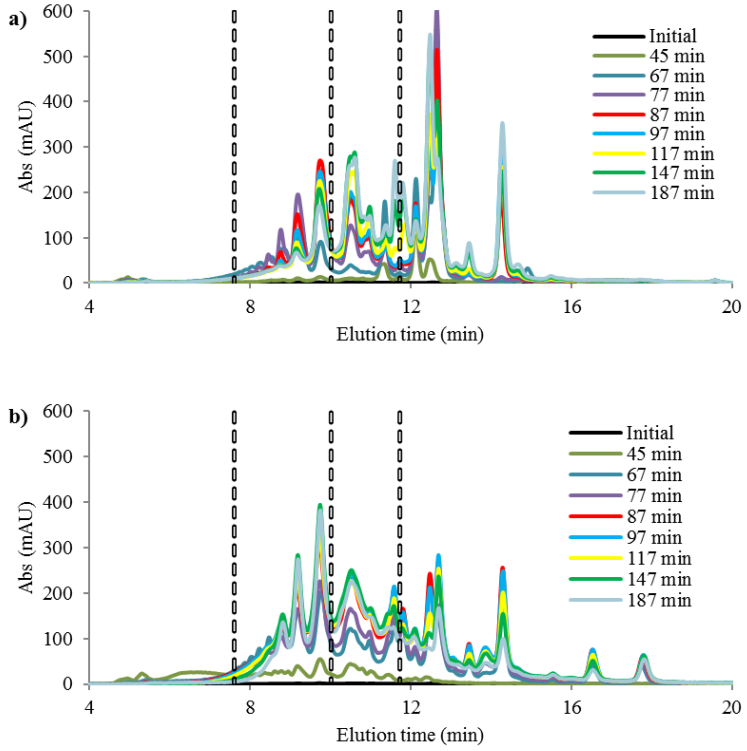


Figure 3. Evolution of the chromatograms for TH (a) and WO (b).

Figure 4 collects the evolution of the fingerprints areas for low, medium and high size polymers, as well as hydrophobic polymers, for thermal hydrolysis and wet oxidation, respectively.

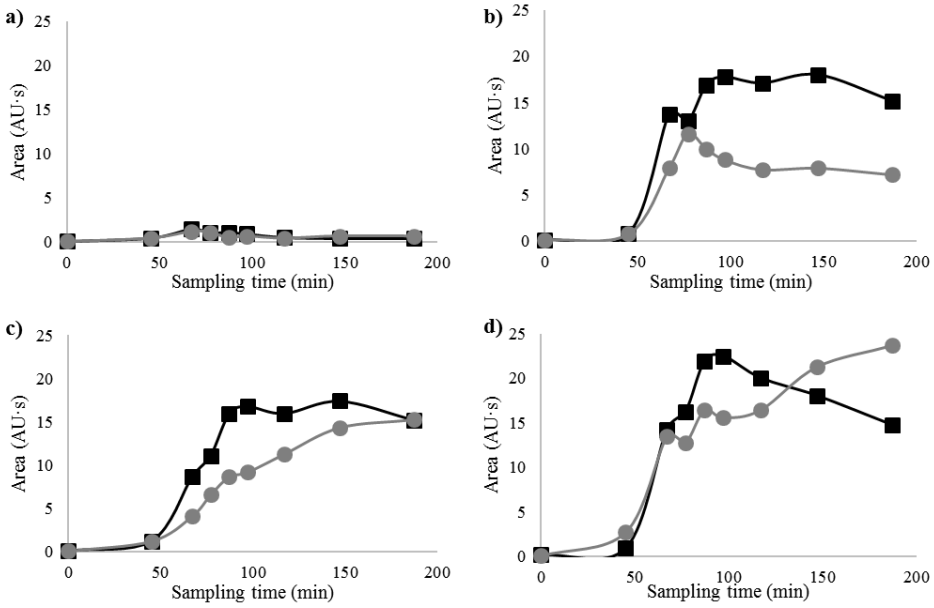


Figure 4. Evolution of the different size of the polymers: a) higher than 150 kDa, b) from 35 kDa to 150 kDa, c) smaller than 35 kDa, d) after total column volume; for TH (●) and WO (■). In all cases, 160 ºC and 40 bar**.**

As expected, the increase in the area corresponding to high size polymers occurred during the first minutes of both treatments (67 min). From this time, these areas decreased, which means that such polymers were broken by hydrolysis or oxidation reactions to form others of lower size. In fact, it must also be pointed out that the area corresponding to these polymers (> 150 kDa) is the smallest of all and their evolution is very similar either in presence of absence of an oxidant atmosphere. These results suggest a fast shortening of high size polymers (<150 kDa) by hydrolysis.

Nevertheless, the behaviour for the rest of categories depended on the type of treatment.

The medium size polymers (35-150 kDa) also initially increased during thermal hydrolysis, reaching a maximum in minute 77, and then decreased, probably due to the hydrolysis of these polymers. This hydrolysis rate is slowing down, until the medium size polymers concentration achieves a constant value (7 AU·s) after two hours of reaction. However, wet oxidation provoked a continuous increase of these polymers until an area of 17 AU·s, this remaining constant from the 87th minute. Regarding low size polymers, both treatments caused a continuous increase in their concentration up to an area of around 15 AU·s. However, this increase was more marked for wet oxidation than for thermal hydrolysis. This behaviour seems to be reasonable, taking into account that an oxidant atmosphere involves a faster breakage towards smaller size polymers.

Finally, the areas corresponding to hydrophobic polymers progressively increased in the case of the thermal hydrolysis, whereas they showed a final reduction when sludge was treated by wet oxidation. The results revealed that the presence of an oxidant atmosphere has two effects of opposite sign on the hydrophobic polymers. On one hand, wet oxidation involved a faster formation of these polymers, probably due to the more aggressive attack of the free radicals to the components of the cell membrane. On the other hand, oxidation reactions also reduce the hydrophobic effect due either to the breakage of these polymers and to the reduction in the hydrophobic character of the polymer, caused by side oxidation reactions of the polymers without breakage. These reactions are also able to generate organic acids, such as acetic acid, propionic acid or iso-butyric acid as well as low molecular weight alcohols, for example, methanol (Baroutian et al. 2016; Baroutian et al. 2015). These compounds are hydrophilic due to their carboxylic group and their low molecular weight. Owing to a smaller value of temperature in our experiment than that found in bibliography, these organic compounds

probably appear at the second half of the experiment and the reaction conditions are not capable to degrade them, as shown in the figure. The behaviour shown in the figure could be also related with the biomolecules present in the sludge. Proteins had been related with higher levels of hydrophobicity (Liu and Fang, 2003), this means that, by the time that proteins are being broken, the hydrophobicity decreases. That can be corroborated by comparing figures 2a and 4d, since when protein concentration begins to decrease, the area of the hydrophobic molecules decreases as well.

According to the size categorization used, the areas corresponding to high, medium and small size hydrophilic polymers and hydrophobic polymers at the optimum time of solubilisation were of 0.5, 10, 9 and 16.4 AU·s for thermal hydrolysis and 1, 17, 16 and 22 AU·s for wet oxidation, respectively, as shown in Figure 5. These findings make evident the higher solubilisation capacity provided by wet oxidation, being its fingerprint area 57% higher than that for thermal hydrolysis. In addition to this, it was observed that the hydrophilic character prevailed in both fingerprints, these polymers being the 61 and 54% of their total area for wet oxidation and thermal hydrolysis, respectively. The higher hydrophilic character obtained with wet oxidation was probably due to the higher content of solubilised carbohydrates, achieved with this treatment (35 mg/gVSSo more) (Liu and Fang, 2003).

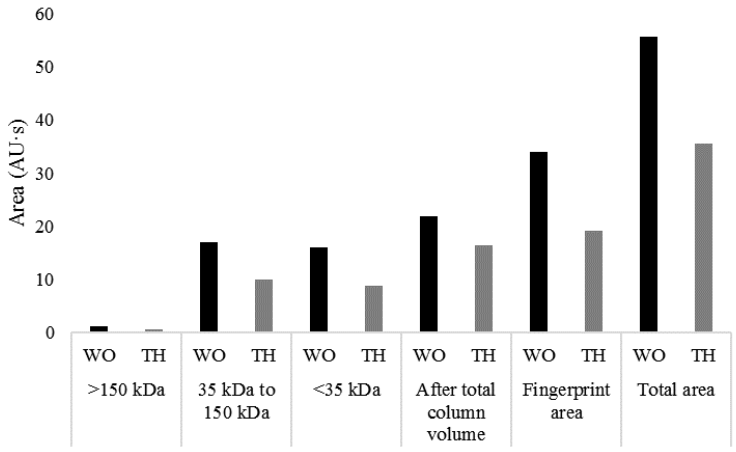


Figure 5. Areas for the different sizes, fingerprint area and total area of the optimal time for WO and TH. In all cases, 160 ºC and 40 bar.

3.3 Protein recovery

Once the products of the thermal treatments have been characterized in terms of compositions and sizes, several separation techniques were tested in order to determine their protein recoveries and selectivities. The separation techniques here used have

traditionally been applied to protein purification with success and they can easily be adapted to full scale. Again, as far as we know, there are no studies dealing with the separation or purification of specific products obtained after the corresponding hydrothermal treatment, neither wet oxidation nor thermal hydrolysis.

Figure 6 shows the recoveries, expressed as percentage of precipitated biopolymer, for proteins, humic acids and carbohydrates, for either wet oxidation or thermal hydrolysis supernatants obtained at the optimum time of solubilisation (87 min) with the different separation methods tested. Recoveries have been calculated according to the equation (1).

As can be observed, the methods that provided a higher protein precipitation were in this order: ammonium sulphate, acetone and trichloroacetic acid (TCA) with yields of 87% (± 1.55), 75% (± 4.19) and 53% (± 5.88) for thermal hydrolysis and of 86% (± 0.49), 77% (± 4.71) and 43% (± 9.17) for wet oxidation, respectively. The results obtained with TCA were not as positive as those reported by Oliveira et al. (1999) for an extracted polymeric matrix from a biofilm of *Pseudomonas fluorescens*. They observed the precipitation of more than 90% of the initial proteins, as well as a minimum effect of TCA on the precipitation of carbohydrates.

As it can be noted, the methods based on the protein precipitation due to pH modification were the least effective, with protein recoveries lower than 35% in all cases. The pH adjustment to 3 was the best option of them, with 33 and 28% of protein recovered from thermal hydrolysis and wet oxidation supernatants, respectively, whereas adjusting pH to 9 involved the lowest recoveries, with 8 and 13%, respectively. When Pervaiz and Sain, (2011) tested several acidic pH (1.5, 3, 4.5 and 5.5) as precipitation methods for a solubilised paper mill sludge by alkaline treatment (pH 12), they reported higher yield of protein recovery than the obtained in this work. However, they also achieved the highest protein recovery at pH 3 (92%). Likewise, Hwang et al. (2008) determined that the higher yield of protein (80%) was achieved at pH 3.3 after assessing different acidic pH (1, 3.3 and 5) with a solubilised sludge by alkali treatment followed by ultra-sonication. In contrast, Wei et al. (2016) recovered the bulk of the amount of protein at pH 1 from of a solubilised sludge by a coupled ultrasonic-acid treatment (1.5W ml^{-1} and pH 2).

The higher protein recoveries by acid precipitation reported in these studies were probably due to the differences between the methods employed for the sludge disintegration. While those works applied either an alkaline treatment or a coupled alkali-

ultrasonic treatment, in this work the sludge was solubilised by hydrothermal techniques. Therefore, although wet oxidation or thermal hydrolysis contributed to a higher degree of sludge solubilisation than the alkaline treatments, these techniques cause an irreversible denaturalisation of the proteins which affects their properties, and consequently, their ability to precipitate.

Finally, it should be taken into account that the comparison between the results here obtained and those published in other studies is difficult due to the different methods of analysis employed, particularly for protein determination (Le et al. 2016). Hwang et al. (2008) and Wei et al. (2016) employed the Lowry method but not considering the interferences caused by humic acids. Therefore, these studies overestimated the amount of proteins recovered. In addition to this, none of these authors determined the presence of carbohydrates on the recovered material.

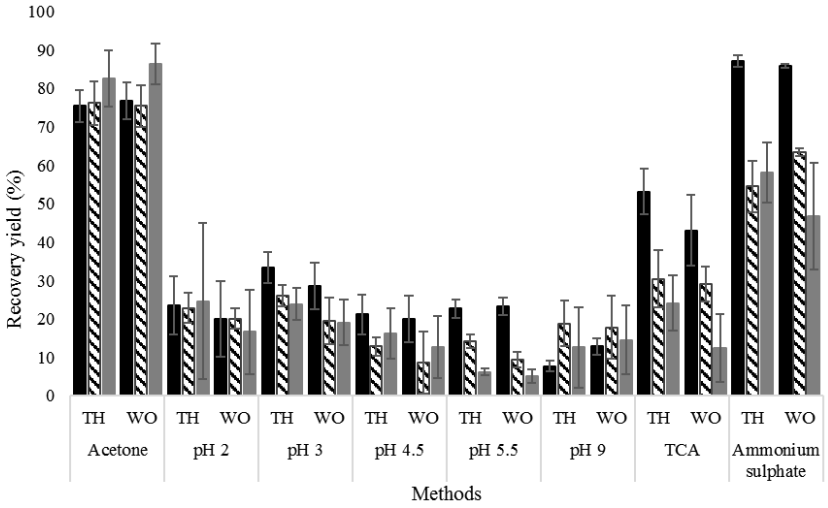


Figure 6. Percentage of precipitation for proteins (■), humic acids (▤) and carbohydrates (■) for each method and sample (TH or WO).

At this point, it is necessary to notice that all the methods tested in this study cause the precipitation of not only proteins, but also humic acids and carbohydrates.

The results of the selectivity factor (α) calculated for proteins against humic acids ($\alpha_{p/h.a.}$) or carbohydrates ($\alpha_{p/c}$) for each method of separation are showed in Figures 7a and 7b, respectively, according to the supernatant employed. For the sake of clarity, the resulting dispersion diagram was divided into four different areas, as shown in the figures.

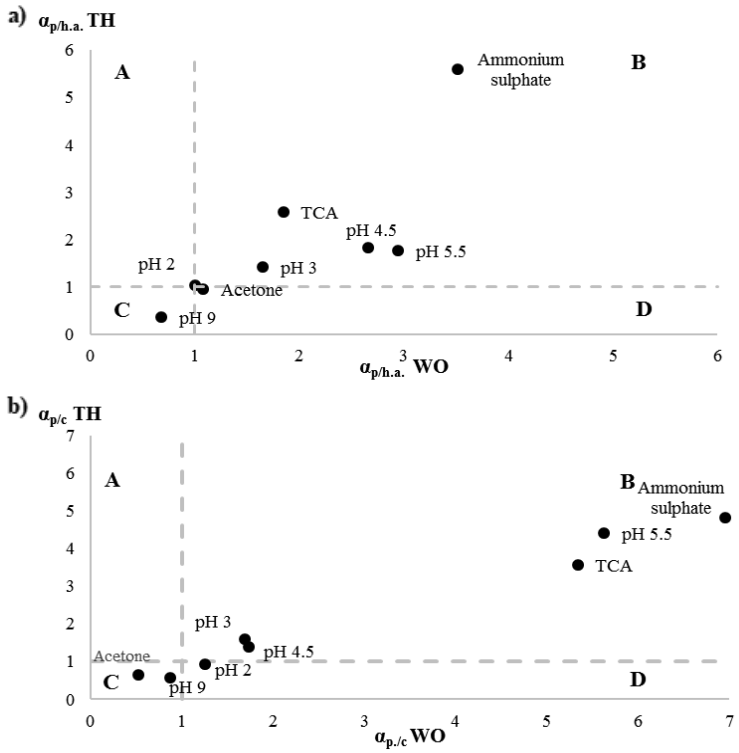


Figure 7a and 7b. Dispersion diagram analysing the different protein recovery areas in base of α value for proteins against humic acids (a) or proteins against carbohydrates (b).

The upper left quadrant (A) (α >1 for TH, α < 1 for WO), collects methods where proteins are concentrated in the precipitate for the TH sample, and in the supernatant for the WO sample. The upper right quadrant (B) (α >1 for TH, α >1 for WO) corresponds to methods where proteins are recovered in the precipitate either for TH or WO samples. In a similar way, methods with α values in the lower left quadrant (C) (α < 1 for TH, α < 1 for WO) show a protein recovery in the supernatant either for TH or WO treatments. Finally, methods in the lower right quadrant (D) (α < 1 for TH, α > 1 for WO) allow to recover proteins in the precipitate for samples of WO, and in the supernatant in the case of sludges treated by TH.

Therefore, the methods which cause the most selective protein precipitations against humic acids and carbohydrates were ammonium sulphate, pH 3, 4.5 and 5.5, and TCA, while pH 9 allows to recover proteins in the supernatant. pH 2 and acetone methods did not offer a good separation.

The selectivity for the proteins separation of carbohydrates or humic acids in function of the hydrothermal treatment employed can be checked in basis to the position

of α with respect to the diagonal Y=X, indicated as a red line in Figures 8a and 8b, and the α value itself. Again, the area of the dispersion diagram must be divided in four areas.

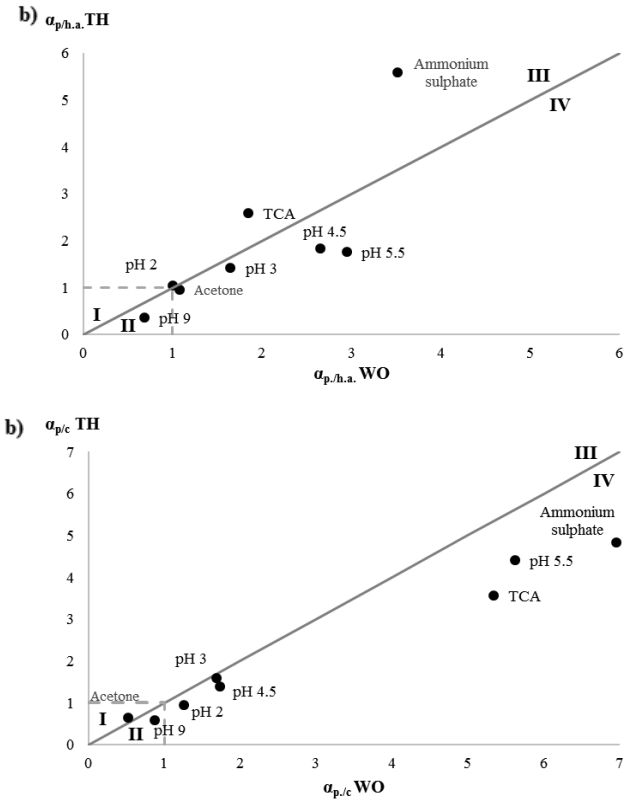


Figure 8a and 8b. Dispersion diagram comparing TH and WO treatments in base of α value for proteins against humic acids (a) or proteins against carbohydrates (b).

The areas I and IV corresponds to protein separation methods that worked better for WO samples than for TH ones, whereas the behaviour in the areas II and III is exactly the opposite, the protein separation is better in sludges treated by TH than in WO treatment.

Attending to the previous explanation, ammonium sulphate, TCA (α values were 2.59 for TH versus 1.84 for WO) and pH 9 (α value of 0.36 for TH against 0.68 for WO) worked better on the TH sample when separating protein and humic acids, while pH 3 (α values were 1.43 for TH versus 1.64 for WO), 4.5 (α values were 1.83 for TH against 2.65 for WO) and 5.5 (α values were 1.77 for TH against 2.95 for WO) worked better on the WO supernatant. No differences were found between TH and WO when acetone (α values were 0.96 for TH versus 1.08 for WO) and pH 2 (α values were 1.04 for TH against 1.00 for WO) were tested as precipitation methods.

In the case of proteins and carbohydrates separation, only pH 9 method was found to work better on the TH sample than on the WO one (α values were 0.59 for TH versus 0.87 for WO). Acetone (α values were 0.65 for TH against 0.52 for WO) and pH 3 (α

values were 1.59 for TH versus 1.69 for WO) showed no differences when applying them to TH or WO samples. All other methods showed better results on the WO sample.

In conclusion, the better separation method is ammonium sulphate, owing to its high selectivity factor either for TH or WO for proteins against humic acids (5.6 and 3.15 respectively) and for proteins against carbohydrates (4.82 and 6.95 respectively). Ammonium sulphate is not only the method with the highest selectivity factor, it is the method that provokes the major percentage of protein precipitation (87% and 85.9% for TH and WO respectively) as well.

4. Conclusions

Hydrothermal treatments, such as thermal hydrolysis and wet oxidation, have been proved as suitable techniques for sludge revalorization. Both treatments allowed to achieve high degrees of solubilisation, in terms of suspended solids, and organic load reduction as COD. Wet oxidation provided better values in this aspect than thermal hydrolysis.

Concerning the soluble biopolymers releasing, five main compounds were detected: proteins, humic acids, carbohydrates, uronic acids and DNA, being the three first biopolymers found to have the highest concentration. In the first half of the treatments, soluble biopolymers concentrations increase until their maximum value, obtaining this concentration higher in the presence of an oxidising atmosphere. In the second half, in wet oxidation treatment, oxidation reactions provoke a decrease in the concentrations, whereas in thermal hydrolysis, the concentrations remained constant to the final of the process. Taking into account the maximum in protein concentration obtained, an optimal treatment time was established, in order to recover them by further experiments. Size analysis demonstrated that wet oxidation provoked a higher and faster breakage of the biopolymers than thermal hydrolysis. Both treatments led to size reduction from big-size molecules to medium-size molecules and small-size molecules, with optimal reaction time selected being medium-size molecules at their higher amount.

Aiming to recover proteins from the supernatant obtained, several methods were tested. Although many of them led to good protein recoveries in terms of amount precipitated, they also provoked humic acid and carbohydrate precipitation. Therefore, taking only into account the protein percentage precipitation is not enough. With this purpose, a selectivity factor was calculated, showing that separation selectivities of the tested methods were not high enough to obtain pure protein from the effluent of the

hydrothermal treatments. Ammonium sulphate addition was found to have the highest selectivity and the highest protein recovery either for thermal hydrolysis or wet oxidation.

With this work, new ways of sludge management have been opened. Recovering added value biopolymers, such as proteins, humic acids or carbohydrates, allows to give a new output to a controversial and highly produced waste, generating adding value and eliminating it simultaneously.

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Appendix A for

‘Protein recovery from solubilized sludge by hydrothermal treatments’

*José L. Urrea[a], Manuel García[a], Sergio Collado[a], Paula Oulego[a], Mario Díaz[a,]**

[a]Department of Chemical and Environmental Engineering,
University of Oviedo, c/Julián Clavería s/n, E-33071, Oviedo, Spain

(3 Pages, 3 Figures)

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*Corresponding author’s e-mail: mariodiaz@uniovi.es

Phone: +34 985 10 34 39; Fax: +34 985 10 34 34

1. Comparison between protein precipitation percentages for each method depending on the hydrothermal treatment.

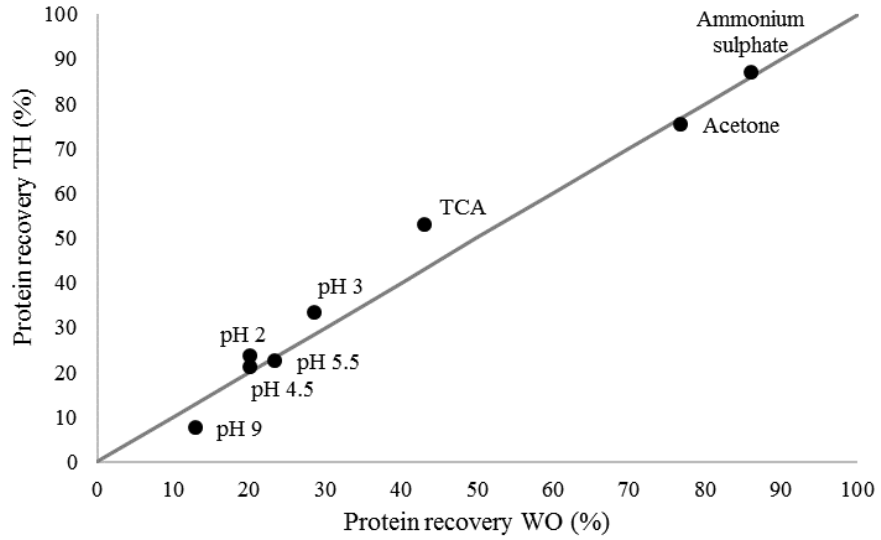

Figure A1. Comparison between protein precipitation percentages for each method depending on the hydrothermal treatment.

2. Comparison between humic acid precipitation percentages for each method depending on the hydrothermal treatment.

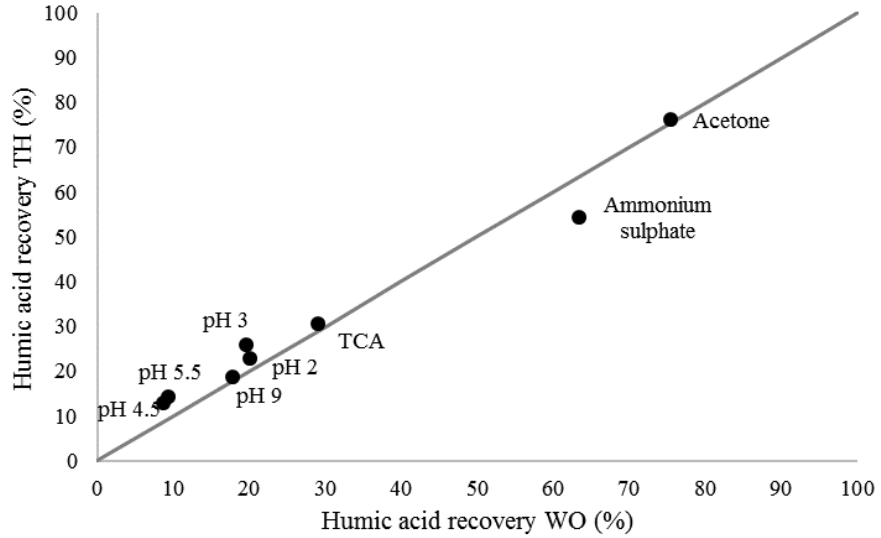

Figure A2. Comparison between humic acid precipitation percentages for each method depending on the hydrothermal treatment.

3. Comparison between carbohydrate precipitation percentages for each method depending on the hydrothermal treatment.

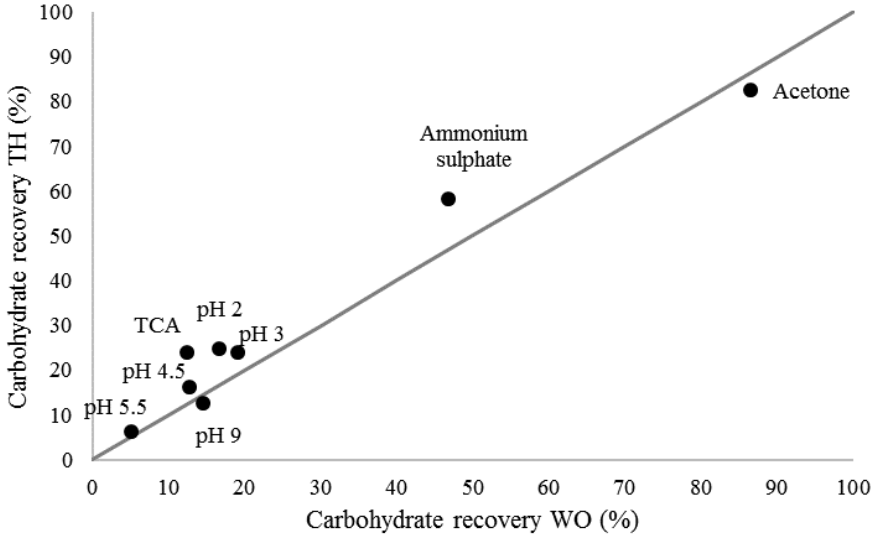

Figure A3. Comparison between carbohydrate precipitation percentages for each method depending on the hydrothermal treatment.

4.3. TRATAMIENTOS HIDROTÉRMICOS DE LEVADURAS

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Una de las claves del desarrollo de una tecnología es la expansión de su aplicación a diferentes campos. En lo relativo a los tratamientos hidrotérmicos, éstos son técnicas versátiles que se pueden aplicar a diferentes sustratos. Dado que uno de los objetivos que persigue esta tesis es la revalorización de residuos mediante la obtención de compuestos de interés, como pueden ser proteínas, se planteó evaluar la aplicación de las técnicas empleadas en lodos de depuradora en otros sustratos de características similares, es decir, biomasas unicelulares ricas en proteínas. Este es el caso de las levaduras de cerveza, cuyo porcentaje en peso de proteínas ronda el 50% (Caballero-Córdoba y Sgarbieri, 2000; Chae et al., 2001; Pacheco et al., 1997).

Durante el trabajo con los lodos de depuradora, se observó el efecto positivo de los tratamientos hidrotérmicos en la solubilización de proteínas, por lo que su aplicación a levadura cervecera es perfectamente factible. Además, algunos de los problemas descritos en el apartado 4.1.3. con la técnica IMAC para recuperar proteínas no se encontrarán en los hidrolizados de levaduras, puesto que no tienen ácidos húmicos que compliquen el proceso de adsorción ni de medida.

Por lo tanto, el objetivo de este capítulo fue estudiar los tratamientos hidrotérmicos de levadura de cervecería, observando el efecto de la presencia de oxígeno en las propiedades físicas, composición y potencial recuperación de productos de valor añadido de los hidrolizados de levadura obtenidos, así como establecer y validar un modelo cinético basado en los resultados obtenidos.

Effects of oxidising atmosphere on brewer's yeast hydrothermal treatment and subsequent biopolymer recovery

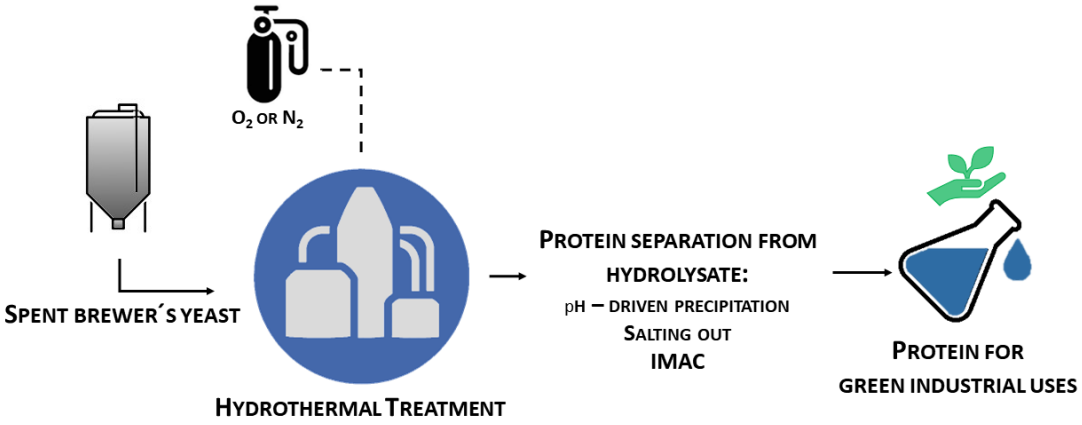
*Manuel García[a], Sergio Collado[a], Paula Oulego[a], Mario Díaz[a]**

[a] Department of Chemical and Environmental Engineering. University of Oviedo.

C/ Julián Clavería s/n. E-33071 Oviedo, Spain. Tel.:34985103439; Fax: 34985103434

* C/ Julián Clavería s/n. E-33071 Oviedo, Spain. Tel.: +34 985 10 34 39; Fax: +34 985 10 34 34 mariodiaz@uniovi.es

GRAPHICAL ABSTRACT



HIGHLIGHTS

The presence of oxygen enhanced yeast disintegration.

Soluble proteins abated at long times when oxygen was present.

Oxygen caused a convergency in the different protein isoelectric points to 3.

Oxygen decreased protein hydrophobicity, worsening saline precipitation.

Oxygen reduced protein recovery but enhanced selectivity in IMAC.

ABSTRACT

Spent brewer´s yeast is an underused by-product, whose current management offers low returns. This article studies, for the first time ever, the hydrothermal treatment of spent brewer´s yeast, paying special attention to the effect of the presence of oxygen on the physical properties, composition and potential recovery of valuable products from the hydrolysed yeast, as previous step towards the employment of these recovered proteins as renewable chemical feedstock for green industrial uses.

Results showed a higher VSS disintegration employing an oxidising atmosphere, as well as a better solubilisation in terms of solid COD reduction, although it also lowered soluble COD but without significantly reducing soluble TOC. Both treatments led to an almost complete solubilisation of protein and carbohydrates from sludge, which were subsequently degraded in presence of oxygen if reaction times were lengthened. Looking at biopolymer recovery, protein precipitation by pH adjustment worked better in hydrolysates obtained in presence of oxygen, with almost 90% of protein precipitation and good selectivities at pH 2.5 and 3. Salting out was more effective, in terms of selectivity, for samples obtained in absence of oxygen and low concentrations of ammonium sulphate. The application of IMAC showed better results on yeast hydrolysates obtained under an inert atmosphere, reaching maximum sorption capacities of almost 100 mg protein/ g dry resin, three times higher than those observed using the hydrolysate produced in presence of oxygen. A new perspective in yeast management during beer production is open as renewable chemical feedstock for protein, although further studies are required to optimise protein recovery and purification with respect to its applications.

KEYWORDS

Hydrothermal treatments, brewer’s yeast, protein recovery, kinetic model, IMAC

1. Introduction

Beer is one of the most popular drinks all around the globe, particularly in Europe. According to EUROSTAT [1], more than 39 billion litres of beer were produced during 2018 in the old continent. It is well known that the main responsible for beer production from the starch fermentation from cereal are yeasts, mainly those from *Saccharomyces* genus. During the fermentation, the cellular growth involves the production and drainage of around 1.7- 2.3 kg of yeast (expressed as dry weight) per tonne of beer, thus generating a huge amount of wet biomass which must be properly managed [2]. In fact, spent yeast is the second major by-product of the brewing industry [3]. Due to its high nutritional value [4], brewer´s yeast has been traditionally used as animal feedstock, since it represents an economically friendly source of proteins [5].

Currently, different strategies focused on the transformation of spent brewer´s yeast into other added-value resources are becoming important. The most explored alternative is the use of spent yeast as a nutritional supplement in human diets; for example, as an ingredient to increase the protein content in vegan diet or in functional foods [2,6–8]. However, although this application is extended, it has an important disadvantage due to the high content of nucleic acids in the yeast, that could lead to high values of uric acid and its associated illnesses [8]. In addition, the huge quantities of yeast produced every year provide enough feedstock for a wider variety of applications. Therefore, the research for new applications for spent yeast is providing new innovative uses for this waste in several fields. As it presents high nutritional values for animals, it also represents a source of proteins and minerals for microorganisms. For this reason, yeast extracts have been used as sole media or along with supplements in several interesting fermentation processes such as the growth of lactic acid bacteria, the production of succinic acid or the use of the extracts as fermentation substrates [9–11]. Other applications are based on the fact that yeasts are cells and, as such, produce the enzymes required in their life cycle. These enzymes also have a commercial interest; for instance, the use of pectinase is being studied in the juice industry [12]. Moreover, other constituents of the yeast, such as monosodium glutamate or some nucleotides, have also been employed after being appropriately treated as flavour enhancers in meat, sauces and other foods [2,5,13,14]. Spent yeast, as other biomasses, also shows adsorbent properties and has been employed in the removal of chromium, lead or dyes, hence being useful in bioremediation and treatment of contaminated industrial wastewaters [15,16].

Nevertheless, most of these routes for the reuse of yeasts require their previous disruption in order to release and solubilise their compounds. Different methods of cellular lysis have been reviewed extensively in the bibliography and employed for this purpose, such as homogenization, thermal lysis, sonication, chemical disruption or enzymatic lysis, being categorized mainly as mechanical or chemical ones [17]. During the mechanical lysis, yeast membrane is physically broken down by using shear forces. These methods are the most popular, with high throughputs, although problems such as heating of sample volume, degradation of cellular products, cell debris and higher cost limit the use of this method. On the other hand, chemical lysis methods use reagents such as buffers, detergents or enzymes to disrupt the cell membrane. In this case, their main drawbacks are that an additional purification step has to be incorporated to remove the corresponding reagent and the difficulty to achieve a completed lysis. Nevertheless, there is another yeast lysis method, the thermal lysis. This one is based on the supply of heat to the cells to denature proteins and lyse the cells. However, the heating for a long period may damage target materials such as proteins and enzymes. It is true that the thermal denaturation has several important consequences, such as a loss of biological activity, the increase in the viscosity of protein solutions, a decrease in the solubility, an increase in the reactivity of side groups and altered surfactant properties and sensitivities to enzymatic proteolysis [18]. These effects may result in problems, but are also recognised as benign and even desirable in non-food applications, such as the use of proteins for the production of coatings, films, adhesives or surfactants [19,20]. It is also well-known that in hydrothermal media under high temperature, carbohydrate and protein produce several chemicals (furfural, 5-hydroxymethyl furfural, and nitrogenous aromatics) which are often inhibitory to microbial growth [21]. In addition, the possibility of treating high volumes with a high percentage of solids, as in the case of the spent brewer´s yeasts, in a short time and with a high yield makes thermal lysis attractive to the supply of proteins from yeast for uses where keeping the protein native structure or the presence of inhibitory compounds to microbial growth are not essential, such as in the production of resins [22], films [23], organic fertilizers [24], adhesives [25] or bioplastics [26].

Hydrothermal treatments, that is to say, the thermal lysis at high temperatures and pressures in absence (thermal hydrolysis) or presence (wet oxidation) of oxygen, have already been successfully applied to different waste biomasses with high protein content, mainly waste activated sludge from wastewater treatment plants and food industry wastes

[27,28]. Nevertheless, the literature dealing with the thermal hydrolysis of spent brewer's yeast in order to obtain valuable biomolecules is extremely scarce. In this regard, Espinosa et *al*. [29] evaluated the hydrothermal treatment of oleaginous yeast biomass at 280 ºC and 500 psi as an alternative bioprocessing strategy for hydrolysis and lipid extraction resulting in fatty acids used for biofuel production. During the treatment, the original biomass lipids, mainly triacylglycerides, were converted into fatty acids and recovered by hexane extraction, thus obtaining a stream of these compounds free of sulphur and low in salts and nitrogen. In fact, only one paper deals specifically with the hydrothermal treatment of Baker´s yeast cells for the production of proteins and amino acids [11]. The purpose of that research was to determine the effect of temperature and hydrolysis time during the thermal hydrolysis under an inert atmosphere on the amount of residual yeast, TOC, and the amount of protein and amino acids in the soluble products, not paying attention to other biopolymers or to the subsequent potential separation of compounds from the hydrolysate. They found that the amount of solubilised protein increased with an increase in temperature, while that of amino acids decreased with increasing temperature. The highest yield of protein and amino acids obtained were 0.16 and 0.063 mg/mg of dry yeast, respectively.

Most of the available works involve thermal hydrolysis at severe conditions to achieve a total liquefaction of the yeast in order to obtain biofuels [30–32] and not proteins, so they will not be here discussed. Only Miao et *al*. [31] studied a sequential hydrothermal liquefaction process to firstly obtain sugar and protein at a lower temperature, then converting the remaining biomass to bio-oil at a higher temperature. They reported that high temperatures (>180 ºC) in the first step increased the production of inhibitory compounds. The authors proposed the use of isolated polysaccharides and protein at low temperature as potential carbon and nitrogen sources for repeated culture of yeasts. Regarding the application of wet oxidation treatments to brewer's yeast, to the best of our knowledge, the literature in this field is inexistent, even when wet oxidation has been proved to be a suitable technique during the treatment of other biomasses with a high protein content [33].

The potential applications of the proteins previously described are in the line of the context of circular economy and green industrial uses, by taking advantage of a waste. However, the first step that has to be taken is the recovery of the proteins. Although the number of protein recovery methods is high, from an industrial point of view, it would be

desirable the use of safe, cheap and/or reusable materials. In this basis, some interesting methods for protein recovery from the hydrolysates are pH driven precipitation, salting out or IMAC (Immobilized Metal Affinity Chromatography). These techniques have been used in other protein recovery strategies from effluents of food industry [34]. The adjustment of pH and the use of salting out have been widely studied and require specific testing in each case to find the isoelectrical point or the salting out point. IMAC is based on the bind of the specific groups of protein to the metal [35]. The most interesting fact regarding IMAC is the cheapness of the technique, since the resin could be reused by eluting protein with a saline solution. Although other methods match the premises of reusability and non-toxicity, as can be the case of membrane filtration, their application to recover protein from hydrolysates is extremely complex. Previous studies in sewage sludge hydrolysate shed light on the size distribution of its components, showing that the solubilized molecules had different sizes that make the separation almost impossible by membrane technologies [36].

Due to the aforementioned, the aim of this article is to study the hydrothermal treatment of spent brewer´s yeast, paying special attention to the effect of the presence of oxygen on the physical properties, composition and potential recovery of valuable products from the hydrolysed yeast, as well as proposing and validating a kinetic model based on the results obtained. These knowledge may well constitute a first step towards the employment of spent brewer´s yeast as renewable chemical feedstock for green industrial uses.

2. Material and methods

2.1. Spent brewer´s yeast

The yeast samples were withdrawn from the bottom of a conical-cylindrical fermenter from an artisan brewery (Cerveza Caleya), situated in Asturias (Spain). The yeast species was identified as *Saccharomyces pastorianus*. It is important to point out that yeast samples did not contain hop or other flavouring compounds. Once collected, the yeast samples were stored at 4 ºC until ready to use. An initial analysis of the yeast showed a high concentration of TSS (more than 150 g/L) and carbohydrates, as expected due to the origin of the sample. It has to be reminded that yeast samples were withdrawn from a beer fermenter containing barley wort as fermentation medium, thus explaining the high initial concentration of carbohydrates. The initial analysis also showed that more than 98% of TSS were VSS, so suspended solids will be treated as VSS throughout the

text. Therefore, before being hydrolysed, spent yeast was washed 3 times with distilled water, in order to remove the remaining liquid corresponding to the fermentation medium. The characteristics of the washed yeast that was charged into the reactor are shown in Table 1.

Table 1. *Initial parameters after washing the yeast samples employed in the experiment.*

Parameter	***Units***	***Value***
pH		5.6 ± 0.5
TSS	*g/L*	26 ± 6
VSS	*g/L*	26 ± 6
SVI	*mL/g*	18 ± 3
TCOD	$g\ O_2/g\ VSS_o$	1.6 ± 0.3
SCOD	$g\ O_2/g\ VSS_o$	0.22 ± 0.07
TOC	$g\ C/g\ VSS_o$	0.07 ± 0.03
Soluble proteins	$g/g\ VSS_o$	0.07 ± 0.04
Soluble carbohydrates	$g/g\ VSS_o$	0.02 ± 0.02

2.2. Hydrothermal treatments

Hydrothermal treatments were carried out in a 1 litre capacity reactor (PARR series 4520 reactor) equipped with a six-bladed turbine stirrer. In order to study the effect of an inert or oxidizing atmosphere on the process, nitrogen or oxygen, respectively, were added at a flowrate of 1200 mL min^{-1}, after both being previously conditioned by means of an upstream humidifier of 2 litre capacity. Both reactor and humidifier were filled at a 70% of their maximum capacity aiming to safety purposes. The pressure was controlled using a back-pressure valve at the end of the gas line, whereas the temperature of both reactor and humidifier, the flowrate and the stirrer speed were regulated by a PID controller.

2.3. Biopolymers recovery

With this purpose, hydrolysates obtained after 80 minutes of treatment were used. This time was selected attending to the biopolymers concentration that will be show in figure 2 (section 3.2). In this figure, it can be observed that protein did not increase in high amounts from minute 80 and carbohydrates were also high at that time. In addition, treatment time was not too long, thus avoiding excessive damage in the biopolymers.

The recovery methods selection strategy was set on the basis of four main premises: one step, low cost, the avoiding of harmful chemicals and feasibility of industrial implementation. Therefore, the methods selected were pH driven precipitation, ammonium sulphate and IMAC sorption.

2.3.1. pH-driven precipitation

Different pH values (2, 2.5, 3, 3.5, 4 and 9) were tested by adding HCl 1 M or NaOH 1 M to the hydrolysates obtained either in presence or absence of oxygen to find out which value yields the higher precipitation of one of the biopolymers with the less precipitation of the other. Precipitate was separated by centrifugation at 10000 g for 10 min. This method has been successfully applied to protein separation strategies and its mechanism involves the shifting of pH near the isoelectric point of the proteins to achieve protein to protein hydrophobic interactions, thus making them precipitate [34].

2.3.2. Saline precipitation

The addition of salts to precipitate proteins is known as salting out. Different percentages of the saturation concentration of ammonium sulphate (0.764 g/mL) were employed: 100%, 90%, 80% and 50%. Precipitate was separated by centrifugation at 10000 g for 10 min.

2.3.3. IMAC sorption

Immobilized Metal Affinity Cromatography (IMAC) was employed, using the Lewatit TP-207 resin (Sigma). Copper was bound to the resin by soaking into a $CuCl_2$ 1M solution for at least four hours. This metal was chosen owing to its high affinity to peptide bonds [37]. Then, different L/S relations between resin and hydrolysates were tested in order to elucidate the resin capacity for protein retention.

The main advantage of IMAC technology is the reutilisation of the resin once protein has been eluted, thus sharply decreasing the recovery step cost.

2.3.4. Recovery and selectivity

The percentage of biopolymer precipitation, or recovery, was calculated according to equation 1:

$$\%_{precipitated} = \left(1 - \frac{\text{supernatant concentration after precipitation}}{\text{supernatant concentration before precipitation}}\right) \text{x100} \qquad (1)$$

A selectivity factor was calculated according to the following equation [38]:

$$\alpha_{p/c} = \frac{\left(\frac{(C_p)_{\text{before precipitation}} - (C_p)_{\text{supernatant after precipitation}}}{(C_c)_{\text{before precipitation}} - (C_c)_{\text{supernatant after precipitation}}}\right)}{\frac{(C_p)_{\text{supernatant after precipitation}}}{(C_c)_{\text{supernatant after precipitation}}}} \qquad (2)$$

2.4. Analytical methods

pH, total and volatile suspended solids (TSS and VSS), sludge volumetric index (SVI), total and soluble chemical oxygen demands (TCOD and SCOD, respectively) were

measured according to the Standard Methods [39]. Total soluble organic carbon (TOC) was determined by means of a TOC-VCSH Analyzer (Shimadzu, Japan) and colour number (CN) was calculated according to the equation proposed by Tizaoui et *al*. [40]:

$$CN = \frac{SAC_{436}^2 + SAC_{525}^2 + SAC_{620}^2}{SAC_{436} + SAC_{525} + SAC_{620}} \quad (3)$$

In equation 1, SAC_i are the spectral absorption coefficients at a wavelength of *i* nanometers, which were determined using an AnalitykJena Spectrophotometer. At this point, it should be pointed out that sCOD, TOC and CN were measured in the supernatant obtained after centrifuging the yeast samples at 10000 g for 10 minutes.

The average oxidation state of carbon (AOSC) is calculated by means of SCOD and TOC values, following the next equation [41]:

$$AOSC = 4 - 1.5 \cdot \frac{SCOD}{TOC} \quad (4)$$

AOSC values can oscillate from -4 to 4, being negative for reduced carbon (-4 is associated to the most reduced carbon molecule, CH_4) and positive when carbon is oxidized (4 is the value given to CO_2, the most oxidized form of carbon). For the sake of a better understanding, it has to be pointed out that AOSC refers to the oxidation state of a mixture, whereas the term employed to indicate the oxidation state of a specific compound is MOC (Mean Oxidation state of Carbon).

The concentration of soluble biopolymers (proteins and carbohydrates) were also determined in the supernatant. The concentration of soluble proteins was measured by the Lowry method, using BSA (Sigma) as standard [42]. Regarding the soluble carbohydrates, its concentration was determined by the phenol-sulphuric method, with D-+-glucose (Sigma) as reference [43].

3. Results and discussion

3.1. Effect of the atmosphere on the physical properties

The evolutions of the pH, VSS, SVI, TCOD, SCOD, TOC, AOSC and CN during the hydrothermal treatment of spent yeast under an inert or oxidising atmosphere are shown in figure 1.

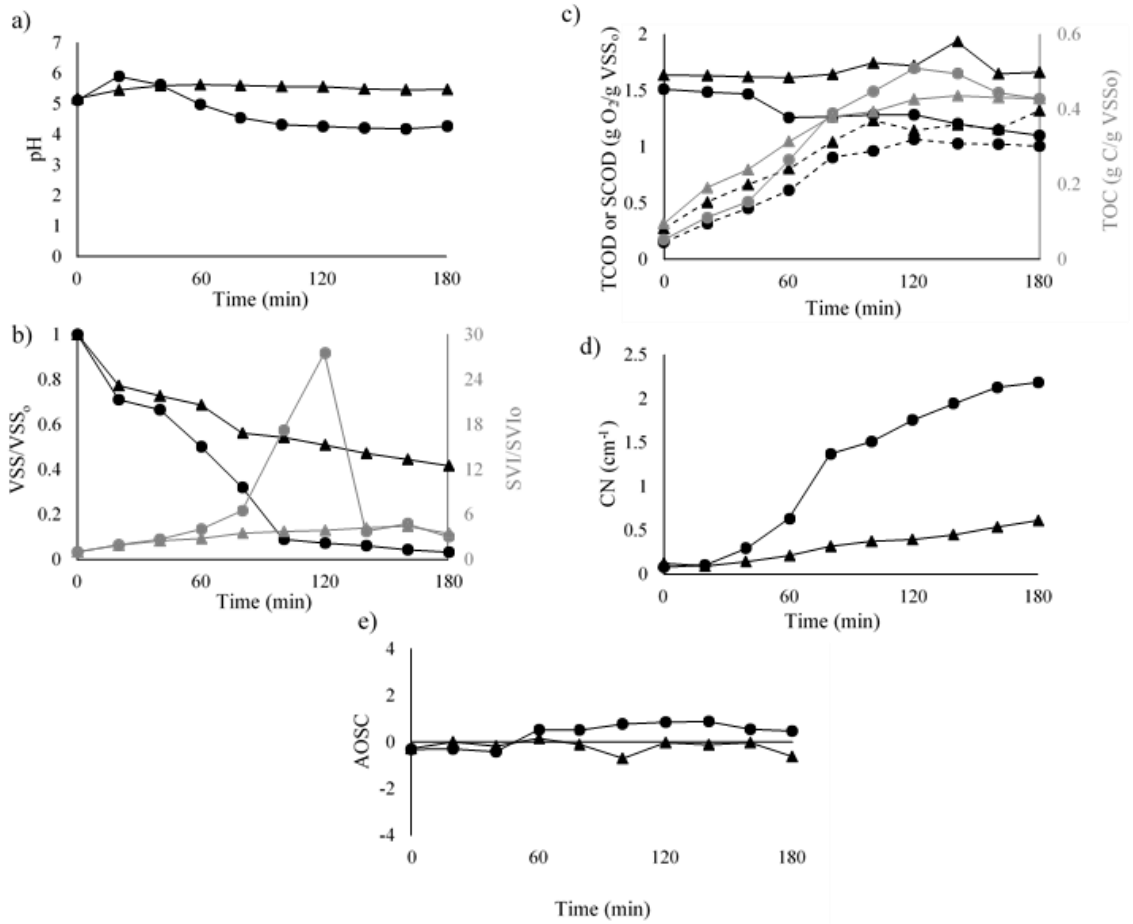


Figure 1. *Evolution of a) pH, b) VSS and SVI (grey), c) TCOD, SCOD (dashed lines) and TOC (grey), d) CN, e) AOSC during wet oxidation (●) or thermal hydrolysis (▲) of brewer´s spent yeast. In all cases: 160 °C and 40 bar, 1200 mL min^{-1} of either N_2 or O_2 and 125 rpm of agitation.*

Before starting the discussion of the results, it is important to take into account that, under an inert atmosphere, the yeast solubilisation via VSS disintegration is supposed to be only based on hydrolysis reactions, accelerated by the use of high temperatures and pressures, these latter to avoid the vaporization of the liquid phase. On the other hand, when an oxidizing atmosphere is selected, oxidation reactions are present as well. These oxidation reactions are more aggressive than the hydrolysis ones, being able to modify to a greater extent the structure of the molecules by generating oxidized functional groups, such as carboxyl, carbonyl, ketone, hydroxyl… [44,45]. Results here obtained were in line with these premises. So, the changes in the pH (fig. 1a) under an inert atmosphere were almost negligible, with only a slight increase in the alkalinity for the first 60 minutes (from 5.17 to 5.63), probably related to the solubilisation of proteins and amino acids. When an oxidising atmosphere was selected, in contrast, the initial increase in the pH attributed to the release of cytoplasmic materials was followed by an acidification due to the oxidation reactions from 5.45 to 4.26 after 120 minutes, with this value remaining

constant until the end of the treatment. Focusing on VSS behaviour (fig. 1b), in both thermal hydrolysis and wet oxidation, the reduction was noticeable after only 20 minutes even at low temperatures, probably due to the solubilisation of soluble microbial products (SMP) and lightly bound extracellular polymeric substances (LB-EPS) [36]. From this point, the effect of the oxidizing reactions was evident. The disintegration of the VSS was 10% higher in presence of oxygen than in its absence after 60 minutes. From this point until the end of the process, the behaviours totally differed. Thermal hydrolysis only provoked a slight VSS decrease from this time to the end of the treatment (180 min), whereas wet oxidation led to an almost complete abatement of the VSS. To illustrate these differences, at minute 180, thermal hydrolysis disintegrated a 59% of the initial VSS, whereas wet oxidation reduced a 97% the initial VSS. To put some light on the mechanism that makes wet oxidation more efficient in VSS destruction, it has to be known the characteristics of the reactions. Hydrolysis reactions require a lower activation energy than oxidation reactions, so the last only occurred when temperature is high, after the initial heating-up of the reactor (around 40 min). This effect was also observed in other works [33]. However, VSS diminution can be related to solubilisation or their complete destruction to carbon dioxide or volatile compounds that could leave the reactor. The SVI can be used to discern between these two options. In thermal hydrolysis, SVI increased during all treatment time, being more than three times higher at the end, due to the biopolymers release. This phenomenon will be discussed later, but briefly, the more the protein concentration, the worse the settleability [46]. However, during wet oxidation, the SVI astonishingly rose from minute 80 to minute 120 (multiplying by 27 the initial SVI value), indicating an intense solubilisation (corresponding with the high decrease in VSS observed) during those minutes. During the final minutes, SVI dramatically dropped, suggesting the oxidative degradation of those biopolymers that hindered the sedimentation, ending with a final SVI value 3 times higher than the initial value.

As can be seen in figure 1c, TCOD did not show significant changes during the hydrothermal treatment of yeast in absence of oxygen, because no oxidation reactions occurred. Meanwhile, the organic load decreased in presence of oxygen and oxidation reactions caused the mineralization of a 30% of the initial TCOD from minute 40, when the high temperature made these reactions prevail over the hydrolysis ones. Moving on to the soluble organic load, it has to be pointed out that the SCOD was higher in absence than in presence of oxygen, even when the VSS disintegration was significantly higher

under an oxidizing atmosphere, as previously explained. This can be easily explained taking into account that oxidation reactions not only caused a faster disintegration of solids but also the mineralization of a fraction of this dissolved organic matter to carbon dioxide. Maximum SCOD concentrations were achieved after 100 minutes of treatment under an inert atmosphere (75% of the initial COD) and after 120 minutes when oxygen was present (70% of the initial COD). These results differ from the initially expectable behaviour, in which wet oxidation should produce a higher SCOD than thermal hydrolysis because the VSS disintegration was also higher under an oxidising atmosphere. The higher SCOD observed under an inert atmosphere can be easily explained taking into account that oxygen not only accelerated the lysis of the yeast, but also partially oxidised the soluble organic load, thus reducing the SCOD.

Regarding TOC, both treatments caused an increase in this parameter during the first minutes of reaction. After this time, TOC concentration remained constant when an inert atmosphere was employed, but decreased in presence of oxygen, due to the formation of carbon dioxide by means of oxidation reactions. Comparative research in sewage sludge between wet oxidation and thermal hydrolysis showed similar results [47]. Unlike the case of SCOD, it should be stressed that an oxidizing atmosphere did led to higher concentrations of soluble TOC than an inert one: 0.51 g C/g VSS_o and 0.44 g C/g VSS_o, respectively (Figure 1c). These differences in the evolutions of SCOD and TOC with or without oxygen suggest that the solubilised compounds were partially oxidized, but not completely mineralized to carbon dioxide, in presence of oxygen. This would involve the accumulation of partially oxidised intermediates in the medium under an oxidising atmosphere and a faster decrease in the SCOD concentrations than in the TOC ones, as observed experimentally.

These partially oxidised compounds would also be responsible for the colour evolution showed in figure 1d. Although either inert or oxidising atmospheres caused an increase in the CN, this was substantially higher in presence of oxygen. In fact, this difference in colour, which was already noticeable after 40 minutes, progressively increased with the time of treatment, with a final CN in presence oxygen 3.6 times higher than in its absence. The increase in colour in absence of oxygen is probably due to the formation of Maillard and Amadori products at temperatures from 140 to 165 °C, which also show a brownish colour [48]. These molecules are reported to appear in materials with high concentration of sugars and proteins, molecules that our sample showed to be

rich in [49]. As time advanced and oxidation reactions began to take place in the wet oxidation experiment, a modification in the nature of the molecules occurred, generating other coloured compounds such as phenolic derivates [50,51].

Finally, and closely related to COD and TOC, AOSC showed in figure 1e represents the oxidation state of the liquid mixture. As can be expected in absence of oxidation reactions, AOSC for the hydrothermal treatment of the yeast under an inert atmosphere did not show significant changes, with a negative value of around -0.1 during all the treatment, thus corroborating the non-oxidative nature of this process. In contrast, a slight increase in AOSC values was observed when the hydrothermal treatment was carried out in presence of oxygen, beginning from an initial reduced mixture (AOSC = -0.3) to a final pool of partially oxidised compounds after 180 minutes (AOSC = 0.47). Due to the oxidation reactions, the molecules released from the cells were enriched in carboxyl, carbonyl, hydroxyl groups… raising their MOC value, as well as reducing the pH of the hydrolysed yeast. This can be checked by comparing figs. 1a and 1e, where AOSC values raised when pH dropped in presence of oxygen, effect not observed for thermal hydrolysis experiments. This phenomenon was attributed to the formation of organic acids, a typical intermediate partially oxidised in wet oxidation treatments [52–54].

3.2. Effect of the atmosphere on the composition

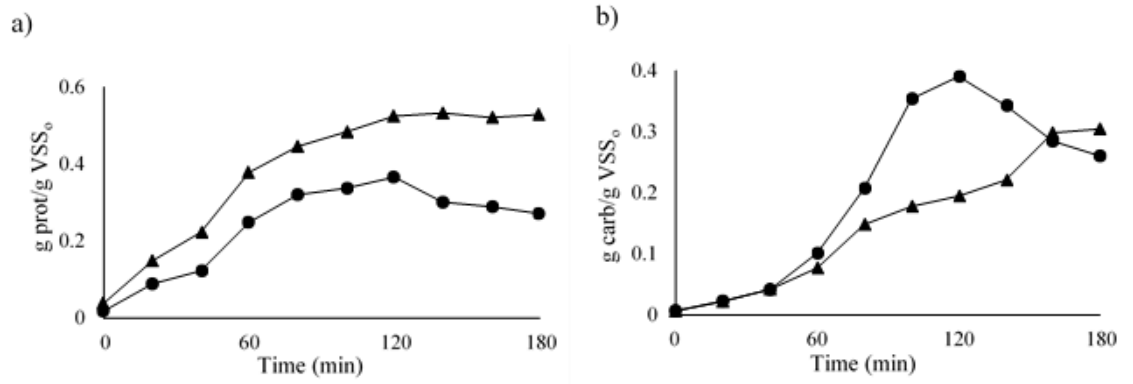


Figure 2. *Evolution of a) proteins and b) carbohydrates during wet oxidation (●) or thermal hydrolysis (▲) of brewer´s spent yeast. In all cases: 160 °C and 40 bar, 1200 mL min^{-1} of either N_2 or O_2 and 125 rpm of agitation.*

Putting now the light in the solubilisation of the biopolymers, both treatments showed a high potential for the yeast lysis and the release of its intracellular content. Proteins were the main biopolymer solubilised from the yeast (fig. 2a), with both wet oxidation and thermal hydrolysis yielding high concentrations of these compounds in the liquid phases with maximum values of 0.36 g protein/g VSS_o and 0.53 g protein/g VSS_o, respectively. It is interesting to note that, according to the bibliography, yeasts are

approximately composed by a percentage of proteins comprised between 47% and 60% [4,13,55]. This involves that, according to the data, almost all the protein in the yeast was solubilised by means of thermal hydrolysis. In the case of wet oxidation, the maximum concentration of solubilised protein was only around a 50% of the total protein in the yeast, even when the oxidising atmospheres caused a higher solubilisation than the inert ones, as was previously proved. Therefore, this lower percentage of soluble protein in presence of oxygen was not due to a lower solubilisation, but to a higher degradation of this biopolymer once solubilised due to the oxidation reactions. The maximum soluble protein concentrations were reached after 120 minutes of hydrothermal treatment, independently of the atmosphere used. Although the protein concentration remained constant after this time until the end of the treatment using an inert atmosphere, the presence of oxygen caused a final decrease in the soluble protein to a final value of 0.27 g protein/g VSS_o, that is a 25% less than the highest concentration achieved under an oxidising atmosphere, thus demonstrating the degradation of these biopolymers by oxidation reactions, but not by hydrolysis ones.

Soluble protein concentrations in the hydrolysed yeast were higher than those obtained in other works also dealing with hydrothermal treatments of yeasts, probably due to the different kinds of sample (baker's yeast was employed instead of brewer's yeast) and different conditions [11]. However, when comparing the data obtained to other hydrothermal treatments of proteinaceous materials, such as deoiled soybean, results were pretty similar, with 50% of protein solubilized after being treated 30 minutes at 200 °C and almost 4 MPa, with these conditions being comparable to those employed in this work [56]. Similar protein solubilization percentages were achieved when treating algae (*Chlorella*) at 150 °C and 0.01 atm [57].

Regarding carbohydrates, the evolutions of their soluble concentrations during the hydrothermal treatments of yeast with nitrogen and oxygen can be observed in figure 2b. It can be noted that an oxidising atmosphere produced a higher, faster solubilisation of carbohydrates than an inert one, which is the opposite to what happened with proteins. A maximum concentration of soluble carbohydrates of 0.39 g carbohydrate/g VSS_o was reached after 120 minutes of reaction in presence of oxygen, the same time at which maximum soluble protein concentrations were observed. As in the case of proteins, soluble carbohydrate concentration also decreased after this time if oxygen was used, ending with a final value of 0.26 g carbohydrate/g VSS_o after 180 minutes of reaction.

On the other hand, soluble carbohydrate concentrations during the treatment under an inert atmosphere increased progressively during all the time of reaction, although at a rate lower than the observed for the first 120 minutes of treatment with oxygen, reaching a final value of 0.30 g carbohydrate/g VSS_o at the end of the treatment (180 minutes). It emerges very clearly from these facts that the presence of oxygen accelerated the solubilisation of carbohydrates, especially at short times of reaction, where the temperature was still low. Nevertheless, oxygen also had effect on the stability of the solubilised carbohydrates, causing their degradation by oxidation if the reaction time was longer. Taking into account that around a 30% of the yeast weight corresponds to carbohydrates, it is also interesting to note that hydrothermal treatments caused a high solubilisation of the inner carbohydrates [58]. It is important to bear in mind that the yeast employed also contained some carbohydrates that came from the fermentation media. Considering the previous statement and the referenced percentage, almost all the intracellular carbohydrates were released employing an oxidising atmosphere, whereas a lower percentage, 90%, was solubilised when thermal hydrolysis was selected as hydrothermal treatment. In studies dealing with hydrothermal treatments in other biomasses, such as sewage sludge, almost complete solubilisations of carbohydrates were achieved [38]. Carbohydrates solubilisation of algal biomass at milder conditions (autoclaving at 120 °C for 40 minutes) yielded a release of the 64% of total carbohydrates, which was lower that the percentages reached in this work with or without and oxidising atmosphere, probably owed to the lower temperature and shorter time of treatment [59]. In the case of wet oxidation for the algae *Ulva*, the carbohydrate solubilization efficiency was 95% after a treatment carried out at 130 °C, 78 minutes, 1.38 bar and pH 1, corroborating the potential of wet oxidation to effectively solubilise carbohydrates from biomasses [60].

Finally, comparing carbohydrate and protein concentration data, it can be deduced that the solubilisation of proteins just by hydrolysis reactions is faster than the carbohydrates one, but the effect of oxidation reactions on solubilisations is more marked in carbohydrates than in proteins. Regarding the stability of the solubilised biopolymers, results suggest that although either proteins or carbohydrates are stable under inert atmospheres, soluble proteins are more susceptible to be degraded by oxidation reactions than the carbohydrates.

3.3. Kinetic modelling

On the basis of the experimental data afore-discussed, a kinetic model involving the concentrations of VSS, TCOD, SCOD, soluble proteins and carbohydrates was proposed and successfully fitted. To this purpose, the fitting parameters were obtained by using Micromath Scientist software, with a least square method for the error minimization.

The kinetic model was deduced from the mechanism proposed in figure 3. To model the changes in the COD, this was divided into three fractions: TCOD, SCOD and CODsf (COD of the solid fraction. The mechanism is based on the direct disintegration of the VSS (or CODsf) into soluble matter, measured as SCOD. This SCOD included the solubilised proteins and carbohydrates from the yeast which, in turn, could be subsequently degraded to other compounds and carbon dioxide, especially in presence of oxygen. In order to tie together COD with biopolymer concentrations, the corresponding yields (grams of biopolymer per gram of CODsf solubilised) were also introduced as fitting parameters in the kinetic model. All these reactions are schematized in figure 3:

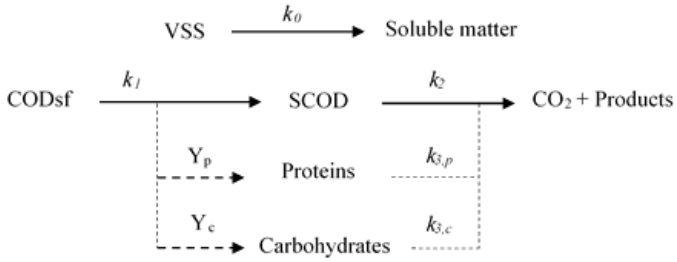


Figure 3. *Proposed mechanism for the hydrothermal treatment of spent yeasts.*

The following kinetic equations can be deduced from the proposed mechanism:

$$r_{VSS} = -k_0 \cdot [\text{VSS}] \quad (5)$$

$$[\text{TCOD}] = [\text{SCOD}] + [\text{CODsf}] \quad (6)$$

$$r_{\text{CODsf}} = -k_1 \cdot [\text{CODsf}] \quad (7)$$

$$r_{\text{SCOD}} = k_1 \cdot [\text{CODsf}] - k_2 \cdot [\text{SCOD}] \quad (8)$$

$$r_{\text{CODsf}} = -k_1 \cdot [\text{CODsf}] \quad (9)$$

$$r_i = Y_i \cdot k_1 \cdot [\text{CODsf}] - k_{3i} \cdot [i] \quad (10)$$

The kinetic model was successfully fitted to the experimental results and the fitting parameters are shown in table 2:

Table 2. *Most relevant fitting parameters for the kinetic model.*

	Thermal hydrolysis	***Wet oxidation***
$\bar{k}_0$ (s^{-1})	0.016 ± 0.001	0.0056 ± 0.0004
r^2	0.975	0.996
$\bar{k}_1$ (s^{-1})	0.0085 ± 0.0005	0.0121 ± 0.0007
r^2	0.978	0.992
$\bar{k}_2$ (s^{-1})	~0	0.0028 ± 0.0003
r^2	0.983	0.994
$\bar{Y}_{protein}$	0.66 ± 0.17	0.43 ± 0.12
$\bar{k}_{3protein}$ (s^{-1})	0.0028 ± 0.0003	0.005 ± 0.004
r^2	0.997	0.98
$\bar{Y}_{carb.}$	0.5 ± 0.2	0.23 ± 0.04
$\bar{k}_{3carb.}$ (s^{-1})	0.006 ± 0.005	~0
r^2	0.97	0.92

The fitting of the model to the experimental data is shown in figure A1.

The results for the kinetic constants showed in table 2 corroborate the previous discussion about a high efficiency of the hydrothermal treatment in VSS degradation, especially under an oxidising atmosphere, fact that was reflected in the high k_0 values, being this parameter three times higher for wet oxidation than for thermal hydrolysis. These facts are also in accordance with the k_1 values obtained for the CODsf conversion to SCOD under inert and oxidising atmospheres.

It is also noticeable that k_{2,N_2} values were negligible for experiments under inert atmosphere; this was expected because k_2 corresponds to the oxidation of SCOD towards to carbon dioxide, which is negligible under an inert atmosphere.

Focusing on the modelling and fitting of the evolution of the two considered biopolymers, proteins and carbohydrates, it can be easily deduced that k_1 value has to be the same obtained for the fitting of CODsf solubilisation data (eq. 7). The first order kinetic models proposed showed a successful fitting to the data collected during the experimentation. Regarding the fitting parameters for the soluble protein evolution, it can be deduced that thermal hydrolysis led to a higher protein production due to the fact that wet oxidation degraded the solubilized proteins more intensely, since its k_{3,O_2} value was

twice the corresponding to thermal hydrolysis value (k_{3,N_2}). Moreover, $Y_{prot.,N_2}$ for thermal hydrolysis was sensitively higher (0.66 for thermal hydrolysis and 0.43 for thermal hydrolysis), supporting the higher soluble protein concentration reported before. Regarding the evolution of soluble carbohydrates, the results were in accordance with the previous evidences. It has to be highlighted that k_{3,N_2} did not reach a noticeable value, thus indicating that thermal hydrolysis did not seem to degrade carbohydrates in a significant amount. In contrast, the value obtained for k_{3,O_2} showed the degradation of the solubilised carbohydrates during the wet oxidation and supported the sharp decrease observed in the experimental points corresponding to the last minutes of treatment (minute 120 to minute 180). The conversion ratio (Y) of CODsf into carbohydrates also showed the better carbohydrate solubilisation under an oxidising atmosphere than under an inert one (0.47 and 0.23, respectively).

3.4. Effect of the atmosphere on the downstream processing

Once yeast has been hydrolysed, many of the applications previously described in the introduction require a subsequent step of separation and purification of either proteins or carbohydrates. As commented, the mechanisms and reactions that yeast suffered during thermal hydrolysis and wet oxidation were different. These differences play an important role not only in the physicochemical properties and composition of the hydrolysate, but also in the subsequent downstream processes, which are focused on the separation of components from the hydrolysate. Due to this, the effect of the kind of atmosphere during the hydrothermal treatment on the separation of proteins from carbohydrates in the hydrolysate obtained was studied in this section. To this end, three different separation techniques were selected: saline precipitation, pH-driven precipitation and IMAC sorption. This selection was based on that these techniques are profusely used at industrial level.

3.4.1. pH-driven precipitation

The pH of yeast hydrolysates from either thermal hydrolysis or wet oxidation were adjusted to values of 2, 2.5, 3, 3.5, 4 and 9 in order to know the amounts of proteins and carbohydrates precipitated at each pH. These results (as percentages of biopolymer precipitated) are shown in figure 4.

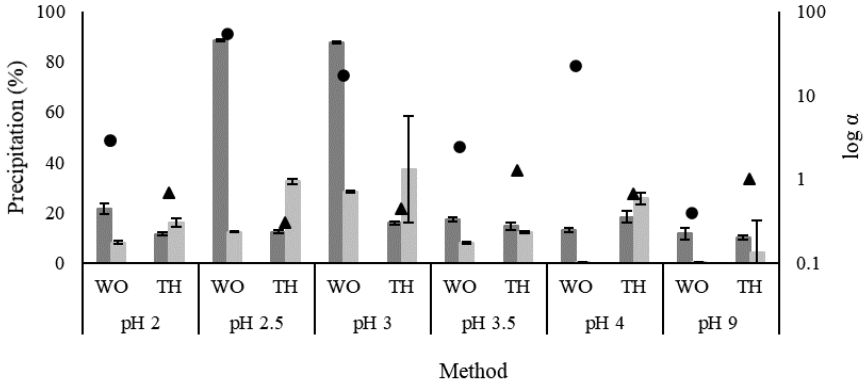


Figure 4. *Percentages of precipitation for protein (■) and carbohydrates (■) precipitation for each pH value and hydrolysate (WO is wet oxidation; TH is thermal hydrolysis). Black points represent the selectivity factor (α) for each method and hydrolysate (● wet oxidation and ▲ thermal hydrolysis).*

As can be easily observed in figure 4, the higher protein precipitations were obtained at acidic pH values, especially when this was from 2.5 to 3. Nevertheless, pH values lower than 2.5 or higher than three involved a poor protein separation. These findings suggest that the mean isoelectric point of the protein pool ranged from 2.5 to 3. This is why low protein recoveries were obtained for pH values outside of this interval. Other authors had reported that more than 80% of protein can be precipitated by pH adjustment at 3.3 of a sewage sludge hydrolysed by a coupled alkaline-ultrasonic treatment [61]. This result was consistent with other studies where pH adjustment was the strategy to recover protein from wastewater, with similar isoelectric points (3.8 – 3.9) and yields (up to 78% for protein recovery) [62]. The pH adjustment to a value of 3 also led to 90% of protein precipitation in papermill sludge hydrolysates [63].

The effect of the atmosphere during the hydrothermal treatment on the protein recovery was also clear. It was observed a higher precipitation of proteins when sludge was hydrolysed under an oxidising atmosphere. It is interesting to notice the excellent precipitation of proteins from wet oxidation hydrolysate at pH 2.5 and 3, with recoveries of 88% and 87%, in comparison to 12% and 16% obtained with the thermal hydrolysis hydrolysate. The better precipitation at acidic pH when an oxidising atmosphere was selected was probably due to the generation of new carboxyl, carbonyl and hydroxyl groups in the structure of the different proteins by the oxidation reactions, thus modifying the initial isoelectric point of each protein towards a converging value 2.5 - 3. In a similar way, the absence of oxidising reactions is the reason why the pH effect on the recovery of proteins is less marked during the precipitation using the hydrolysate from thermal hydrolysis.

Regarding the carbohydrates, it was also observed a preferential precipitation of these at pH values between 2.5 and 3, particularly when the hydrolysate from thermal hydrolysis was used. The higher carbohydrate recoveries at these pH values were due to the presence of glycoproteins, as well as the afore-mentioned Maillard and Amadori products [49]. Anyway, the percentages of carbohydrates precipitated at different pH values was less marked than for the case of proteins, probably due the former were less susceptible to oxidation reactions.

To elucidate at which pH value the separation was more selective, $\alpha_{p/c}$ parameter was calculated for each pH value and each hydrolysate. As can be easily deduced from figure 4, selectivities were significantly higher if the hydrolysate from wet oxidation instead of the thermal hydrolysis one was used. It is also interesting to note that selectivities for the hydrolysate from wet oxidation were always higher than 1, with the exception of the corresponding one to pH 9, whereas the values for the hydrolysate from thermal hydrolysis were lower or slightly higher than 1. This means that the presence of an oxidising atmosphere during the hydrothermal treatment of the spent yeast highly improved the selective precipitation of proteins by pH adjustment, thus obtaining protein precipitates with a lower percentage of impurities due to carbohydrates, particularly for pH values of 2.5, 3 and 4. It has to be reminded that these pH values were also the corresponding ones to a highest recovery of protein. On the other hand, protein precipitates from hydrolysed yeast under inert atmosphere were lower in amount (lower recovery) and decidedly less pure, containing an appreciable proportion of carbohydrates (low selectivity). In fact, carbohydrates were the main component in the precipitates from thermal hydrolysis at these pH values were selectivities were lower than 1. Previous research in sewage sludge hydrolysates, avoiding the differences with brewer's yeast hydrolysates, showed a worse selectivity in acidic pH for wet oxidation samples, although separation in thermal hydrolysis samples was not good, as in the present study [38].

3.4.2. Saline precipitation

The effect of the atmosphere during the hydrothermal treatment of yeast on the subsequent separation of proteins in the hydrolysate obtained was also studied using saline precipitation as purification technique. The figure 5 shows either the protein or carbohydrates recoveries using different concentrations of ammonium sulphate.

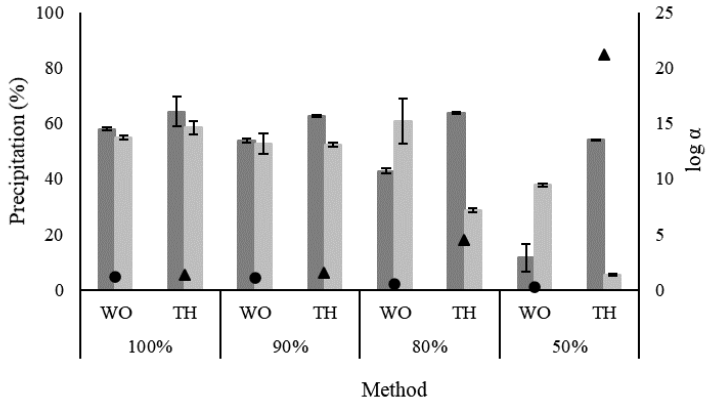


Figure 5. *Percentages of precipitation for protein (■) and carbohydrates (■) precipitation for each $(NH_4)_2SO_4$ concentration and hydrolysate (WO is wet oxidation; TH is thermal hydrolysis). Black points represent the selectivity factor (α) for each method and hydrolysate (● wet oxidation and ▲ thermal hydrolysis).*

As can be seen in the figure 5, the results revealed there was no significant differences in the proteins and carbohydrates recoveries at the highest concentrations of ammonium sulphate (> 90%), regardless the atmosphere used during the hydrothermal treatment used. Thus, the values for 100% and 90% were 57% and 64%, 54% and 62%; for hydrolysates obtained in presence or absence of oxygen, respectively. Nevertheless, for ammonium sulphate saturations lower than 90%, some discrepancies in protein and carbohydrate behaviour became evident, particularly if the hydrolysate was obtained under oxidising atmosphere. Thus, although protein recoveries for hydrolysates from thermal hydrolysis remained approximately constant at an average value of 60% for all the saline concentrations, this parameter gradually increased from 12 for 50% $(NH_4)_2SO_4$ saturation to 58% for 100% saturation, when the hydrolysate from wet oxidation was employed. The reason for these discrepancies was again related to the formation of new carboxyl, carbonyl and hydroxyl groups in the protein structure due to the oxidising atmosphere. These polar groups reduced the hydrophobic interactions and increased the hydrophilic ones of the proteins with the cellular environment, leading to the protein precipitation at higher ammonium sulphate concentrations.

Regarding carbohydrate recovery, this achieved a maximum stable value of around 55% for ammonium sulphates concentrations above 90% saturation, regardless the atmosphere used during the hydrothermal treatment. For salt concentrations lower than 90% saturation, an increase in the ammonium sulphate concentration did involve an increase in the precipitation, being the carbohydrate recovery always higher for the wet

oxidation hydrolysate than for the thermal hydrolysis one for the same concentration of salt tested.

Moving on to selectivity, the corresponding values obtained for each hydrolysate and salt concentration tested are showed in figure 5 as well. As can be seen, the selectivities obtained for saline precipitation were clearly lower than the corresponding to the pH-driven one. The selectivity values obtained for ammonium sulphate concentrations equal or higher than 90% saturation were always around one for both hydrothermal treatments, indicating a non-selective precipitation of proteins and the obtaining of precipitates highly impurified by carbohydrates. In fact, carbohydrates turned out to be the main biopolymer precipitated for ammonium sulphate concentrations lower than 80% saturation when the yeast hydrolysed under an oxidising atmosphere was employed. The highest selectivity was observed at the lowest ammonium sulphate concentration tested (50%) and with the hydrolysate from thermal hydrolysis. Nevertheless, these conditions also coincided with the lowest recovery observed. Although salting out is a very common technique to purify proteins, to the best of our knowledge, there are no studies dealing with the separation of proteins and other biopolymers in hydrolysates from hydrothermal treatments but previous works in sewage sludge. In this case, the precipitation percentages of proteins were higher in both thermal hydrolysis and wet oxidation, although selectivity was worse, probably due to the different feedstock employed [38].

3.4.3. IMAC sorption

Eventually, the effect of the atmosphere during the hydrothermal treatment of spent yeast on the protein recovery and selectivity during the sorption of these on a copper-functionalized resin using the hydrolysates obtained was also discussed (figure 6).

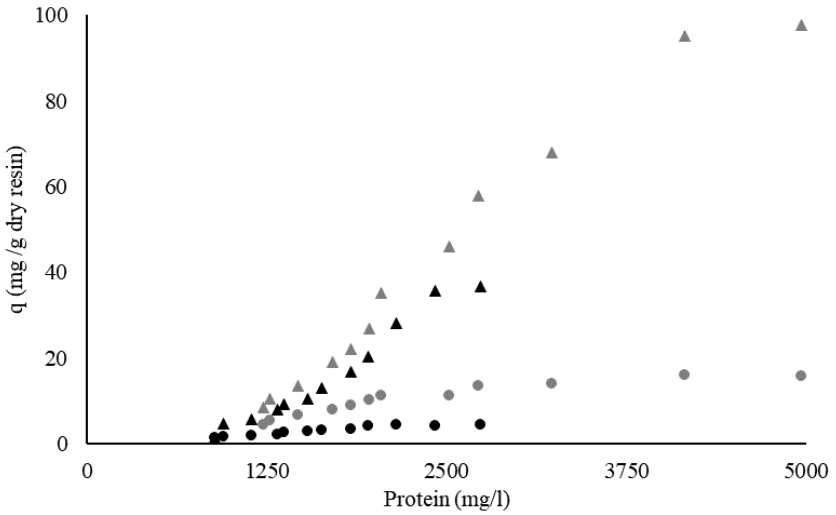


Figure 6. *Adsorption isotherms. In all cases: grey for thermal hydrolysis samples, black for wet oxidation samples, ▲ indicates protein data and ● is carbohydrates data. Hydrolysates were obtained after 80 minutes at 160 °C and 40 bar.*

Sorption isotherms showed a preferential adsorption of proteins by IMAC resins for both kind of hydrolysates, although carbohydrates were also adsorbed in small amounts, probably due to the presence of glycoproteins and the formation of Maillard and Amadori products [49]. In this sense, other authors reported that during the use of IMAC resins for protein sorption, these were also able to bind impurities such as aromatic rings similar to the amino acid side chains [64].

The atmosphere selected during the previous hydrothermal treatment also played a crucial role. As can be seen in figure 6, either proteins or carbohydrates obtained by a hydrothermal treatment under an inert atmosphere shower greater affinity for the IMAC resin than those obtained from the treatment in presence of oxygen. Thus, the maximum sorption capacities for proteins or carbohydrates when hydrolysate from thermal hydrolysis was used were 98 and 16 mg/g dry resin, respectively. On the other hand, these parameters were reduced to 37 mg proteins /g dry resin and to 4 for mg carbohydrates /g dry resin if the previous hydrothermal treatment of the spent yeast was carried out in presence of oxygen. The reason for these behaviours is again related to alterations in the molecular structures of proteins and carbohydrates during the hydrothermal treatments. In this line, wet oxidation was known to be more aggressive than thermal hydrolysis, thus leading to greater changes of proteins and reducing their affinity for the IMAC resin in a higher degree. In any case, the theoretical maximum sorption capacity, 429 mg protein per gram of resin was not achieved, due to the thermal denaturation caused during the hydrothermal treatment [65].

Regarding selectivity, this parameter was calculated by dividing the $q_{prot.}$ by $q_{carb.}$ (figure 7).

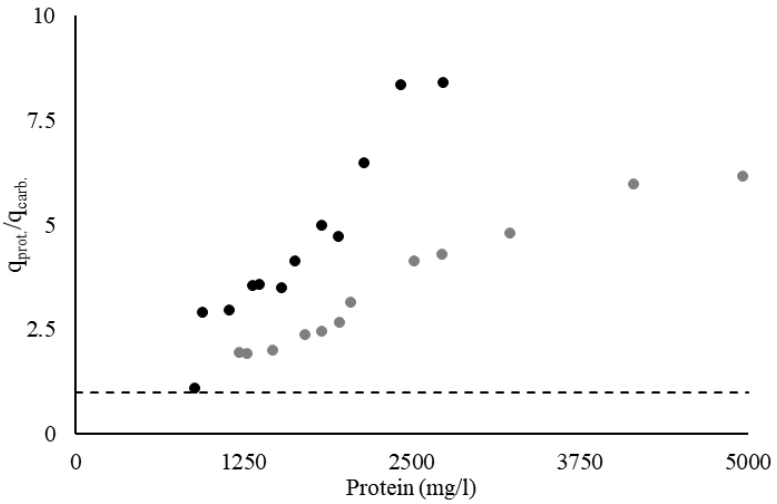


Figure 7. *Selectivity for the IMAC sorption. Grey represents thermal hydrolysis data and black wet oxidation data .*

As can be observed, the IMAC resin selectivity (as $q_{prot.}/q_{carb.)}$ was higher for hydrolysates obtained in presence of oxygen, which means a lower retention of carbohydrates per gram of protein adsorbed, that is to say, a lower amount of impurities (as carbohydrates) attached to the resin. This higher selectivity for proteins from the wet oxidation hydrolysates can be explained in two points. Firstly, attending to wet oxidation ability to degrade complex molecules like glycoproteins or Maillard products, thereby releasing the carbohydrate monomers or oligomers from protein and avoiding their indirect binding to the resin. Secondly, Klinke et *al.* [66] reported the formation of phenols, furans and carboxylic acids during the wet oxidation of wheat straw, a waste also rich in carbohydrates. Therefore, if carbohydrates were transformed into the mentioned molecules, less carbohydrates were able to bond to the resin. It is important to point out that this is not an absolute concept, only a few percentage of the carbohydrates suffered the above-mentioned reactions.

It can be also observed that the higher the resin to hydrolysate mass ratio, the lower the selectivity, regardless of the hydrolysate employed. This fact is related to a higher availability of sorption sites per molecule of biopolymer.

In summary, it is possible to obtain a stream of 0.373 g prot/g VSS_o from spent brewer's yeast, with an 86% of purity, by means of IMAC technique application after a thermal hydrolysis treatment. When pH-driven precipitation was the selected method, only 0.281 g prot/g VSS_o could be recovered at pH 3, being its purity of 88%, however this results where only achieved in wet oxidation hydrolysates. Salting out yielded a

stream of 0.240 g prot/g VSS_o with a purity of 97%. This allows spent brewer's yeast to become a renewable and cheap source of biopolymers. Proteins can be used in the fabrication of resins, bioplastics or fertilizers. Meanwhile, the remaining carbohydrates could also be employed as a fermentation media. Therefore, the application of hydrothermal treatments followed by biopolymers recovery by IMAC allows a complete revalorization of the spent brewer's yeast, making it competitive against its traditional uses as animal food or food additive. However, as a first approach, this study only dealt with one treatment condition. The evaluation of other temperatures and pressures could provide useful information in order to find the optimal treatment conditions and balancing the waste minimization and products recovery. Regarding the possibilities of scale-up of the process, this should not be very difficult, taking into account either the hydrothermal treatment or the separation methods here tested have already been successfully implemented at industrial scale for other substrates [44,67–72]. In this sense, it is interesting to note that it was previously found out that batch laboratory degradation rates during hydrothermal treatments were usually significantly lower than those found in industrial continuous stirred operation, due to the degree of backmixing and the synergistic effects [73]. Obviously, the final operation conditions of both the yeast hydrothermal treatment and the hydrolysate downstream processing should be adapted to the product specifications, which will depend on the proposed green industrial use for the recovered protein.

4. Conclusions

In this work, the application of hydrothermal treatments to spent brewer's yeast was assayed as potential source of protein for green industrial uses. The presence of an oxidising atmosphere was found to be a key factor since it strongly enhanced the VSS disintegration and the solubilisation in terms of solid COD reduction, but also lowered soluble COD without significantly reducing soluble TOC for long treatment times.

Both thermal hydrolysis and wet oxidation effectively solubilized proteins and carbohydrates, although if time was lengthened, the presence of oxidation reactions was able to degrade both biopolymers. In the basis of the collected data, a first-order kinetic model including the conversion of volatile suspended solids into soluble matter and the transformation of the solid fraction into SCOD and, particularly, into proteins and carbohydrates, was proposed and successfully fitted.

The potential recovery processes for these biomolecules were also highly influenced by the presence of oxygen in the previous treatment. It was found that oxidation reactions led to a convergency in the different protein isoelectric points to a value near three, which allowed an excellent and selective precipitation of proteins at pH values between 2.5 and 3. In contrast, the higher diversity of functional groups provided by thermal hydrolysis favoured a selective protein precipitation by salting out, especially at low ammonium sulphate concentrations. This variety of functional groups also made a difference in subsequent IMAC processes, favouring the recovery, although selectivity was higher when the hydrothermal treatment was carried out in presence of oxygen.

5. Acknowledgements

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APPENDIX A

Effects of oxidising atmosphere on brewer's yeast hydrothermal treatment and subsequent biopolymer recovery

Manuel García[a], *Sergio Collado*[a], *Paula Oulego*[a], *Mario Díaz*[a]*

[a] Department of Chemical and Environmental Engineering. University of Oviedo. C/ Julián Clavería s/n. E-33071 Oviedo, Spain. Tel.:34985103439; Fax: 34985103434

* Mario Díaz. Department of Chemical and Environmental Engineering. University of Oviedo. C/ Julián Clavería s/n. E-33071 Oviedo, Spain. Tel.: +34 985 10 34 39; Fax: +34 985 10 34 34 mariodiaz@uniovi.es

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Figure A1. Fitting of the model to the experimental data. In a) lines represent the calculated values; ● represent the experimental points for wet oxidation experiment; ▲ represent the experimental points for thermal hydrolysis experiment. In b) fitting for thermal hydrolysis and c) fitting for wet oxidation; in both cases: ● TCOD experimental values, ■ SCOD experimental values, ▲ CODsf experimental value and — calculated value for each COD fraction. In d) proteins and e) carbohydrates; in both cases: ● wet oxidation experimental points, ▲ thermal hydrolysis experimental points, — thermal hydrolysis calculated values, - - wet oxidation calculated values. Black symbols and lines represent CODsf values (experimental and calculated values, respectively), grey symbols and lines represent proteins or carbohydrates values (experimental and calculated values, respectively).

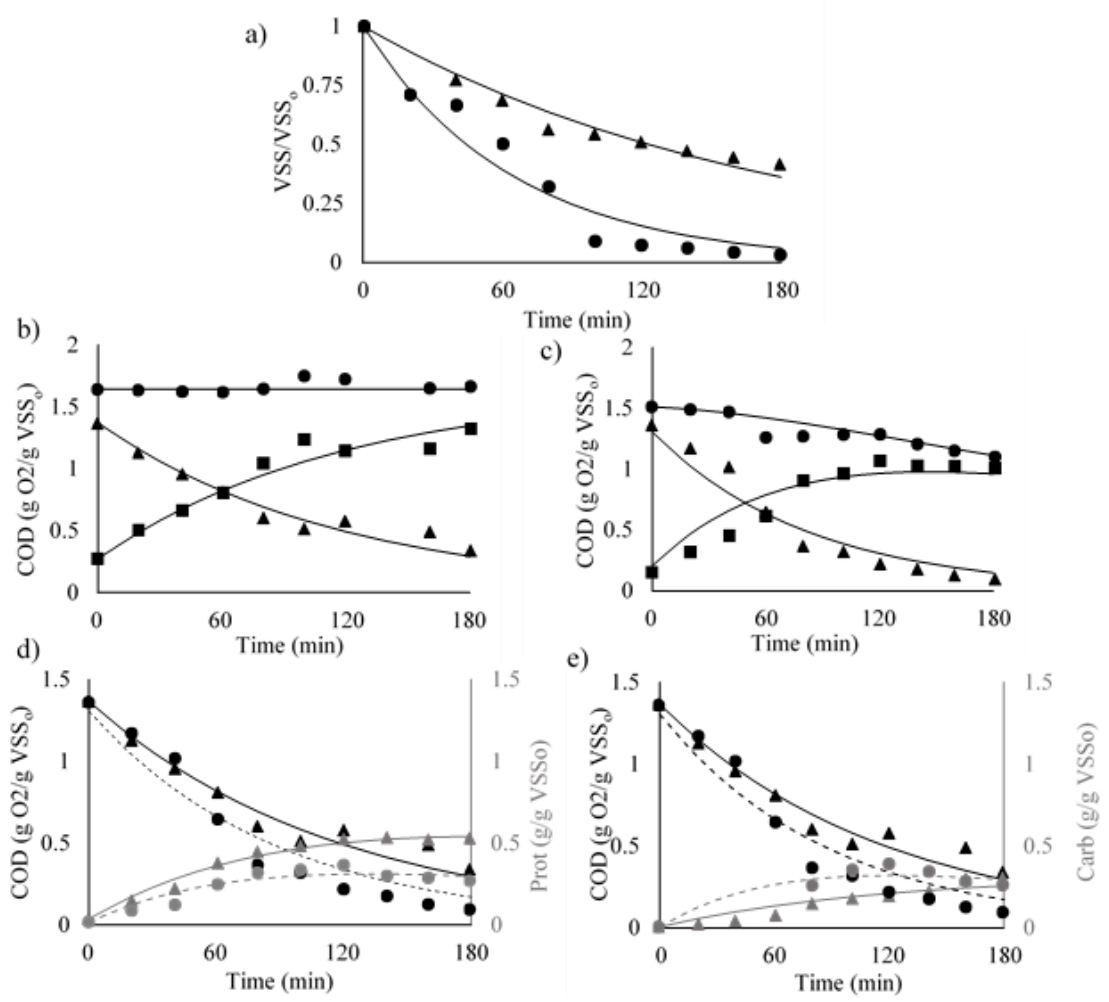

Figure A1. Fitting of the model to the experimental data. In a) lines represent the calculated values; ● represent the experimental points for wet oxidation experiment; ▲ represent the experimental points for thermal hydrolysis experiment. In b) fitting for thermal hydrolysis and c) fitting for wet oxidation; in both cases: ● TCOD experimental values, ■ SCOD experimental values, ▲ CODsf experimental value and — calculated value for each COD fraction. In d) proteins and e) carbohydrates; in both cases: ● wet oxidation experimental points, ▲ thermal hydrolysis experimental points, — thermal hydrolysis calculated values, - - wet oxidation calculated values. Black symbols and lines represent CODsf values (experimental and calculated values, respectively), grey symbols and lines represent proteins or carbohydrates values (experimental and calculated values, respectively).

4.4. OXIDACIÓN HÚMEDA DE ÁCIDO HÚMICO

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Los ácidos húmicos son moléculas procedentes de la degradación bioquímica de otras moléculas, como proteínas, vitaminas, lípidos o azúcares (Stevenson, 1994). Por tanto, son moléculas complejas y ubicuas, y que, como se mencionó en los apartados 4.1.3. y 4.2., pueden interferir en los procesos de recuperación de proteínas, debido a las características que comparten (de Melo et al., 2016).

Como se pudo comprobar en el trabajo previo, la HT no afecta a los ácidos húmicos, mientras que la OH conduce a su degradación en ciertas condiciones. Puesto que si los ácidos húmicos están presentes en los hidrolizados de lodos de depuradora afectan a la recuperación de proteínas, especialmente en la OH, es necesario conocer los cambios que sufren durante este tratamiento y cómo afectan a dichos cambios diferentes parámetros de operación, como son la temperatura, la presión y el pH.

Es de gran interés para trabajos futuros entender qué cambios sufren los ácidos húmicos durante la OH. Por ello, el objetivo de este capítulo es caracterizar la OH de ácido húmico comercial en base a la evolución de su concentración, DQO, TOC, color y productos generados, atendiendo a los efectos de diferentes temperaturas, presiones y valores iniciales de pH.

The wet oxidation of aqueous humic acids

*Manuel García[a], Sergio Collado[a], Paula Oulego[a], Mario Díaz[a]**

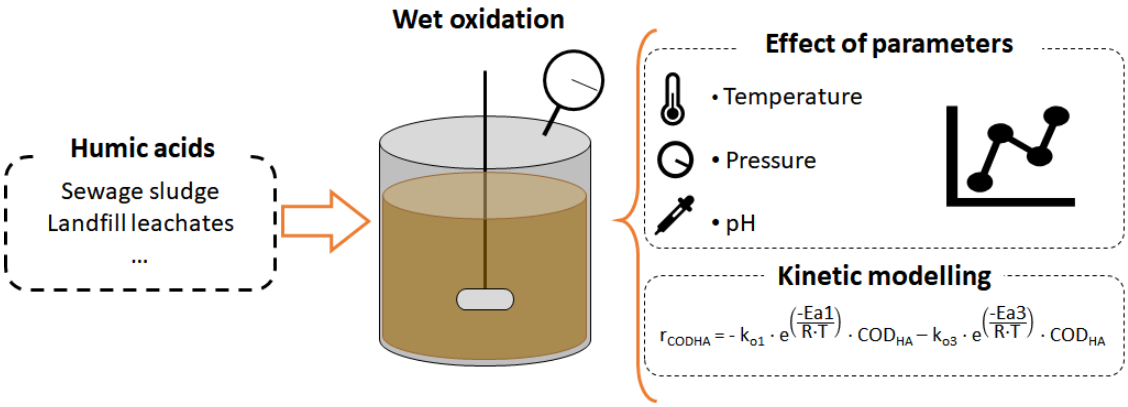
[a] Department of Chemical and Environmental Engineering. University of Oviedo.

C/ Julián Clavería s/n. E-33071 Oviedo, Spain. Tel.:34985103439; Fax: 34985103434

* Mario Díaz. Department of Chemical and Environmental Engineering. University of Oviedo.

C/ Julián Clavería s/n. E-33071 Oviedo, Spain. Tel.: +34 985 10 34 39; Fax: +34 985 10 34 34 mariodiaz@uniovi.es

GRAPHICAL ABSTRACT



HIGHLIGHTS

Higher temperatures enhanced formation of acetic and oxalic acids

No effect of oxygen pressure (65-95 bar) on the removal

Alkaline medium: higher HA removal but more refractory intermediates

Alkaline medium favours the formation of formic, lactic, maleic and pyruvic acids

A lumped kinetic model was successfully fitted to the experimental data

ABSTRACT

Humic acids are highly distributed in aqueous environments. This article examines in depth the advanced oxidation of humic acid aqueous solutions, in order to understand more complex oxidation processes such as those of the sewage sludge or landfill leachate, or the matrix effects triggered by the humic acids of natural organic matter (NOM) in the oxidation of other aqueous compounds as herbicides.

Humic acids were efficiently oxidized; higher temperatures (180-220ºC) involved higher mineralization, the formation of intermediates with lower colour and also led to a higher concentration of organic acids at the end of the treatment, particularly acetic and oxalic ones. Nevertheless, humic acid wet oxidation was not sensitive to changes in the pressure, at least in the range tested (65-95 bar), but the initial pH (4-13) was found to be a key factor. Thus, alkaline media accelerated the humic acid removal, but more refractory intermediates were generated, and the organic acids, excepting malic acid, were more stable than in neutral or acidic media. Eventually, a lumped kinetic model was proposed and successfully fitted to the experimental data, including the effect of all the operating variables studied.

KEYWORDS: humic acid, lumped kinetic model, NOM, organic acids, wet oxidation

1. Introduction

Humic acids (HAs) are extremely complex molecules. Its structure, which usually comprises quinones, phenolic, carboxylic, enolic and ether functional groups, but also peptides and carbohydrates [1], is characteristic of its biological origin, age and environment (among other factors). One of the main characteristics of these compounds is that they are partially soluble in water, totally insoluble in acidic media, but fully soluble in alkaline media.

HAs naturally occur in soils [2], but can also be found in both sea and land waters [3], constituting the main components of the Natural Organic Matter (NOM) [4]. Their high distribution throughout the environment is not surprising, taking into account that they derive from the highly transformed part of the residues of dead plants, animals, microorganisms and their degradation products, although the specific mechanisms of the formation of humic acids as well as their structure are still a subject of discussion and controversy [5].

Regarding their occurrence due to anthropogenic activities, humic acids are mainly found in some wastewaters in higher concentrations to those reported in terrestrial soil, natural water, and sediments. So, humic acids are present in sewage sludge due to the hydrolysis of organic residues and as a result of the cellular lysis during post-production processing. The humic acids from NOM also get adsorbed to extracellular polymeric (EPS) matrix of sludge by different functional groups like carboxylic and phenolic ones [6–8]. Because of the different determination methods and influent sources used, the percentage of humic substances in the sludge composition varies in the range from 8% to 29% (expressed as % volatile solids) [9]. Humic acids are not anaerobically biodegradable, therefore, as well as increasing the disposal cost of sewage sludge, their presence decreases the biomethane production potential and generates a more polluted concentrate during the sludge anaerobic digestion [10,11].

Humic acids are also the main pollutants in landfill leachates. Specifically, the organic load, and toxicity, of mature leachates is largely caused by them, due to their very poor biodegradability [12]. This fact, together with the high volumes generated, explains the potential dangers of landfill leachates and the necessity to treat it so as to meet the standards for discharge in receiving waters [13].

Even when humic acids are naturally found in waters as a component of the NOM, their presence is also linked to some environmental concerns, mainly related to drinking

water quality and its treatment processes, such as a deterioration in its organoleptic properties, higher costs of desalination and disinfection or the production of harmful disinfection by-products [14,15].

Ultimately, it is evident that the removal of humic acids is necessary, whether they are present in sewage sludge, leachates or as NOM. Different methods have been studied to this end such as fungal biodegradation [12,16], adsorption [17,18] or biosorption [6], coagulation [19,20]… Among them, advanced oxidation processes appear as ones of the most promising technologies for the removal of humic acids [21]. These processes are based on utilizing in-situ generated hydroxyl and/or sulphate radicals for the degradation of pollutants [22]. This technology is found to be particularly effective for the degradation of recalcitrant compounds, such as humic acids, by increasing biodegradability and reducing toxicity. Although available literature about advanced oxidation of humic acid-containing waters (sludges, leachates, NOM…) is abundant, to the best of our knowledge, there are no studies specifically dealing with the advanced oxidation of pure humic acids, even when this information should be very useful by several reasons. For example, this knowledge enables one to study the mechanisms involved during the oxidation of humic acid, avoiding the matrix effects caused by the other compounds present in the real wastewater. Similarly, taking into account that humic acids (as part of NOM) are ubiquitous [23] in natural aquatic and terrestrial environments, they are also the main responsible for the matrix effects during the oxidation of other compounds, such as emerging contaminants or micro-pollutants in drinking water treatment [24]. Therefore, in order to understand either the oxidation of humic acids in wastewaters or their matrix effects on the oxidation of other pollutants in natural waters, examining in depth the advanced oxidation of purified humic acid is highly recommended as a first step towards achieving these objectives.

In consequence, the aim of this work is to investigate, for the first time ever, the oxidation of humic acid by hydroxyl radicals, using wet oxidation as AOP and paying special attention to the effect of the main process on either the reaction pathway or the kinetic model.

2. Material and methods

2.1. Reagents

The commercial humic acid (CAS 1415-93-6) used in the experiments was provided by Sigma-Aldrich. A 1000 ppm humic acid stock solution was prepared with

distilled water. This concentration was chosen because it is an average value among those found in old landfill leachates, a waste that is mainly composed of humic acids [25].

2.2. Experimental setup

Wet oxidation experiments were performed with a PARR 4520 series semi-batch reactor (Parr Instrument Company, Illinois, USA), of one litre capacity. The reactor was equipped with a six-bladed stirrer. The gas line included a gas humidifier of two litre capacity upstream. For security, both reactor and humidifier were filled only to 70% of their maximum capacity. A PID controller allowed to select and maintain a constant temperature in both reactor and humidifier. Pressure was handled by a back-pressure valve located at the end of the gas line. A schematic view of the reactor setup can be found in Urrea et *al* [26].

Temperatures assayed during wet oxidation experiments ranged from 180 to 220 °C and pressures, from 65 to 95 bar, these being typical in conventional wet oxidation processes [27]. Different initial pH values between 4 and 13 were tested as well, which were adjusted using HCl 1 M or NaOH 1 M.

2.3. Analytical methods

Chemical Oxygen Demand (COD) and pH measurements were carried out according to Standard Methods [28]. Colour was evaluated in terms of colour number (CN), which was calculated using equation 1 [29]:

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

where SAC_i corresponds to the spectral absorption coefficient at a wavelength of *i* nanometres. The absorbance at each wavelength was determined with an AnalitykJena Spectrophotometer. HA concentration was spectrophotometrically measured according to Lowry modified method [30] with an AnalitykJena Spectrophotometer. Total organic carbon (TOC) was obtained using a TOC analyser (Shimadzu TOC-VCSH, Japan). The Average Oxidation State of Carbon (AOSC) was obtained by applying equation 2 [31]:

$$AOSC = 4 - 1.5 \times \frac{TOC}{COD} \quad (2)$$

Attending to AOSC, is important to point out that in the text will be used the term MOC as well, which stands for Mean Oxidation number of Carbon. The difference between AOSC and MOC is that AOSC is referred to a mixture whereas MOC is used for pure compounds.

Concentrations of organic acids were determined by HPLC (Agilent 1200, Agilent Technologies Inc., California, USA) equipped with a Refractive Index Detector (RID). The column employed was a Coregel ION300 (Concise Separations, San Jose, USA), using 0.450 mM sulphuric acid (pH 3.1) as mobile phase at a flow rate of 0.3 mL min^{-1}. The following organic acids were detected and measured: formic, acetic, lactic, malic, maleic, oxalic and pyruvic.

3. Results and discussion

3.1. Effect of temperature

At first, the effect of temperature on the humic acid wet oxidation was analysed. In this regard, figure 1 shows the most relevant results obtained at three different temperatures.

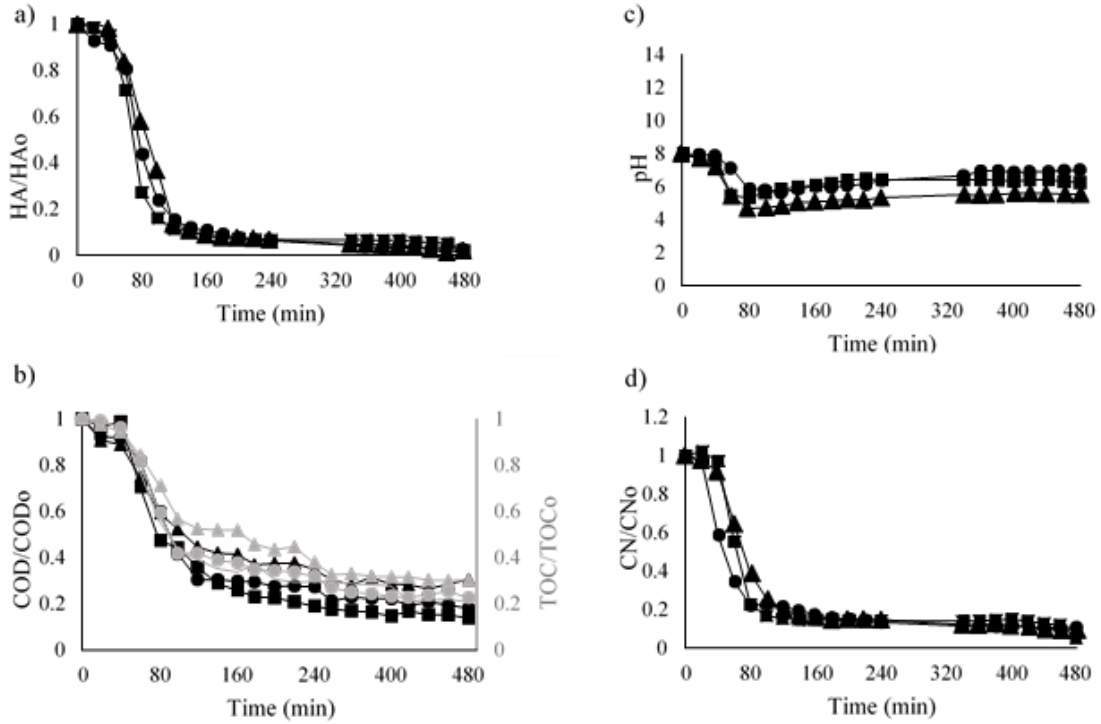


Figure 1. Evolution of: a) HA concentration, b) TOC and COD, c) pH, d) colour number during the wet oxidation of humic acids at three different temperatures: 180 ºC (▲), 200 ºC (●) and 220 ºC (■). In all cases: 80 bar, initial pH 8 and initial concentration of 1 g/l.

From figures 1a, 1b, 1c and 1d, it is easily deduced that the evolution of pH, COD, TOC, CN and HA concentration showed three distinguishable stages: an initial phase, where there was no variation; a second stage, where the corresponding parameter sharply decreased; and a final stage, where its value remained almost constant.

It should be noted that the first minutes, where no variations were observed, corresponded to the heating phase until the operating conditions were reached. The main differences in the evolution of these parameters at different temperatures were found in the second stage when oxidation reactions prevailed. The third stage was probably related to the formation and accumulation of compounds highly refractory to oxidation [32].

Focusing on the organic load, expressed as either COD or TOC (figure 1b), this did not significantly depend on temperature during the preheating period, that is to say, the first 40 minutes of treatment. However, its decrease during the second stage was remarkable, being the removal faster at high temperatures. Regarding the third stage, the values at the end of the experiment corroborated the effect of temperature on the mineralization: the higher the temperature, the higher the organic load removed (70%, 82%, 86% of COD reduction at 180 °C, 200 °C and 220 °C, respectively; and 70%, 78%, 80% of TOC eliminated at 180 °C, 200 °C and 220 °C, respectively). These values are in the range of those reported in other works carried out with molecules with similar structures to humic acids, such as lignin, with COD removals around 80% [33]. However, the TOC removals reported for lignin were lower than those here obtained for HA, probably owing to the fact that lignin is chemically more complex than humic acids.

Attending directly to HA concentration, an increase in temperature also led to a faster abatement of this; at minute 100 (the end of drop stage), 84% HA was degraded at 220 °C, 76% at 200 °C and only 60% at 180 ºC. In fact, humic acid was almost completely degraded at all temperatures if a sufficiently long reaction time is employed. This behaviour also proved that the remaining organic load is mainly due to refractory products and not to unreacted HA. The HA solutions are brownish, so its degradation is highly related to the evolution of colour number, as can be seen in figures 1a and 1d. As expected, higher temperatures led to faster CN reductions. However, there is not a direct proportionality between both parameters during the experimentation (figure S1a). It was found in the first stages that CN decreased faster than the HA concentration for all the temperatures assayed. This phenomenon could be surprising; however, it has been demonstrated that the colour of a humic acid solution is related to their size: the higher the size, the higher the colour [34]. In the final stages of the treatments (near point 0,0 in figure S1a), it can be observed that the curves went into the CN zone, indicating the presence of coloured intermediates. In this sense, a slight increase in colour was also detected during the first minutes of wet oxidation, especially at the highest temperature,

whereas the humic acid concentration slightly decreased at this time, thus suggesting the formation of highly coloured intermediates in the first instants of reaction. Other authors have reported similar behaviours when carrying out WO of debarking wastewater, which contains important amounts of lignin [35]; or phenol itself [36], for example. These molecules, as well as humic acids, are initially oxidized into benzoquinones which are highly coloured and highly toxic [37]. The fact that COD lowered faster than TOC also suggested the presence of intermediates since oxygen was being consumed faster than carbon was being released.

Regarding pH (figure 1c), it suffered an initial decrease, reaching a minimum at 100 minutes. This drop on pH values is associated with the formation of organic acids and intermediates with low pKa, that decreased the global pH value, as will be subsequently evidenced by HPLC measurements. From minutes 100 to 480, pH increased, especially for experiments at 200 °C and 220 °C. Similar results were found in other works [38,39]. This phenomenon is explained by the reaction routes of WO, which include the formation of the mentioned organic molecules and CO_2 [40]. It is crucial to point out that the reactions mentioned are not sequential, but a balance between the generation of acidic molecules and their release from the liquid phase as CO_2 after being totally oxidized. This fact is supported by the findings of other authors, who reported CO_2 generation even in the first moments of the wet oxidation of phenol solutions [40].

These results were contrasted through the measurement of acids by HPLC (table S1). It was observed the predominance of acetic acid at the end of the reaction. This molecule was also observed as a final product in almost all the studies involving wet oxidation [33,41,42]. Increasing the temperature had a positive effect on the formation of acetic, lactic, maleic and oxalic acid and a negative one on formic, malic and pyruvic. The AOSC is a parameter that is strongly related to organic acids. As expected, AOSC was increased in all temperatures tested, being this increase higher at higher temperatures, corroborating the results mentioned for organic acids production, which have positive MOC values (excepting acetic acid which have a MOC = 0).

In conclusion, higher temperatures involved an increase in COD and TOC reductions and led to a faster formation of organic acids, except for formic, pyruvic and malic acids, which were less stable at high temperature.

3.2. Effect of pressure

Figure 2 show the main results obtained during the WO of humic acid at different pressures.

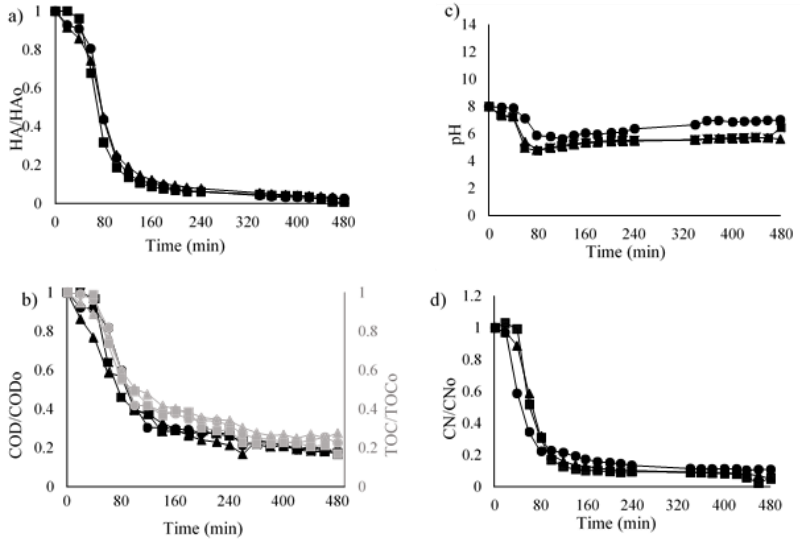


Figure 2. Evolution of: a) HA concentration, b) TOC and COD, c) pH, d) colour number during the wet oxidation of humic acids at three different pressures: 65 bar (▲), 80 bar (●) and 95 bar (■). In all cases: 200 °C, initial pH 8 and initial concentration of 1 g/l.

As in the case of temperature, the results for pH, COD, TOC, CN and HA concentration during the wet oxidation of commercial humic acid at different pressures showed again the previously explained three phases. Nevertheless, the effects of different pressures on the evolution of these three stages were negligible (figure 2b). So, an 84% of COD reduction was achieved at the end of the experiments, regardless of pressure. These results were similar to those reported by other authors, where pressure was not found to be significant in the COD removal of other organic materials, like sewage sludge, which is rich in humic acids [43,44].

The effect of pressure on the non-identified intermediates was also negligible, since a 49%, 51% or 54% of final COD values (at 65 bar, 80 bar and 95 bar, respectively) were attributed to the organic acids identified.

The evolution of pH (figure 2c) showed again similar values for 65 bar and 95 bar and a totally different evolution for 80 bar, probably due to the different distribution of acids obtained, predominating at this pressure those with higher pKa, such as malic acid.

To sum up, the use of higher pressures during the wet oxidation of humic acid did not show improvements in the evolution of either COD, TOC or CN.

3.3. Effect of initial pH

Finally, the effect of the initial pH on the wet oxidation of humic acid was analysed as well, testing initial pH values ranging between 4 and 13 (figure 3).

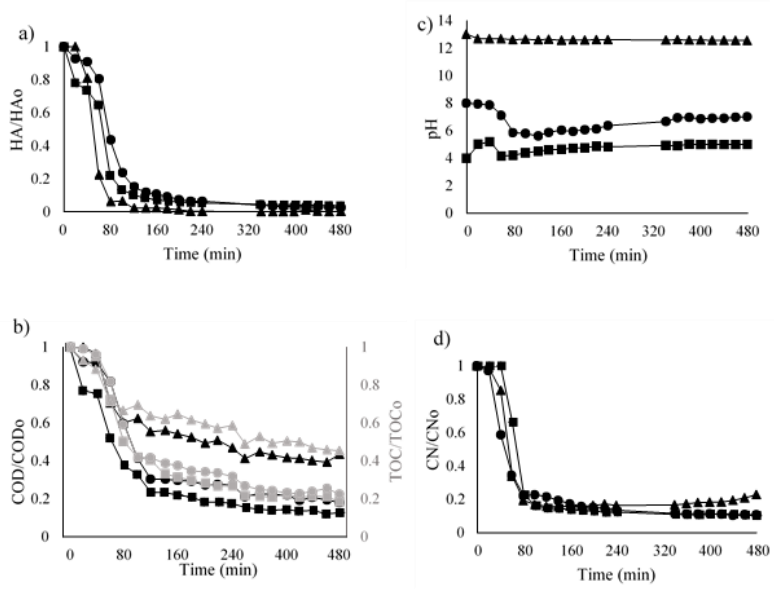


Figure 3. Evolution of: a) HA concentration, b) TOC and COD, c) pH, d) colour number during the wet oxidation of humic acids at three different initial pH values: 13 (▲), 8 (●) and 4 (■). In all cases: 200 °C, 80 bar and initial concentration of 1 g/l.

The results revealed that initial pH was the variable with the highest impact on the wet oxidation of commercial humic acid. As in the case of temperature and pressure sections, the evolutions of COD, TOC and HA followed the three phases already explained. In terms of COD, an acidic initial pH of 4 showed the highest mineralization (88% of initial COD was abated after 480 minutes), whereas the higher the initial pH, the lower the COD reduction, with COD reductions of 72% and 60% at initial pH values of 8 and 13, respectively. This behaviour was also corroborated by the TOC evolution; the reductions in this parameter were 55%, 78% and 82% for initial pH values of 13, 8 and 4, respectively. Studies dealing with the WO of phenolic compounds also obtained lower COD and TOC reductions in alkaline media [45].

Surprisingly, just attending to humic acid concentration, adjusting initial pH to 13 led to its fastest removal, achieving a complete abatement of the compound in 120 minutes, whereas for the other pH values tested, although degradation was high, the removal rates were slower (95% of HA degraded for initial pH 4 and 8 in 120 minutes) than at an initial pH of 13. In all cases, colour was quickly reduced (80% of the initial CN

after 100 minutes). However, it is interesting to point out that some colour remained at the end of the treatments, especially in those experiments carried out in alkaline medium. In fact, at pH 13, CN even increased in the last minutes. This is associated with the formation of coloured intermediates (phenolic derivates), as has been already mentioned during the discussion about the effect of temperature. Comparing humic acids and colour number evolutions (figure S1c), it was found that the removal of humic acid was faster than the colour reduction when an initial pH of 13 was selected, whereas this tendency reversed when the initial pH was 8. At acidic pH, the reduction of HA and colour was proportional during the experiment, indicating the no formation of a significant amount of coloured intermediates at acidic pH, thus also suggesting a less toxic degradation pathway of humic acid at lower pH [46].

These compounds (benzoquinones, hydroquinones…) are easily oxidized toward colourless molecules in neutral or acidic media, but their stability is increased at alkaline pH. In this light, it has been reported that phenolic molecules were much more slowly degraded at strong alkaline conditions [47], thus maintaining the colour we detected even at long reaction times. This increase can be linked with the increase in the CN at the same time.

The evolution of the oxidation state, measured as AOSC, was similar for the three pHs assayed. As the experiment advanced, the generation of oxidized molecules, such as organic acids, raised AOSC values. This increase was particularly high for the experiment at pH = 4 since it started at negative a value (-0.7) and at the end of the experiment it turned into 0.75. The surprisingly low initial value could be due to the low pH, that led to the precipitation of a part of humic acids, lowering the COD value and therefore the AOSC value.

Regarding organic acids production, an alkaline medium strongly favoured the generation of some organic acids such as formic, lactic or pyruvic; whereas an acidic one led to worse generation, even some acids were not detected, for instance, pyruvic acid. These behaviour has already been reported in other works and is due to the effect of pH in the dissociation of the acids [45]. Moreover, our results coincide with works where phenol was subjected to WO. Santos et. *al* reported an enhancement in the production of formic, oxalic and pyruvic acid employing a catalytic WO treatment [48]. For example, at acidic pHs, formic acid and not formate was present, thus being easily degradable via

non-oxidative thermal treatment and also via oxidation but, at the dissociated form, it is hard to degrade, because the only possible way is an oxidation reaction [49].

As seen in the previous paragraphs, there is a visible difference in the behaviour of all the parameters when an alkaline medium was employed. Excepting for HA, all parameters showed worse results, in terms of mineralisation and structure breakage. Moreover, the production of some organic acids was enhanced. This is mainly related to two phenomena. On the one hand, it has been reported that alkaline pH strongly affected the reaction pathways in wet oxidation experiments, since WO reactions are known to occur via free radical reactions [50] and the generation rate of these radicals is slower at alkaline pH [51]. On the other hand, humic acids tend to form pseudomicelles as pH decreases [52], so they are more difficult to attack.

In conclusion, the effect of pH on the WO of humic acid is clear. An alkaline pH led to worse mineralization and humic acids were completely degraded into coloured intermediates. In contrast, it allowed to obtain a higher concentration of organic acids at the end of the treatment. An acidic pH caused high COD and TOC reductions, but it also led to a lower final concentration of organic acids.

3.4. Kinetic modelling

Based on the findings, a lumped kinetic model was proposed, assuming the sequences of reactions shown in Figure 4. According to the reaction pathway proposed, the commercial HA is initially oxidized into non-identified intermediates (reaction 1) or directly oxidized into organic acids and CO_2, (reaction 3). In turn, the non-identified intermediates can be further degraded to produce organic acids (reaction 2). Finally, organic acids can be completely mineralized into CO_2 (reaction 4).

The concentrations of humic acids, non-identified intermediates and organic acids were expressed as COD in order to simplify the calculations. The COD of HA and organic acids were calculated by using their theoretical COD, which can be easily obtained from the stoichiometry for the complete oxidation of the corresponding molecule to CO_2 and H_2O. The COD values for CO_2 (COD_{CO_2}) and non-identified intermediates (COD_{Int}), these were calculated according to the following equations:

$$COD_{Int} = COD_{T} - COD_{HA} - COD_{OA} \quad (3)$$

$$COD_{CO_2} = COD_{TI} - COD_{Tt} \quad (4)$$

where COD_{Int} is the COD of intermediates, COD_T is the total COD at any time, COD_{HA} and COD_{OA} are the COD values due to humic acid and organic acids, respectively, COD_{TI} is the initial total COD value and COD_{Tt} is the total COD value at each time.

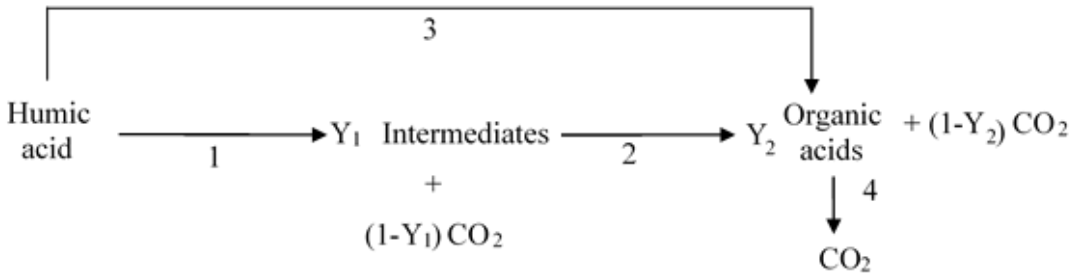


Figure 4. Scheme of the reactions proposed for the WO of humic acids. Numbers in the reactions indicate: 1, humic acids oxidation into non-identified intermediates and CO_2; 2, oxidation of the intermediates into organic acids and CO_2; 3, oxidation of humic acids directly into organic acids and CO_2; 4, oxidation of organic acids into CO_2. Y_1 and Y_2 are coefficients that represent the part of humic acid and intermediates that are converted into intermediates and organic acids respectively.

The software employed to obtain the kinetic constants was Micromath Scientist, by adjusting the model to the data obtained from the experiments at different temperatures or initial pH values.

3.1.1. Effect of temperature

According to the reaction pathway proposed, the next kinetic equations were deduced:

$$r_{COD_{HA}} = - k_{o1} \cdot e^{\left(\frac{-Ea1}{R \cdot T}\right)} \cdot COD_{HA} - k_{o3} \cdot e^{\left(\frac{-Ea3}{R \cdot T}\right)} \cdot COD_{HA} \quad (5)$$

$$r_{COD_{Int}} = Y_1 \cdot k_{o1} \cdot e^{\left(\frac{-Ea1}{R \cdot T}\right)} \cdot COD_{HA} - k_{o2} \cdot e^{\left(\frac{-Ea2}{R \cdot T}\right)} \cdot (COD_{Int} - COD_{Int,R}) \quad (6)$$

$$r_{COD_{OA}} = Y_2 \cdot k_{o2} \cdot e^{\left(\frac{-Ea2}{R \cdot T}\right)} \cdot (COD_{Int} - COD_{IntR}) + k_{o3} \cdot e^{\left(\frac{-Ea3}{R \cdot T}\right)} \cdot COD_{HA} - k_{o4} \cdot e^{\left(\frac{-Ea4}{R \cdot T}\right)} \cdot COD_{OA} \quad (7)$$

It is important to point out that, as can be checked in figure 1b, some COD remained after the wet oxidation treatment. This residual value was taken into account during the modelization, as COD_{IntR}. The proposed model was successfully fitted to the experimental data, as can be seen in figures S2a, S2b and S2c.

Table 1. Parameters of the Equations (5, 6 and 7), estimated from the kinetic constants. In all temeperatures COD_{IntR} / COD_{TI} was 0.1.

Reactions (i)	$\overline{K_{0i}}$ **(s^{-1})**	$\overline{Ea_i}$ ***(J/mol)***	$\overline{Y_i}$
1	$(3.4 \pm 0.1) \times 10^5$	$(6.17 \pm 0.01) \times 10^4$	0.32 ± 0.05
2	$(3.4 \pm 0.2) \times 10^4$	$(5.07 \pm 0.07) \times 10^4$	0.40 ± 0.10
3	*Not applicable.*		
4			

From the fitting parameters obtained (table 1), several findings can be deduced. Thus, the main reactions of the mechanism were the direct oxidation of humic acid to non-identified intermediates (1) and the subsequent oxidation of these to organic acids (2). The low value of the kinetic constants 3 and 4 revealed that the direct transformations of humic acid into organic acids (3), as well as the degradation of organic acids into CO2 (4), were no significant. The values obtained for Y_1 and Y_2 in combination with the high *k* for reactions 1 and 2 and the low *k* value obtained for reaction 4, suggest that the oxidation of the humic acid into intermediates is the main responsible for the CO_2 generation.

3.4.2. Effect of pressure

As explained in previous paragraphs, pressure did not produce noticeable effects on the COD values. For this reason, the effect of pressure was not modelled, since in all cases the results would have been similar to the results obtained for the modelling of the wet oxidation at 200 °C, 80 bar and initial pH equal to 8.

3.4.3. Effect of pH

Regarding the modelling of the experimental data obtained at different initial pH values (section 3.3), and due to the strong effect of an alkaline or acidic media in the WO of humic acid, it is necessary to take into account the effect of the different initial pH values tested on the kinetic model. The high effect of the pH on the wet oxidation suggests that the reaction mechanisms may well be different depending on the pH, so activation energies previously calculated for pH 8 cannot be extrapolated to pH 4 or, particularly, pH 13. Therefore, the data at different pH were employed to fit the kinetic model, assuming that the temperature remained constant during the experimentation, thus obtaining the pseudo-first kinetic constants instead of the corresponding pre-exponential factors and activation energies. Therefore, the next equations were proposed:

$$r_{COD_{HA}} = - k_1 \cdot COD_{HA} - k_3 \cdot COD_{HA} \quad (8)$$

$$r_{COD_{Int}} = Y_1 \cdot k_1 \cdot COD_{HA} - k_2 \cdot (COD_{Int} - COD_{Int,R}) \quad (9)$$

$$r_{COD_{OA}} = Y_2 \cdot k_2 \cdot (COD_{Int} - COD_{IntR}) + k_3 \cdot COD_{HA} - k_4 \cdot COD_{OA} \quad (10)$$

This model was successfully fitted to the experimental data, as can be observed in figures S3a, S3b and S3c. Table 2 shows the values of the kinetic constants calculated at the different pH values tested.

Table 2. Parameters of the Equations (8, 9 and 10), estimated from the kinetic constants. COD_{IntR} values from figure 3b are showed as well.

Parameter	***pH 4*** $\bar{x}$	***pH 8*** $\bar{x}$	***pH 13*** $\bar{x}$
k_1	5.9×10^{-2}	5.2×10^{-2}	2.0×10^{-1}
k_2	2.3×10^{-2}	8.4×10^{-2}	2.5×10^{-3}
k_3	~0	~0	~0
k_4	~0	~0	1.4×10^{-2}
Y_1	0.29	0.32	0.48
Y_2	0.35	0.40	0.05
COD_{IntR}	0.02	0.11	0.23

Observing the results in table 2, it can be corroborated that the alkaline medium enhanced the oxidation of the humic acid, being k_1 at pH 13 two-fold higher than at pH 8 or pH 4. However, reaction 2 is less favoured at higher pH values. As in the case of the effect of temperature, the conversion of humic acid to CO_2 was found negligible at any pH. Unexpectedly, at alkaline pH values, the final oxidation of organic acids to carbon dioxide (reaction 4) was significant, owing to the decomposition of organic acids to formic acid, a molecule that cannot be easily oxidized at alkaline pHs [45].

In alkaline medium, the production of carbon dioxide was mainly associated with the oxidation of the non-identified intermediates, as Y_2 coefficient shows. Nevertheless, the production of CO_2 from the direct oxidation of humic acids was also significant in acidic medium. The effect of the different pH in the structure of humic acids and the formation of free radicals has been profusely discussed in the effect of pH section, so those explanations can also be applied here.

4. Conclusions

Results revealed that humic acid can efficiently be removed by means of wet oxidation for all the conditions tested. It was found that the higher the temperature, the faster the humic acid removal and the mineralization, although around a 10% of the initial COD always turned out to be refractory to oxidation. High temperatures also involved a

higher decolourization of the medium, enhancing the formation of acetic and oxalic acids as final products and reducing the stability of formic and pyruvic ones

On the other hand, pressure effect was negligible during the humic acid wet oxidation in the range tested (65 bar – 95 bar), not observing better mineralization or a faster humic acid degradation.

Finally, the initial pH turned out to be the key parameter during the wet oxidation of humic acids. When oxidation was carried out in an alkaline medium, humic acid was faster oxidized, but also gave place to more refractory intermediates than those obtained at either neutral or acidic media, as well as favoured the formation of organic acids as final products.

Finally, a reaction pathway based on the sequential oxidation of humic acids → quinone-like compounds → organic acids → carbon dioxide was proposed on the basis of the experimental observations and employed to deduce the corresponding kinetic model, which was successfully fitted to the experimental data.

5. Acknowledgements

The authors are grateful for the financial support from the Employment, Industry and Tourism Office of Principality of Asturias (Spain) through project GRUPIN IDI/2018/000127. Authors also acknowledge the financial support from the Spanish Ministry of Economy and Competitiveness (MINECO) through Project MCIU-19-RTI2018-094218-B-I00 and FEDER funds from European Union.

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SUPPLEMENTARY MATERIAL

THE WET OXIDATION OF AQUEOUS HUMIC ACIDS

*Manuel García[a], Sergio Collado[a], Paula Oulego[a], Mario Díaz[a]**

[a] Department of Chemical and Environmental Engineering. University of Oviedo.

C/ Julián Clavería s/n. E-33071 Oviedo, Spain. Tel.:34985103439; Fax: 34985103434

* Mario Díaz. Department of Chemical and Environmental Engineering. University of Oviedo.

C/ Julián Clavería s/n. E-33071 Oviedo, Spain. Tel.: +34 985 10 34 39; Fax: +34 985 10 34 34 mariodiaz@uniovi.es

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intermediates COD, (- ·) is the calculated COD of organic acids and (· ·) is the calculated COD loss as CO_2.

Figure S3. Fitting of the model to the experimental data for the different initial pH values: a) initial pH = 8, b) initial pH = 13 and c) initial pH = 4. In all cases ● represents experimental points of total COD, ■ is the experimental values for COD of intermediates, ▲ is the COD of organic acids, ♦ shows the COD loss as CO_2, (—) is the calculated total COD, (- -) is the calculated intermediates COD, (- ·) is the calculated COD of organic acids and (· ·) is the calculated COD loss as CO_2.

S.1. Comparison between humic acids and CN decrease

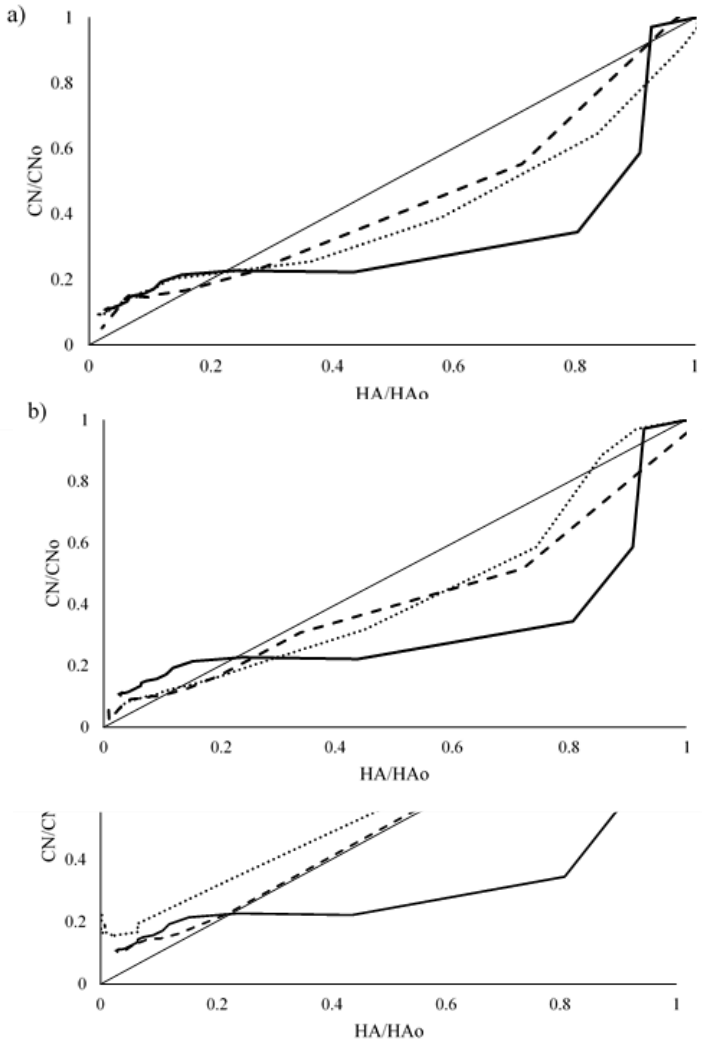
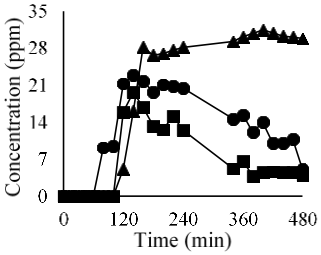
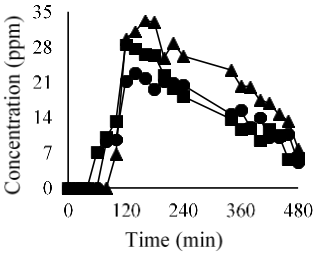
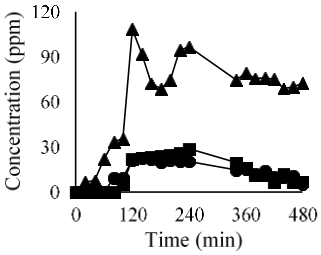
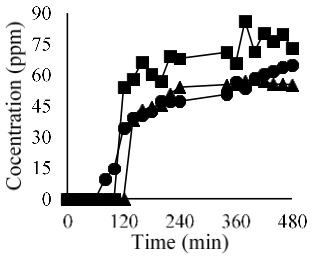
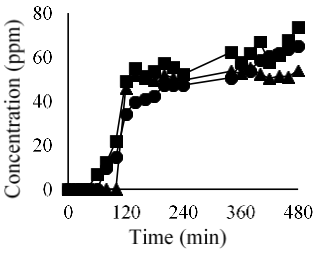
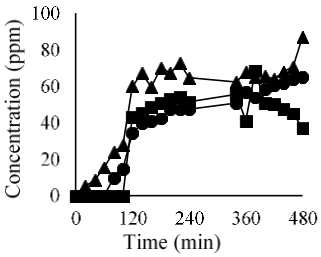
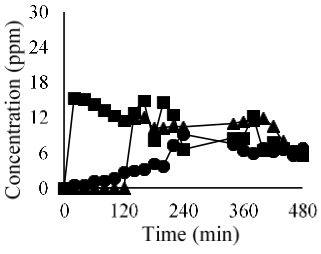
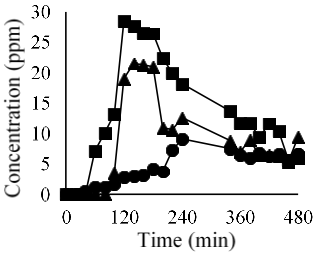
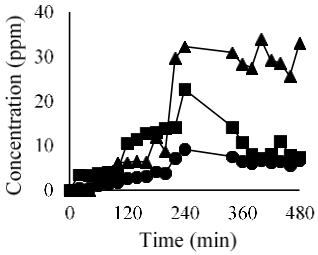
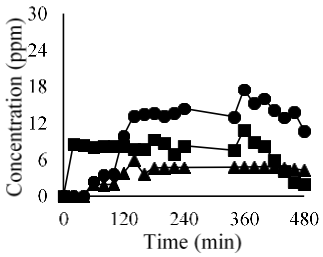
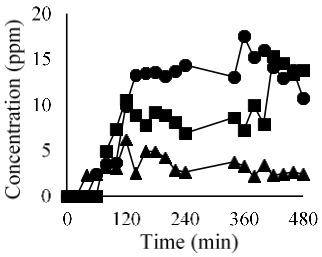
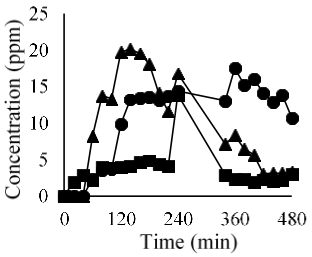
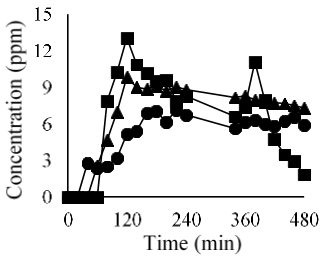
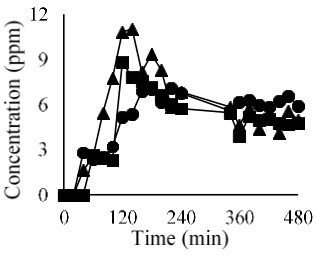
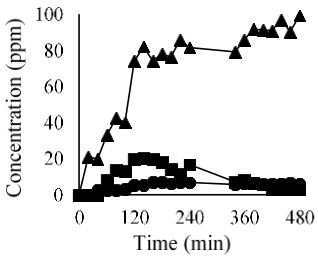


Figure S1. Effect of a) temperature: 200 °C (**—**), 220 °C (**- -**) and 180 °C (**· ·**), in all cases pressure was 80 bar and initial pH was 8; b) pressure: 80 bar (**—**), 95 bar (**- -**) and 65 bar (**· ·**), in all cases temperature was 200 °C and initial pH was 8; and c) pH: 8 (**—**), 4 bar (**-**

-) and 13 (· ·), in all cases temperature was 200 °C and pressure was 80 bar; on the relationship between HAs and CN.

S.2. Organic acids production

Table S1. Effect of the different conditions on the evolution of the concentration of the measured organic acids. In effect of temperature: ● is 200 °C, ■ is 220 °C, ▲ is 180 °C; in all cases pressure was 80 bar and initial pH was 8. In effect of pressure: ● is 80 bar, ■ is 95 bar, ▲ is 65 bar; in all cases temperature was 200 ºC and initial pH was 8. In effect of pH: ● is initial pH = 8, ■ is initial pH = 4, ▲ is initial pH = 13; in all cases temperature

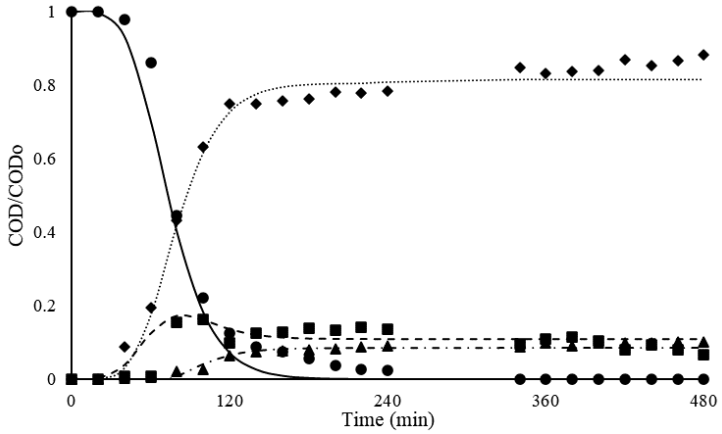
Acid	*Effect of temperature*	*Effect of pressure*	*Effect of initial pH*
Formic			
Acetic			
Lactic			
Malic			
Maleic			

was 200 °C and pressure was 80 bar. Y axes have been deliberately adjusted to improve the view of the figures.

S.3. Modelling

S.3.1. Effect of temperature

a)



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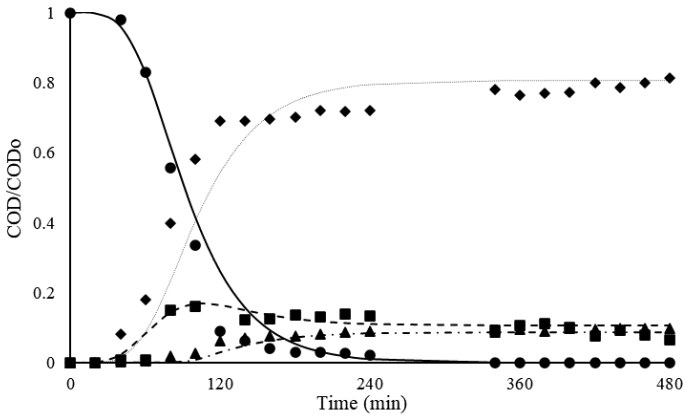

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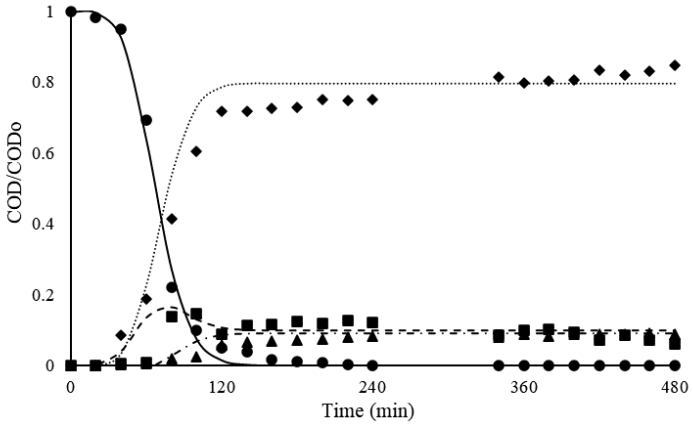
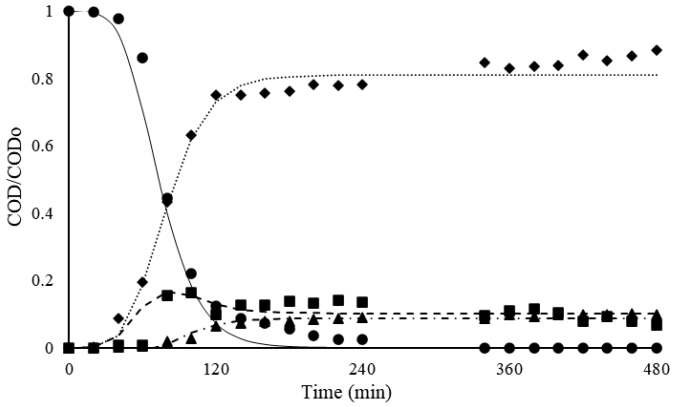


Figure S2. Fitting of the model to the experimental data for the different temperatures: a) 200 °C, b) 220 °C and c) 180 °C. In all cases ● represents experimental points of total COD, ■ is the experimental values for COD of intermediates, ▲ is the COD of organic acids, ♦ shows the COD loss as CO_2, (—) is the calculated total COD, (- -) is the calculated

intermediates COD, (- ·) is the calculated COD of organic acids and (· ·) is the calculated COD loss as CO_2.

S.3.2. Effect of pH

a)



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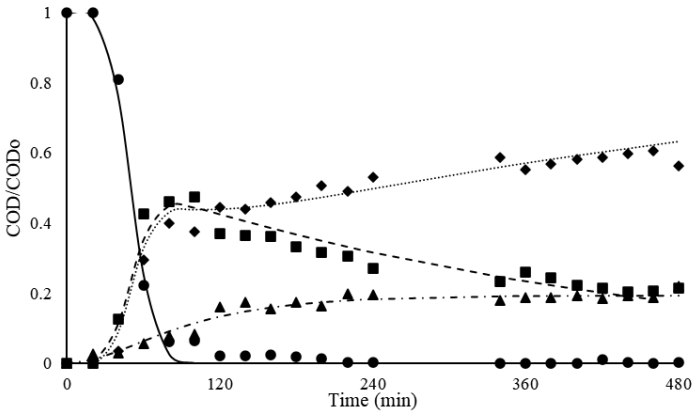

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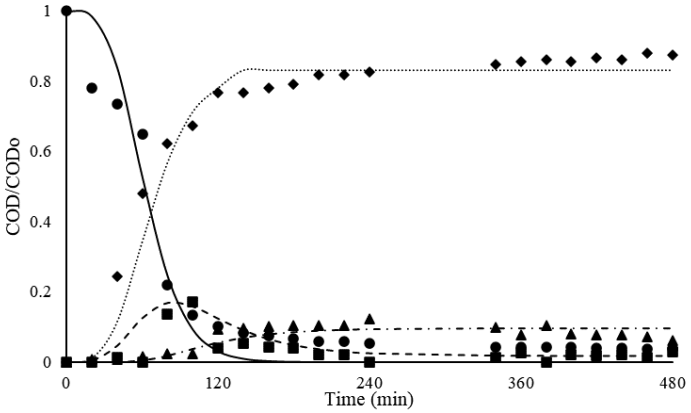


Figure S3. Fitting of the model to the experimental data for the different initial pH values: a) initial pH = 8, b) initial pH = 13 and c) initial pH = 4. In all cases ● represents experimental points of total COD, ■ is the experimental values for COD of intermediates, ▲ is the COD of organic acids, ♦ shows the COD loss as CO_2, (—) is the calculated total

COD, (- -) is the calculated intermediates COD, (- ·) is the calculated COD of organic acids and (· ·) is the calculated COD loss as CO_2.

4.5. USO DE HIDROLIZADOS DE LODOS. PRODUCCIÓN DE ENZIMAS

Publicación: Manuel García, Sergio Collado, Paula Oulego, Mario Díaz. Proteases and laccases production by *Bacillus licheniformis* from hydrolysed sludge
Estado: enviado para ser considerada su publicación

Con la información obtenida en la caracterización de los tratamientos hidrotérmicos de los lodos de depuradora, se pudo comprobar que los hidrolizados son ricos en proteínas y carbohidratos, así como en ácidos húmicos. Estas moléculas son potenciales fuentes de carbono, por lo que es factible utilizar el hidrolizado como medio para fermentaciones que permitan obtener productos de interés. Es necesario indicar que los hidrolizados utilizados se obtuvieron al tiempo adecuado de tratamiento obtenido en el trabajo del aparatado 4.2. Por ello, se plantea la inoculación del hidrolizado con un microorganismo capaz de sobrevivir en los hidrolizados, para lo que debe ser capaz de asimilar proteínas y ácidos húmicos, es decir, secretar proteasas y lacasas que le permitan utilizar las proteínas y los ácidos húmicos. Además, estas enzimas tienen alto valor comercial, pues las proteasas son ampliamente usadas, por ejemplo, en la industria de detergentes, en industria texil, industria química; y la lacasa se puede usar en descontaminación de aguas textiles o en industria alimentaria (Razzaq et al., 2019; Rodríguez Couto y Toca Herrera, 2006).

Uno de los microorganismos que cumple estas características es *Bacillus licheniformis* CECT 20. Además, es de nivel de bioseguridad 1, su genoma está secuenciado y su cultivo es sencillo.

Por lo tanto, el objetivo de este capítulo es evaluar la posibilidad de utilizar el hidrolizado obtenido tras la OH y la HT de lodos de depuradora como medio de fermentación para obtener enzimas de interés, abriendo así otra vía para la revalorización del lodo.

La publicación inicial de este trabajo fue como comunicación oral en el congreso "5th EUROPEAN CONFERENCE ON SLUDGE MANAGEMENT (ECSM'2019), OCTOBER 2019, Liège", a partir de la cual fue seleccionada para un número especial en *Journal of Environmental Management*.

Proteases and laccases production by *Bacillus licheniformis* from hydrolysed sludge

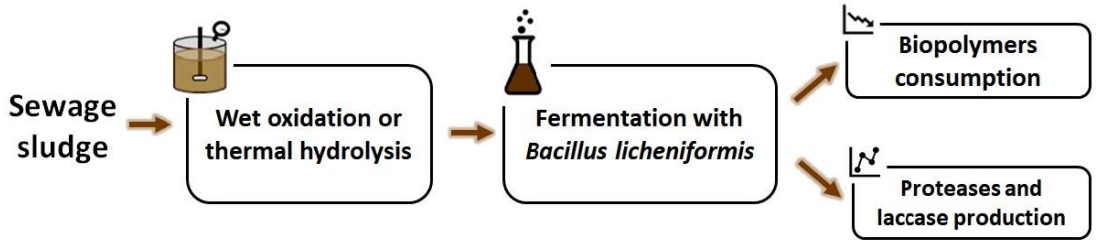
*Manuel García[a], Sergio Collado[a], Paula Oulego[a], Mario Díaz[a]**

Department of Chemical and Environmental Engineering. University of Oviedo.

C/ Julián Clavería s/n. E-33071 Oviedo, Spain. Tel.:34985103439; Fax: 34985103434

* Mario Díaz. Department of Chemical and Environmental Engineering. University of Oviedo.

C/ Julián Clavería s/n. E-33071 Oviedo, Spain. Tel.: +34 985 10 34 39; Fax: +34 985 10 34 34 mariodiaz@uniovi.es



HIGHLIGHTS

B. licheniformis grows on raw hydrothermally hydrolysed sludge.

Oxygen during sludge solubilisation generated inhibitory compounds for *B. licheniformis*.

Preferential assimilation: carbohydrates → proteins → humic acids.

Hydrolysate from thermal hydrolysis favoured protease production.

Hydrolysate from wet oxidation favoured laccase production.

ABSTRACT

Currently, new perspectives focused on recovering valuable compounds are being opened in the management of sewage sludge. One of these is the application of hydrothermal treatments to break the sludge and produce soluble biomolecules, which in turn can be used as substrates for different microbial bioprocesses. In this work, the use of sewage sludge hydrolysates, obtained by hydrothermal treatments in presence (wet oxidation) or absence (thermal hydrolysis) of oxygen, as culture media for the production of enzymes with industrial interest, such as proteases and laccases, by *Bacillus licheniformis* CECT 20 has been assessed for the first time.

Results revealed that this species was able to properly develop using sludge raw hydrolysates as substrate, with any kind of nutrient supplementation, sterilization, or previous dilution. Hydrolysate from thermal hydrolysis was more easily assimilated by *B. licheniformis* than the corresponding to wet oxidation because the presence of oxygen during the hydrothermal treatment promoted the formation of inhibitory substances. *B. licheniformis* exhibited a preferential assimilation of simple carbohydrates during the lag phase, proteins in the log phase and humic acid concentration only during the last hours of fermentation. Maximum protease activity (878 U/mL) was obtained after 72 h using hydrolysate from thermal hydrolysis, whereas the highest laccase activity (234 U/OD) was reached after 192 h of fermentation of the wet oxidation hydrolysate. It was also deduced that the *B. licheniformis* metabolic machineries related to the production of proteases and laccases are enhanced by the presence of complex carbohydrates and, in the case of the laccases, also by a high denaturation of the proteins during the hydrothermal treatment.

Therefore, new ways for sludge management are opened with this study, achieving two main goals of the sector: sludge minimization by hydrothermal treatments and its revalorization by enzyme production.

KEYWORDS

Protease, laccase, sewage sludge, *Bacillus licheniformis*, fermentation, hydrothermal treatments

Introduction

Sewage sludge generation is an issue that usually goes unnoticed, but in fact, it is an important environmental problem of large dimensions. For instance, more than 13 million tons of dry sludge will be produced in the UE in 2020 (Collivignarelli et al., 2019). Due to its characteristics of low dewaterability, high organic loads (Neyens and Baeyens, 2003) and the potential presence of hazardous materials, such as heavy metals (Pathak et al., 2009) or pathogenic organisms, sludge management represents a hard challenge, not only in operational terms but also in the economic balance, since it reach in many cases the 50% of the total operation cost of a conventional wastewater treatment plant (WWTP) (Neyens et al., 2004). Traditionally, several methods have been carried out to treat the sewage sludge, such as landfilling, incineration, anaerobic digestion, composting… However, these are mainly focused on the sludge stabilization as a primary aim, thus obtaining final products with a low added value.

Currently, sludge management is open to new strategies that allow not only the sludge minimization but also a higher valorisation. Some of them include acid pretreatment coupled with dark fermentation to obtain hydrogen (Ilgi and Onur, 2020), microbial electro-hydrolysis to recover both volatile fatty acids and hydrogen (Naresh Kumar et al., 2019), multi-step treatment ($FeCl_3$ mediated coagulation, alkaline fermentation and Mg^{2+} precipitation) to recover volatile fatty acids and phosphorus (Chen et al., 2019) or enzymatic treatment to produce bioethanol or lactic acid in sludge from paper industry (Kaur et al., 2020). In terms of management strategies, the origin of sludge plays an important role in the choice of the most adequate treatment and subsequent revalorization steps.

In this regard, it is interesting to remind that sewage sludge, as a biological material, is mainly composed of biomolecules, such as proteins, humic acids or carbohydrates (Chen et al., 2007), so it can be a cheap rich fermentation medium for the growth of a wide range of microorganisms with industrial relevance, once it has been hydrolysed. Among the available technologies for the solubilisation of the sludge, ones of the most attractive are the hydrothermal treatments, which are based on the cell lysis by means of high temperature and pressure conditions (above the normal boiling point of water), usually over 150 °C and 40 bar, in absence (Thermal Hydrolysis, TH) or presence (Wet Oxidation, WO) of oxygen (Hii et al., 2014). Due to conditions employed, hydrothermal treatments can affect negatively to the economic balance of the WWTP.

For this reason, strategies to revalorize the effluents are currently being studied, for example, by recovering chemicals or biopolymers (García et al., 2017; Suárez-Iglesias et al., 2017). From the point of view of a subsequent fermentation, the hydrothermal treatments, in addition to a high solubilisation, also have other advantages such as obtaining an already sterilized liquid or with an increased biodegradability (Donoso-Bravo et al., 2011; Suárez-Ojeda et al., 2007).

Bibliography dealing with the use of thermally hydrolysed sludge as a fermentation medium for the microbial production of relevant metabolites beyond the conventional anaerobic digestion to produce biogas (Bertanza et al., 2015) is scarce. Available studies include the production of interesting metabolites such as biohydrogen (Guo et al., 2008), polyhydroxyalkanoates (Zhang et al., 2019), volatile fatty acids (Morgan-Sagastume et al., 2011) or bioethanol from sewage sludge previously saccharified by microbial action (Manyuchi et al., 2018).

In this regard, it is surprising to notice that the growth of *Bacillus* species using hydrolysed sludge as culture medium has scarcely been studied. *Bacillus* species are the preferred microorganisms for industrial bioprocesses due to their advantageous features, such as their good growth on cheap carbon sources, robustness and availability or their superior capacity of proteins and enzymes secretion (Gu et al., 2018; Longo et al., 1999). Particularly, *Bacillus licheniformis* is one of the most attractive species of this genus, due to its wide enzymatic machinery. Genome analysis has proved that *B. licheniformis* is able to produce enzymes to break down different complex carbon sources, such as cellulose, hemicellulose or chitooligosaccharides. Moreover, this microorganism is also able to extract nitrogen from proteins, oligopeptides or amino acids by means of the production of proteases (Rey et al., 2004). Furthermore, *B. licheniformis* is known to produce laccases, oxidases capable of oxidising several phenolic and polyphenolic substrates by reducing oxygen to water (Thurston, 1994). Among the huge range of potential metabolites produced by *Bacillus licheniformis*, proteases and laccases are highly demanded by industry due to their wide array of applications and great biotechnological potential. Thus, proteases are commonly used in management of proteinaceous wastes, detergents, food industry, leather industry or even in molecular biology (Razzaq et al., 2019). For their part, laccases are useful in the treatment of polluted wastewaters such as dye manufacturing effluents or water and soil bioremediation (Cho et al., 2011; Muthukumarasamy et al., 2015). Other applications of

laccases include delignification in papermills or drug detection (Mayer and Staples, 2002).

Therefore, the aim of this article is to assess the use of thermally hydrolysed sewage sludge as a low-cost substrate for the simultaneous production of enzymes of industrial relevance (proteases and laccases) by fermentation with *Bacillus licheniformis.*

Material and methods

Characteristics of the sludge used in the experiments

A secondary sludge thickened by flotation and collected in a WWTP in Asturias (Spain) was used in this study. This sludge showed the following initial characteristics: pH = 6.74±0.1; total suspended solids (TSS) = 35.2±0.5 g/L; volatile suspended solids (VSS) = 30±1 g/L; total chemical oxygen demand (tCOD) = 39.70±0.02 g O_2/L; soluble chemical oxygen demand (sCOD) = 5.10±0.05 g O_2/L; and soluble total organic carbon (TOC) = 665±2 mg C/L. Attending to initial concentrations of soluble biopolymers, the sludge presented 6.11±2.61 mg of soluble proteins per g of VSS, 18.6±4.59 mg of soluble humic acids per g of VSS and 5.00±2.25 mg of soluble carbohydrates per g of VSS.

Hydrothermal treatment of the sludge

Sludge was subjected to a WO treatment at 160 °C and 40 bar, with these parameters being typical for this kind of treatments (Hii et al., 2014). The selected conditions respond to a balance between sludge solubilisation and biopolymers damage: mild conditions reduce the solubilisation of the sludge whereas the structure and properties of the solubilised biopolymers could be affected if the treatment is too severe. Oxygen was employed as oxidizer during wet oxidation treatments whereas nitrogen provided an inert atmosphere during the thermal hydrolysis treatment.

A PARR 1520 series reactor of 1 litre of capacity equipped with a 6-bladed stirrer was employed. Before being injected into the reactor, the corresponding gas stream was previously bubbled throughout a humidifier upstream to avoid water losses in the reactor due to evaporation. Both reactor and humidifier were filled until a 70% of their maximum capacity attending to safety considerations. The reactor pressure was manually controlled by a backpressure valve placed at the end of the gas line. Temperatures of humidifier and reactor, as well as the stirrer speed (150 rpm) and the gas flowrate (1200 mL/min), were controlled by a PID controller.

Regarding the hydrothermal treatment time, this was selected attending to data obtained in previous studies carried out in our group (García et al., 2017). It should be

pointed out that proteases work recognising specific amino acid sequences in proteins. Therefore, if the sludge hydrothermal treatment is carried out during an excessive time, these enzymes will not work properly due to the protein denaturation and breakage. This fact can also be applied to other biomolecules, such as humic acids or carbohydrates: if the WO treatment significantly changes the structure of the biopolymer, it will be less probable that the corresponding enzyme recognises its structure. For this reason, the most adequate hydrothermal treatment time in order to use the hydrolysate obtained as substrate turned out to be 70 minutes, since this represents the maximum concentration of protein solubilized and it does not involve a high denaturation of the biopolymers generated.

Microorganism selection

Due to the predictable high concentrations of proteins and humic acids in the sludge hydrolysate, a microorganism with both protease and laccase activities was selected. In order to simplify the downstream processing, it was proposed that at least one of the enzymes should be intracellular. Other requirements taken into account during the selection of the microorganism were a rapid growth, low nutritional demands and level 1 of security. On this basis, *Bacillus licheniformis* CECT 20 was selected as model strain for this study.

Hydrolysate fermentation

As previously explained, the medium chosen for the fermentation was the sludge hydrolysate corresponding to 70 minutes of hydrothermal treatment. The hydrolysates thus obtained showed the characteristics indicated in table 1:

Table 1. Initial concentrations of soluble biopolymers in sludge after being hydrolysed for 70 minutes at 160 °C and 40 bar.

	TH hydrolysate	***WO hydrolysate***
Proteins (mg/l)	5548.61 ± 400.24	3400.89 ± 126.04
Humic acids (mg/l)	4235.19 ± 1100.07	3165.24 ± 168.46
Carbohydrates (mg/l)	1870.64 ± 392.67	1876.31 ± 332.54

Before being inoculated, the hydrolysate was withdrawn from the reactor and centrifuged at 10000 g for 10 minutes in order to removed non-solubilized solids. Medium autoclaving was not required because the effluent from the WO was already sterilised during the hydrothermal treatment. A volume of 95 mL of this hydrolysate was placed in

a 500 mL flask. The inoculum consisted on 5 mL of a culture of *B. licheniformis* after 12 hours of growth in Nutrient Broth, this time corresponding to the exponential phase, at 37 °C and 150 rpm. For all the fermentation experiments, temperature and shaker speed were kept constant at 37 °C and 150 rpm, respectively

Different batch bacterial cultures were carried out to assess the effects of the presence of oxygen during the hydrothermal treatment of the sludge and/or the initial dilution of the raw hydrolysate before being inoculated on either the *B. licheniformis* growth or the enzymatic activities. Parallel, for each set of experiments, a negative control (hydrolysate without microorganism) was also monitored to ensure the sterility of the hydrolysate and the stability of its components. Additionally, *B. licheniformis* was also grown in a synthetic medium composed by commercial BSA, humic acid and glucose or starch as model compounds of proteins, humic acids and carbohydrates, respectively, in concentrations similar to those observed in the raw sludge hydrolysate. These were 5 g/L of BSA (Sigma-Aldrich), 2 g/L of humic acids (Sigma-Aldrich) and 2 g/L of glucose or starch (Sigma-Aldrich). The aim of these experiments with a defined medium was to analyse the role of each biopolymer on the growth of *B. licheniformis*. It has been reported that the carbon source is a determinant factor for the production of the studied enzymes by several authors (Muthukumarasamy et al., 2015; Prakasham et al., 2006); this why two different carbohydrates, glucose or starch were employed as carbon source in the preparation of the synthetic media

Analytical methods

Suspended solids, COD and pH were measured according to the Standard Methods (*APHA*, 1998). COD concentrations were determined by means of a DR2500 spectrophotometer (Hach Company, USA).

Concentrations of soluble protein and humic acids were determined by the Lowry modified method (Frølund et al., 1995; Lowry et al., 1951) which allows the simultaneous determination of these two biopolymers. BSA (Sigma) and humic acid from soil (Sigma) were used as protein and humic acid standards, respectively. Carbohydrates concentrations were obtained by the Dubois method (DuBois et al., 1956), using D-(+)-glucose (Sigma) as standard.

Proteases activity was determined in the centrifuged supernatant of the culture (13200 rpm, 5 minutes) by means of the azocaseine method (Ginther, 1979; Romero et

al., 2001). A unit of protease activity was defined as the amount of enzyme necessary to increase the absorbance at 420 nm in 0.1 units in 1 h.

Regarding the laccase activity, authors have reported that laccase is an intracellular enzyme of *B. licheniformis* (Sharma et al., 2007). Hence, before measuring laccases activity, a previous cell breakage by sonication was required. To this end, 1 mL of the culture medium was centrifuged at 13200 rpm for 5 minutes. The supernatant was discarded and the pellet resuspended and subjected to three pulses of 10 seconds, with 15 seconds between each pulse to avoid the heating of the sample and the denaturalisation of the enzyme. After centrifuging the mixture at 13200 rpm for 5 minutes, 25 µL of supernatant were added to 1.5 mL of ABTS and absorbance at 420 nm was monitored until it was stable (Niku-Paavola et al., 1990). One unit of laccase activity was established as the amount of enzyme that oxidizes 1 µmol of ABTS per minute.

Results and discussion

Effect of the hydrothermal pretreatment

As a starting point, it was firstly determined if *B. licheniformis* was able to grow in the raw hydrolysates obtained by either thermal hydrolysis or wet oxidation of the sludge. The results obtained are shown in figure 1.

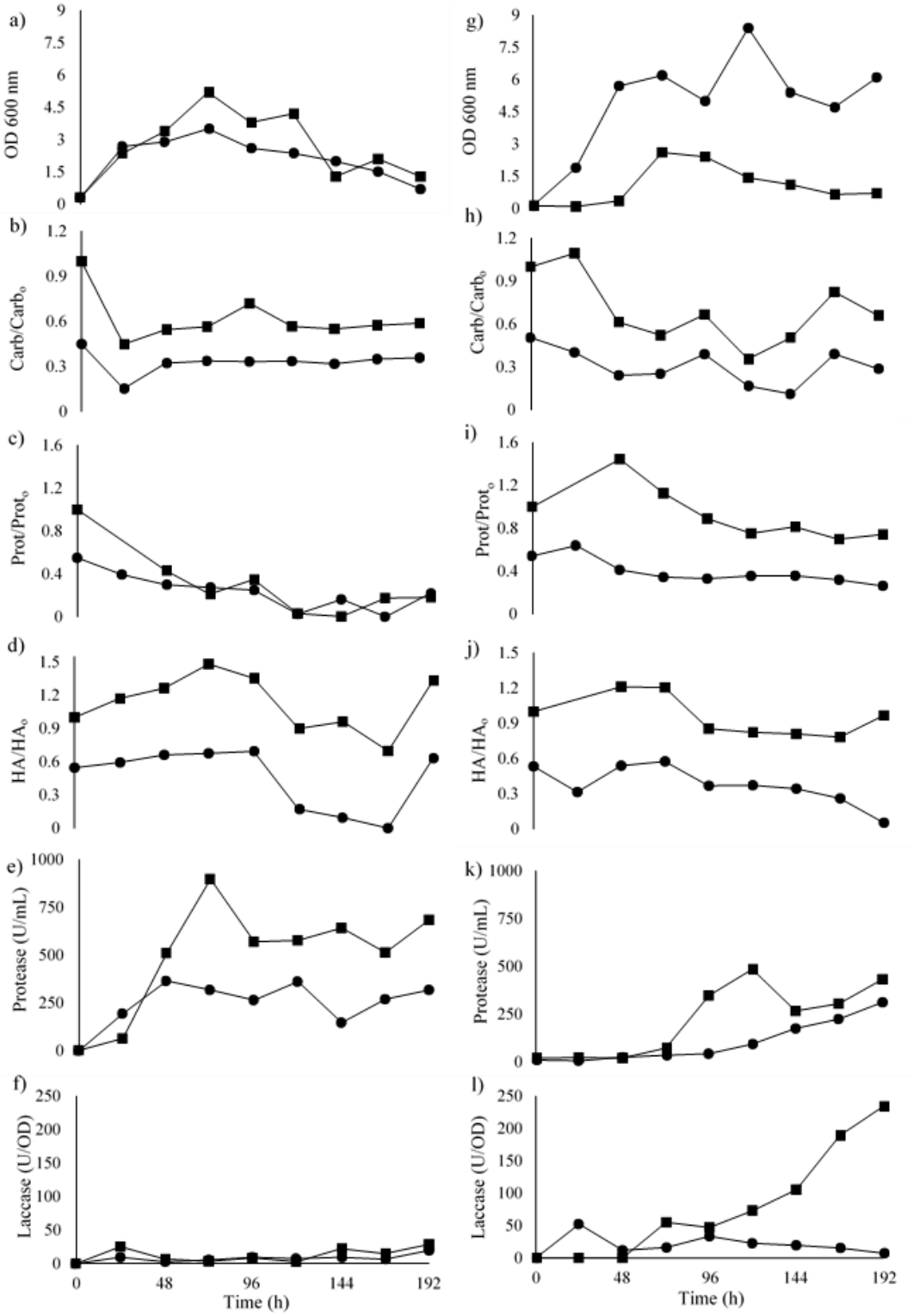


Figure 1. The most relevant parameters of *B. licheniformis* (OD at 600 nm, carbohydrates, proteins, humic acids, proteases activity and laccase activity) in batch culture using sludge hydrolysate obtained by hydrothermal treatment after 70 minutes at 160°C and 40 bar in absence (left column) or presence of oxygen (right column). Both hydrolysates were employed either undiluted (■) or diluted with sterilised water (●). All batch cultures were carried at 37°C and 150 rpm.

As expected, *B. licheniformis* was able to grow using both raw hydrolysates as substrates, with any kind of nutrient supplementation or previous autoclaving of the media. Results also suggested that the hydrolysate obtained by thermal hydrolysis is more easily assimilated by *B. licheniformis* than the corresponding to wet oxidation. So, the presence of oxygen during the hydrothermal treatment generated a hydrolysate that only allowed *B. licheniformis* to grow to the half of the maximum bacterial concentration (as optical density) reported for the fermentation of the hydrolysed sludge under an inert atmosphere (see figure 1a and g).

Regarding the growth curves, both followed the expected logistic trend with a final decay period. From an industrial point of view, it is also interesting to notice that the lag phase, corresponding to the delay before the start of exponential growth and associated to the adaptation required for *B. licheniformis* to begin to exploit new environmental conditions, was no detected when the hydrolysate from thermal hydrolysis was employed as medium. In this case, as can be seen in figure 1a, a significant increase in the optical density was observed, even for the first time tested (24 h), whereas the lag phase was delayed until 48 h when the sludge was hydrolysed under an oxidizing atmosphere (figure 2b). Thus, taking into account that the lag phase for the fermentation of the wet oxidation hydrolysate lasted longer than for the thermal hydrolysis one, it can be concluded that the most suitable medium for the development of *B. licheniformis* is obtained by means of hydrothermal treatments in absence of oxygen. But thermal hydrolysis not only reduces lag phase, but also increases the specific growth rate, reaching the stationary phase in only 24 hours, in comparison with the 72 hours required in the case of using the wet oxidation hydrolysate. Finally, growth curves also suggested that the final death phase was also accelerated when the sludge used as medium was previously hydrolysed in presence of oxygen.

In order to know what carbon source was preferentially employed by the *B. licheniformis* during the batch cultures, the concentrations of carbohydrates, proteins and humic acids were also followed during the fermentation of both sludge hydrolysates (see figures 1b, c, d, h, i and j). When the hydrolysate from thermal hydrolysis was employed as medium, the results showed in figures 1b, 1c and 1d suggested a sequential use of the different biopolymers present in the medium. Thus, the microorganism exhibited a preferential assimilation of carbohydrates and, mainly, proteins at the start of the fermentation, whereas significant decrease in humic acid concentration was only

observed during the last hours of fermentation. In fact, *B. licheniformis* halved the initial concentration of proteins in the first 24 hours and decreased in a 25% the concentration of carbohydrates at the same time. Regarding the latter, it was observed a fast slight decrease in their concentration during the first hours of fermentation and then, remained constant. This evolution in carbohydrates may well correspond to the presence of a significant fraction of simple sugars, such as the reducing ones, which were quickly consumed by *B. licheniformis*, whereas the more complex carbohydrates cannot be properly metabolized, thus remaining unaltered in the medium. On the other hand, the protein uptake turned out to be more marked and progressive, with *B. licheniformis* having assimilated around a 90% of the initial protein at the end of the fermentation. Either the carbohydrate or protein consumptions took mainly place during the exponential growth phase. Results suggests that the stationary phase for the growth of *B. licheniformis* in the sludge hydrolysate from thermal hydrolysis appears when the depletion of proteins is almost complete. It is at this point that the bacteria start to metabolize significant amounts of humic acid. Thus, humic acids consumption began after 96 hours of incubation, sharply reducing their concentration in the following hours. However, at the final hours, an appreciable rise in humic acids concentration was detected, probably related to the release of these compounds during associated to the bacterial death observed during the latter part of the experiment, as can be observed in figure 1a.

Nevertheless, moving on to the *B. licheniformis* fermentation of the sludge hydrolysate from wet oxidation, several differences about the uptake of substrates were observed. The first difference is a higher assimilation of carbohydrates by the bacteria when the sludge was hydrolysate under an oxidizing atmosphere. This behaviour can be explained on the basis of a higher proportion simple sugars, which would be more easily assimilated by the *B. licheniformis*, when wet oxidation is used for the sludge solubilisation. The presence of an oxidising atmosphere during the hydrothermal treatment provokes a higher breakage of the complex carbohydrates to smaller ones than an inert atmosphere (Urrea et al., 2018). Although almost 50% of the initial carbohydrates were consumed in the first 48 hours, protein and humic acid concentrations did not decrease until after this time. So, carbohydrates were mainly metabolized during the lag phase, whereas protein uptake was only noticeable when the exponential growth of the *B. licheniformis* started. Another difference with the thermally hydrolysed sludge is that only around a 34% of the initial protein was metabolized by *B. licheniformis* when it was

grown in the hydrolysate from wet oxidation, in comparison to the 90% previously commented for thermal hydrolysis. This fact can be attributed to a higher protein denaturation when the sludge is solubilised in presence of oxygen instead of in absence of it, thus reducing the effect of the secreted proteases by the bacteria on the lysis of complex proteins to simple peptides, which can then be properly metabolized by the microorganism. Other studies dealing with *Bacillus* species showed that protease activity was higher in presence of complete proteins (for example, soybean meal) instead of proteoses (for example, peptone or soy tryptone) (Joo et al., 2003, 2002). Regarding the humic acid concentration, its evolution during the fermentation of the hydrolysate from wet oxidation was very similar to the reported for proteins: its uptake took mainly place during the exponential growth and the amount metabolized was lower if the sludge is previously hydrolysed under an oxidizing atmosphere. Regarding this latter fact, humic acids are constituted by a core of phenolic molecules surrounded by aliphatic chains. Studies dealing with humic acid biodegradation have demonstrated that microbes attack firstly the aliphatic structures of the humic acid molecule, which are externally located and easiest to oxidize due to steric reasons (Filip and Tesařová, 2004).

Finally, the production of metabolites can also be related with either the bacterial growth or the substrate uptake. The evolution of proteases and laccases during the fermentation of hydrolysates from thermal hydrolysis or wet oxidation by *B. licheniformis* is presented in figures 1e, f, k and l. As expected, the presence of both proteases and laccases was confirmed in all experiments. Regarding proteases, enzymatic activity was strongly dependent of the treatment employed to obtain the hydrolysate. In fact, the maximum proteases activity for the sludge hydrolysate under an inert atmosphere doubled the value obtained when an oxidizing atmosphere was used (898 U/mL and 485 U/mL, respectively). It was also found that proteases are mainly generated during the exponential growth of *B. licheniformis*, which is also in line with the highest protein uptake observed. Thus, protease activity was detected in the first day or after 72 hours of incubation during the fermentation of hydrolysates from thermal hydrolysis or wet oxidation, respectively. Due to this, proteases can be properly considered as primary metabolites, being directly involved in normal growth, development, and reproduction of *B. licheniformis*. Moreover, expression analysis of the proteome of *B. licheniformis* demonstrated that, under nitrogen starvation, the genes that were activated were related to obtaining nitrogen from non-conventional sources, such as nitrates or nitrites (Voigt et al., 2007). The no

presence of proteases during the lag phase can be explained taking into account that *B. licheniformis* showed an initial preference for carbohydrates, as previously explained, hence bacteria did not need to deploy its proteolytic machinery until simple sugars were depleted. This can be experimentally observed in Figure 1k, where the consumption of carbohydrates slowed down after 48 hours, point at which protease activity began to be significant. Moreover, transcriptomic and proteomic studies showed that the genes responsible for metabolism of alternative carbon sources, e.g. proteins, were activated in glucose starvation, that could be the case for the culture after 72 h (Voigt et al., 2007). It is interesting to point out that the proteases activity during the fermentation of the hydrolysate from thermal hydrolysis gradually increased during the log phase of the *B. licheniformis* growth, reaching a maximum of 898 U/mL after 72 h of cultivation, then decreasing until a final activity of around 685 U/mL, which remained approximately during the rest of the fermentation, corresponding to the stationary and death phases of the growth curve (see figure 1a), where the proteins concentration in the medium was too low. This behaviour can be taken as an indicator of the high stability and low deactivation of the proteases by *B. licheniformis*. Regarding the hydrolysate from wet oxidation, evolution of proteases was analogous, although their production was lower and slower than using thermal hydrolysis for the sludge solubilisation, as previously commented.

Focusing on the laccases, some discrepancies with proteases evolution were easily observed (figures 1f and l). So, contrarily to proteases, laccase activities were higher when the bacteria were cultured in a sludge previously hydrolysed under oxidizing atmosphere instead of an inert one, with maximum values of 190 and 60 U/mL, respectively. Despite the low activities observed, it is interesting to recall that laccases are intracellular enzymes and the optical densities were also low. Therefore, expressing laccase activities as U/OD, it can be concluded that wet oxidation led to a laccase production of 55 U/OD after 72 hours of culture, doubling the maximum activity reported for the hydrolysate obtained in absence of oxygen (28.8 U/OD). The lower laccase activity observed when an inert atmosphere was used during the solubilisation could be related with the higher protein denaturation proposed when using an oxidizing one. It seems reasonable to propose that *B. licheniformis* changes their metabolism towards the most easily assailable substrate, thus explaining the higher proteases but lower laccases activities during the hydrolysate fermentation from thermal hydrolysis, where the proteins were less denatured due to the absence of an oxidizing atmosphere.

Moreover, it should be borne in mind that for both enzymes, their activities are pH-dependent: proteases work better at alkaline pH values, whereas laccases are more active at acidic pH values, close to 4 (Koschorreck et al., 2008; Martins et al., 2002). Although pH evolution during the fermentations is not shown, this decreased in the first days of culture (from pH = 7.2 to 6.2) and then slightly increased (until pH = 6.8). As can be seen, these values of pH differed from the corresponding ones to the highest activity for each enzyme; so, a small acidification or basification of the medium will enhance significantly laccase or protease activities, respectively.

Effect of the sludge hydrolysate dilution

In this regard, although it has been proved that the hydrothermally hydrolysed sludge is a complex medium with high contents of carbohydrates, proteins and humic acids, thus suggesting an easy bioassimilation, the generation of some toxic compounds during the hydrothermal treatment cannot be excluded. For this reason, the bacterial growth of *B. licheniformis* was also tested in the corresponding hydrolysates diluted with distilled autoclaved water (with a dilution factor of 2), in order to recognising potential inhibitory effects related to the composition of the media. Figure 1 (● symbols) shows the most relevant experimental data obtained during the fermentation of diluted sludge hydrolysates, previously obtained by either wet oxidation or thermal hydrolysis.

When the fermentation was carried out using the TH hydrolysate after being diluted, the *B. licheniformis* maximum growth was lower than using the raw hydrolysate, as could be expected on the basis of a lower nutrient concentration. Nevertheless, optical density measurements for experiments with WO hydrolysates revealed that *B. licheniformis* grew better in the diluted hydrolysate than in the undiluted one. In fact, the maximum cellular concentrations in the diluted hydrolysate was twofold higher than for the undiluted one, which suggests the presence of one or more inhibitory substances in the hydrolysate. These compounds would be formed during the hydrothermal treatment of the sludge in presence of an oxidizing atmosphere but not under an inert one. This could be the case of furfural, which is known to inhibit microbial growth (Yoo et al., 2015). But the dilution of these inhibitory compounds not only had effect on the maximum bacterial concentration and on the specific growth rate, but also on the length of the initial lag phase. As can be seen in figure 1g, the dilution of the hydrolysate from wet oxidation before being inoculated with *B. licheniformis* drastically reduced the time required for the acclimation, from nearly 48 h to less than 12 h.

Focusing on biopolymer uptake by *B. licheniformis* during the fermentation of hydrolysate from thermal hydrolysis, the previous dilution evidently reduced by half the concentrations, but beyond this, the evolutions of biopolymer in the diluted and the undiluted media were similar, although some facts should be stressed. One of them is that the final concentration of proteins after the fermentation of either the diluted or undiluted hydrolysate from thermal hydrolysis is 770 mg/L. However, as previously explained more, than 95% of the protein was metabolized in the undiluted hydrolysate, so these similar trends in the protein concentration at the end of the experiments with or without dilution are not so surprisingly. It is also interesting to note that the initial slight decrease in the carbohydrate concentration reported for the fermentation of the undiluted hydrolysate from thermal hydrolysis was not observed when the hydrolysate was diluted before being inoculated. This decrease was attributed to the preferential assimilation of the small amount of simple sugars generated during the solubilisation of the sludge. When the hydrolysate is diluted, the concentration of these simple sugars is even lower, so the change in the total concentration of carbohydrates due to the metabolization of these simple sugars is almost negligible.

When it comes to the effect of the previous dilution of the hydrolysate from wet oxidation on the substrate uptake, the concentrations of either proteins, carbohydrates and humic acids during the fermentation are shown in figures 1 h, i and j. For all the biopolymers, the trends in the evolution of their concentrations were analogous, so the effect of the inhibitory compounds does not seem to be connected with the uptake rate of substrate by the microorganism, but with the viability of the microorganism.

Concerning the proteases and laccases activities, the effect of the dilution on the *B. licheniformis* fermentation of the thermally hydrolysed sludge was clear, halving the protease activity, as it was to be expected. In the case of the laccases, their activity also decreased due to the dilution, although this effect is less evident than for proteases, due to their low activity. Nevertheless, the activity results were less foreseeable when the diluted hydrolysate from wet oxidation was fermented. Thus, proteases activity only decreased slightly when the medium was diluted, which suggest that although the bacterial growth was higher than using the raw hydrolysate, the specific protease production per bacterium was reduced. On the contrary, the laccases activity was highly favoured by the initial dilution of the medium, with a faster and higher production of these enzymes. As can be seen in the figure 1l, the previous dilution of the hydrolysate from

wet oxidation allowed the production of laccases since the beginning of the fermentation, and not after 48 h, as in the case of the undiluted one. The maximum laccase activity was also reached by dilution of the hydrolysate before being inoculated, with 190 U/mL, whereas activities no higher than 160 U/mL were detected for the raw hydrolysate. If laccase activities are expressed by unit of optical density instead of by millilitre, then it can be concluded that the activity is similar in the lag and log phases with peaks of 60 U/OD in both diluted and undiluted hydrolysates. However, at death phase (from 120 h) the activity was remarkably higher in the undiluted WO hydrolysate (more than 200 U/DO) whereas in the previously diluted hydrolysate the laccase activity was decreasing to negligible values. This surprising rise in the specific laccase activity was probably due to the higher concentration of laccase substrates, such as humic acids, which concentration was still high in the non-diluted hydrolysate at the final stages. In contrast, it decreased in the case of the diluted hydrolysate.

Synthetic hydrolysate fermentation

Finally, in order to increase the knowledge and understanding of the hydrolysed sludge by *B. licheniformis*, two defined media were prepared with commercial BSA (Sigma Aldrich), humic acid (CAS number 1415-93-6) and starch or glucose (Sigma Aldrich) at concentrations close to those measured in the sludge hydrolysate obtained by thermal hydrolysis for proteins, carbohydrates and humic acids, respectively. It is important to note that starch and glucose were selected as models of non-reducing or reducing sugars. The main results for the fermentation of both synthetic media by *B. licheniformis* are shown in Figure 2.

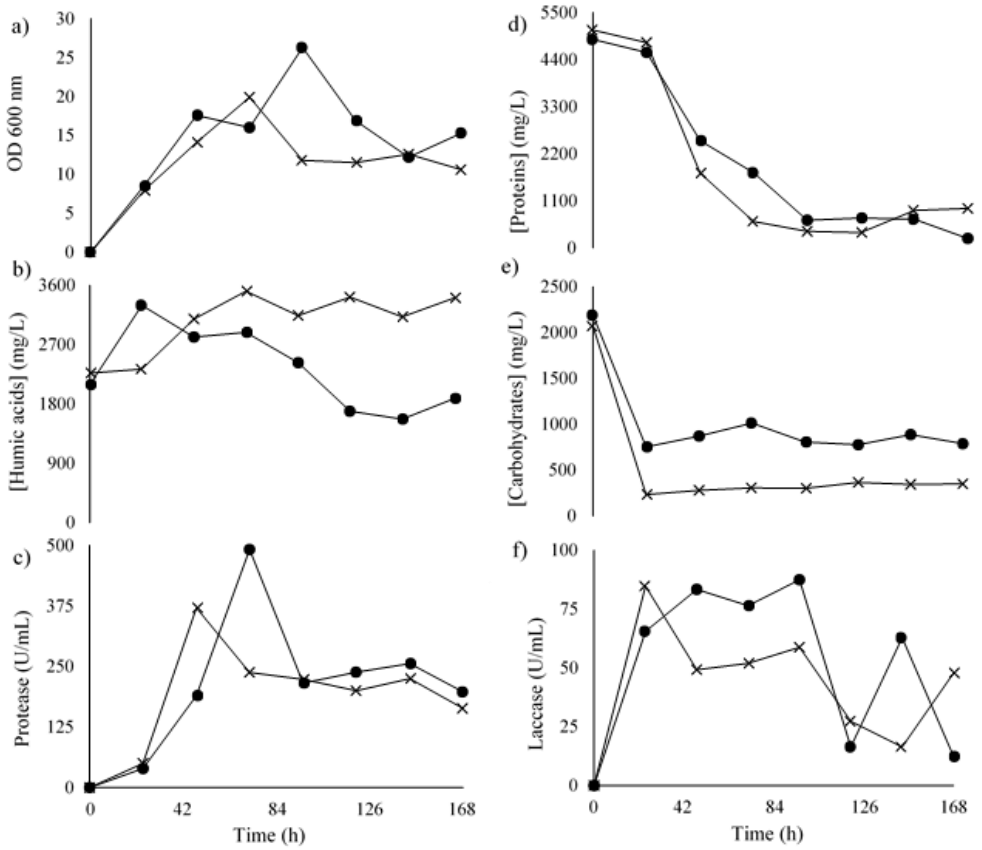


Figure 2. Results for cultures in synthetic medium. a) evolution of OD 600 nm, b) evolution of protein concentration of the medium, c) evolution of humic acids concentration of the medium, d) evolution of carbohydrates concentration of the medium, e) evolution of protease activity and f) evolution of laccase activity. In all cases: (●) starch as carbohydrate (x) glucose as carbohydrate, incubation at 37 °C and 150 rpm.

It was clearly observed that the bacterial growths for both defined media were higher than in the real sludge hydrolysates from either thermal hydrolysis or wet oxidation, diluted or not. Thus, the maximum optical density in media with glucose or starch were 19.9 and 26.3, respectively, which at least doubled the optical densities obtained using sludge hydrolysates as media. These findings seem to support our previous proposal that the changes in the biopolymers structure during the hydrothermal treatment of solubilisation have negative effects on the *Bacillus* growth.

The use of the synthetic medium also confirmed the preferential use of substrates and the triauxic growth here reported for the *B. licheniformis* fermentation of sludge hydrolysates, as can been seen in figures 2b, c and d. In such a way, the carbohydrate model compounds (starch or glucose) were the preferential carbon and energy source for the *Bacillus*, since their concentration dramatically dropped in the first 24 hours. It should be also stressed that around a 65% of starch and 90% of glucose were consumed, which is in accordance with a higher uptake of reducing sugars than of more complex

carbohydrates, such as starch. As in the case of the hydrolysates from sludge, the consumption of proteins in the defined media is evident once the removal of the corresponding carbohydrate stopped. Thus, the protein uptake in the defined media by the microorganism began after 24 hours and around 85% of the initial BSA was assimilated before the 96th hour of cultivation. Eventually, concerning the humic acids, a considerable increase in their concentration was initially observed in both synthetic media, coinciding with the period which protein uptake predominates in (figure 2b). This performance is attributed to the formation of peptides during the breakage of proteins due to proteases attack, which were detected as humic acids by the Lowry modified method. However, from 72 hours to the end of the experiment, humic acid concentration remained approximately constant when glucose was used as carbohydrate model, whereas its concentration decreased if starch is added to the synthetic medium instead of glucose. These results seem to suggest that the metabolic machinery related to the production of laccases is enhanced by the presence of complex carbohydrates in the medium. In this sense, some authors have also reported that laccases activity decreases when glucose is present in the medium, thus reducing the humic acid consumption (Singh et al., 2014).

Focusing now on the enzymes production, it is interesting to point out that, although the *B. licheniformis* growth and the assimilation of either carbohydrates or proteins were boosted in the synthetic media, this did not result in an increase in the production of enzymes. In fact, the proteases activities measured during the fermentation of the defined media were similar to those obtained with the hydrolysate from wet oxidation and even lower than the corresponding ones to the fermentation of the sludge hydrolysed by thermal hydrolysis. In turn, the use of starch as carbohydrate increased the production of proteases. According to these facts, it seems reasonable to propose that the more complex the carbohydrate, the higher the secretion of proteases by *B. licheniformis*. That is to say, bacteria would only promote the production of proteases if the preferential carbon source, the carbohydrates, cannot be easily assimilated. A similar behaviour was also observed for the production of laccases by *B. licheniformis* in synthetic media, where their production was hampered by the presence of easily assimilated carbohydrates, as starch, and/or no denatured protein, as BSA.

Conclusions

The use of the hydrolysate obtained by hydrothermal treatment of sewage sludge as low-cost substrate for the simultaneous production of enzymes (proteases and laccases)

by fermentation with *Bacillus licheniformis* is promising and feasible. This approach achieves two main goals in the sludge management: its minimization by hydrothermal treatment and its revalorization by enzyme production.

Results revealed that *B. licheniformis* was able to grow properly in the thermally hydrolysed sludge (160 ºC, 40 bar, 70 min, with an inert or an oxidising atmosphere) with any kind of nutrient supplementation or previous autoclaving of the media. It was found that hydrolysates obtained by thermal hydrolysis are more easily assimilated by *B. licheniformis* than the corresponding to wet oxidation. Thus, the selection of an inert atmosphere instead of an oxidising one during the hydrothermal treatment of sludge not only shortened the lag phase, but also increased the specific growth rate, and slowed down the death kinetics during the fermentation. The microorganism exhibited a preferential assimilation of simple carbohydrates and proteins at the start of the fermentation, whereas significant decrease in humic acid concentration was only observed during the last hours of fermentation.

Maximum protease activities were also achieved solubilising the sludge under an inert atmosphere (898 U/mL). Proteases were mainly generated during the exponential growth of *B. licheniformis*, which is also in line with the highest protein uptake observed and showed a high stability and low deactivation. On the contrary, laccase activities were higher when *B. licheniformis* were cultured in a sludge previously hydrolysed under oxidizing atmosphere instead of an inert one, with maximum values 55 U/OD after 72 hours of fermentation.

Finally, by means of fermentations carried out by synthetic media simulating the real sludge hydrolysates, was concluded their production was favoured by the absence of easily assimilated carbohydrates and, in the case of laccases, by the presence of highly denatured proteins, both conditions being very common in thermally hydrolysed sludge.

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4.6. PRODUCCIÓN DE PRODUCTOS NO-ENERGÉTICOS A PARTIR DE HIDROLIZADO DE LODOS DE DEPURADORA

Publicación: Manuel García, Sergio Collado, Mario Díaz. Fermentation processes of hydrolysed sewage sludge for production of non-energy products.
Estado: enviado para ser considerada su publicación

Las características del hidrolizado de lodo hacen de él un sustrato atractivo para la obtención de distintos metabolitos por vía fermentativa. A este respecto, la producción de productos energéticos, como biogás o biohidrógeno, a partir de lodos es un campo ampliamente estudiado, del cual se pueden encontrar excelentes revisiones (Bozkurt y Apul, 2020; Elalami et al., 2019; Fu et al., 2021; Kor-Bicakci y Eskicioglu, 2019; Liu et al., 2019; Wong et al., 2014). Sin embargo, durante los últimos años, la producción de metabolitos no energéticos por vía fermentativa a partir de hidrolizados de lodos está ganando interés.

El objetivo del presente capítulo es recopilar y discutir los artículos más recientes que tratan sobre el uso de hidrolizados de lodos de depuradora como sustratos para fermentaciones enfocadas a la obtención de diferentes bioproductos, prestando especial atención al efecto de las condiciones de operación en su producción, e indicando sus principales aplicaciones.

Fermentation processes of hydrolysed sewage sludge for production of non-energy products

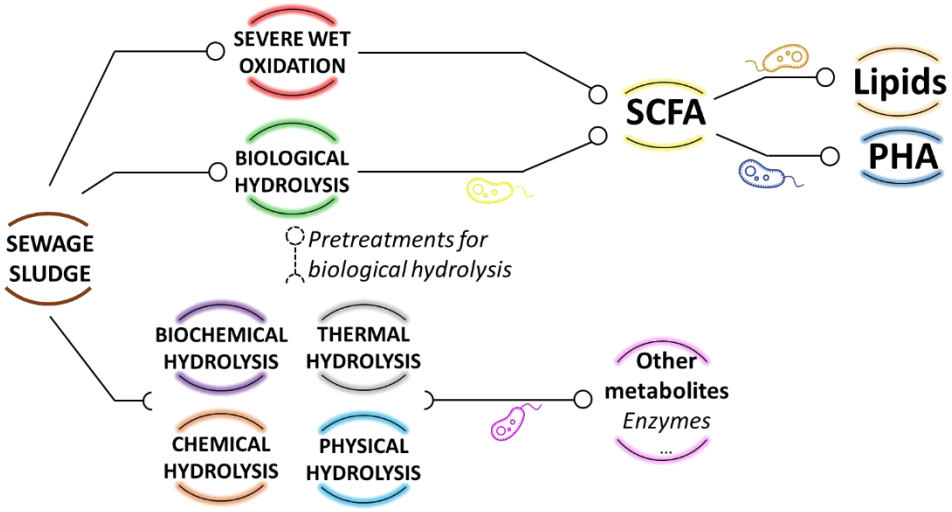
*Manuel García[a], Sergio Collado[a], Mario Díaz[a]**

[a] Department of Chemical and Environmental Engineering. University of Oviedo. C/ Julián Clavería s/n. E-33071 Oviedo, Spain. Tel.:34985103439; Fax: 34985103434

* Mario Díaz. Department of Chemical and Environmental Engineering. University of Oviedo. C/ Julián Clavería s/n. E-33071 Oviedo, Spain. Tel.: +34 985 10 34 39; Fax: +34 985 10 34 34

mariodiaz@uniovi.es

Graphical abstract



Highlights

Main non-energetic products from sludge fermentation are SCFAs.

Production of complex metabolites (enzymes or lipids) is promising but understudied.

Hydrolysis and solubilisation are critical in final product yields and profiles.

Positive synergies by coupling thermal pretreatment and pure culture fermentation.

Assisted biological hydrolysis is the most employed pretreatment.

Abstract

Hydrolysis and solubilization of sewage sludge processes are important tools to obtain small and medium molecules with different application perspectives. Although the production of biomethane and other products like biohydrogen from sludge as biofuel alternatives have been profusely studied, the current perspectives are mainly focused on the use of the sludge hydrolysate to produce non-energy bioproducts and biomaterials. In this review, the most recent bibliography dealing with the use of sludge hydrolysates as fermentation media for the bioproduction of new non-energetic products with industrial interest is here revised and discussed.

In this regard, the main research effort has been focused on the bioproduction of short chain fatty acids, due to their direct use in industrial applications, or as carbon source for polyhydroxyalkanoates-producing microorganisms. The use of sludge hydrolysates as fermentation media using pure cultures to produce more complex biomolecules, such as enzymes or lipids, is gaining interest, but it is yet an undervalued topic.

Literature has been divided into processes where hydrolysis and fermentation stages took place simultaneous or separately, centring then on the effect of the main operational conditions on the yields and properties of the corresponding metabolites produced. In general, the main limiting step of this kind of processes is the proper solubilisation and hydrolysis of the sludge, to improve the bioassimilation of nutrients and, subsequently, productivities and compositions of the metabolites obtained. Biological and/or thermal pretreaments are the options more profusely employed, frequently assisted by different promoters such as oxidants, surfactants or cation exchange resins.

1. Introduction

Wastewater treatment plants (WWTPs) are the main responsible for decontaminating wastewaters resulting from human activities. As a consequence of this depuration process, huge amounts of sewage sludge, ranging from 3.1 L to 8.2 L per inhabitant and day, are generated [1]. As a reference to provide an idea of how much sludge is generated, more than 10 million tons of sludge are expected to be produced in 2020 only in EU, in the basis of the statistical data collected in recent years [2]. These large quantities of sludge require a proper management, since this waste comprises microorganisms and suspended and colloidal matter, thus involving potential hazards, due to the presence of, among other pollutants, heavy metals or pathogens, such as viruses [3]. In fact, the management of this sludge usually comprises between 20% and 60% of the operational costs in a WWTP [1].

The traditional uses for sewage sludge have included land application (with or without previous composting), landfilling or energy recovery. Nevertheless, it should be noted that: i) land application has to compete with other waste streams, the demand is variable and the legislation about this topic is becoming increasingly strict, ii) landfilling is an unsustainable outlet due to concerns over pollution, loss of recyclable materials and loss of void for those wastes which cannot be recycled and iii) incineration is a high cost/high technology option and is currently only likely to be cost-effective for large cities and it does not have a high level of public acceptability due to concerns over gas emissions [4]. Other sludge management strategy commonly used in the WWTPs is the anaerobic digestion, that is to say, the sludge self-digestion to produce biogas [5,6]. However, the intermediate steps of this process require long times and well controlled conditions.

As can be seen, conventional sludge management strategies are only focused on stabilising the sludge and reducing its volume, obtaining low value energy products at its best. In this sense, some more recent alternatives include the conversion of the sludge into bio-oils and biochars from pyrolysis; biofuels, syngas and chemicals from gasification as well as recovering minerals such as struvite [7,8]. Nevertheless, analysing the sludge composition, these approaches clearly underestimate its potential. As previously indicated, sludge is composed by microorganisms and dissolved organic matter. Therefore, focusing on its biochemical composition, sludge comprises a complex mixture of proteins, carbohydrates and lipids, an important proportion of humic substances,

nutrients and minerals [9–11]. Hence, sludge is now emerging as a renewable source for biomolecules with a higher added value than biogas or compost. This approach will allow to put WWTPs into the circular economy context, by taking advantage of a waste that it is currently underused. In a long term and with adequate treatments, WWTPs will be turned into bio-factories, where wastes will be minimized in favour of obtaining green feedstocks [12–14], thus matching the objectives recently proposed by the EU in its New Circular Economy Action Plan [15].

It is interesting to note that many of the either conventional or new strategies for sludge valorisation require an initial pretreatment to solubilise the biomolecules from the microorganisms or the sludge flocs [16]. With the objective of achieving an appropriate sludge disruption, different technologies have been applied, including either biological, thermal, chemical, physical or biochemical treatments. Although this is a well-documented field, a brief review of these technologies will be here carried out, in order to put into perspective their different hydrolysis mechanisms.

Maybe the most employed hydrolysis treatment is the biological one. In this method, the own sludge microorganisms are responsible for the reactions that break cells and extracellular polymeric substances (EPS) structures via enzymatic reactions [17]. The second method for sludge solubilisation is the thermal one, which is based in the supply of heat to the cells to achieve protein denaturation and membrane disintegration, provoking the cytoplasmatic content release. Usually, thermal methods also involve high pressures to raise the water boiling point thus allowing higher temperatures. These methods are known as hydrothermal ones. A modification of these is also possible by introducing in the mixture an oxidant, being the process defined as wet oxidation. The particularity of these processes lies on the reactivity of the oxygen at high temperatures, being able to enhance the solubilisation, although it can also transform the molecules structure by the generation of carboxyl, carbonyl, hydroxyl and other functional groups which include oxygen in their structure [18].

On the other hand, chemical methods are based on the addition of reagents that could alter different parameters of the environment to achieve either EPS structure or membrane integrity disruption as well as both of them. This is the case of ozone, surfactants or other reagents ($Ca(OH)_2$, CaO, NaOH…) [19,20]. Physical methods employ shear forces to achieve the cell breakage thus releasing their inner contents. These shear forces can be generated from several sources: homogenizers, mills, pressure jets or

ultrasounds [21,22]. Finally, biochemical treatments, including the use of hydrolytic enzymes such as proteases, cellulases or amylases, which are also capable of destroying the sludge structure, solubilising the EPS and, if an adequate enzyme cocktail is employed, destroying the cell wall as well [9,23].

Once the sludge has been hydrolysed and the target molecules have been solubilised, the first approach that can be made is their direct recovery from the hydrolysate in a more or less pure form to exploit their commercial value. In this way, the recovered proteins can be used in the formulation of coatings, biofilms, adhesives, surfactants or organic fertilizers [24–28]. Humic acids also have commercial value, being used as fertilizers or surfactants [29]. Lipids can be employed as feedstock for biodiesel production [30]. The phosphorus present in the sludge has been already recovered as struvite as previously commented. With this aim, several approaches have been made, although results were not as promising as to unseat the conventional treatment methods, since low recoveries or low purified streams were usually obtained [31–34]. More research in this field is then required to develop better strategies with higher recoveries and purities for further green uses of these biopolymers.

Other approaches that are being deeply studied and that also fulfil the circular economy goal involve the use of these sludge hydrolysates as fermentation media for the bioproduction of different metabolites with industrial interest. In this regard, depending on the hydrolysis method employed, the hydrolysate will show different characteristics, that will make them more suitable for the bioproduction of certain products with respect to others. When it comes to choosing a hydrolysate as fermentation medium, by revising the products obtained from the fermentation of sludge hydrolysates, it can be concluded that the cornerstone is the content of short chain fatty acids (SCFAs) in the hydrolysates. Therefore, a little reclassification of the hydrolysis methods has to be done. The only change is the separation of extreme versions of oxidative hydrothermal treatments (long times and severe temperature and pressure conditions), which can also be carried out to produce effluents with a high proportion of SCFAs, thus emulating the sludge hydrolysates obtained by biological processes [35,36]. Figure 1 shows a scheme of the different hydrolysis methods, the main characteristics of the hydrolysate and the final products that could be obtained with each treatment, in order to improve the understanding of which hydrolysate is supposed to be better for its use as substrate for subsequent fermentations.

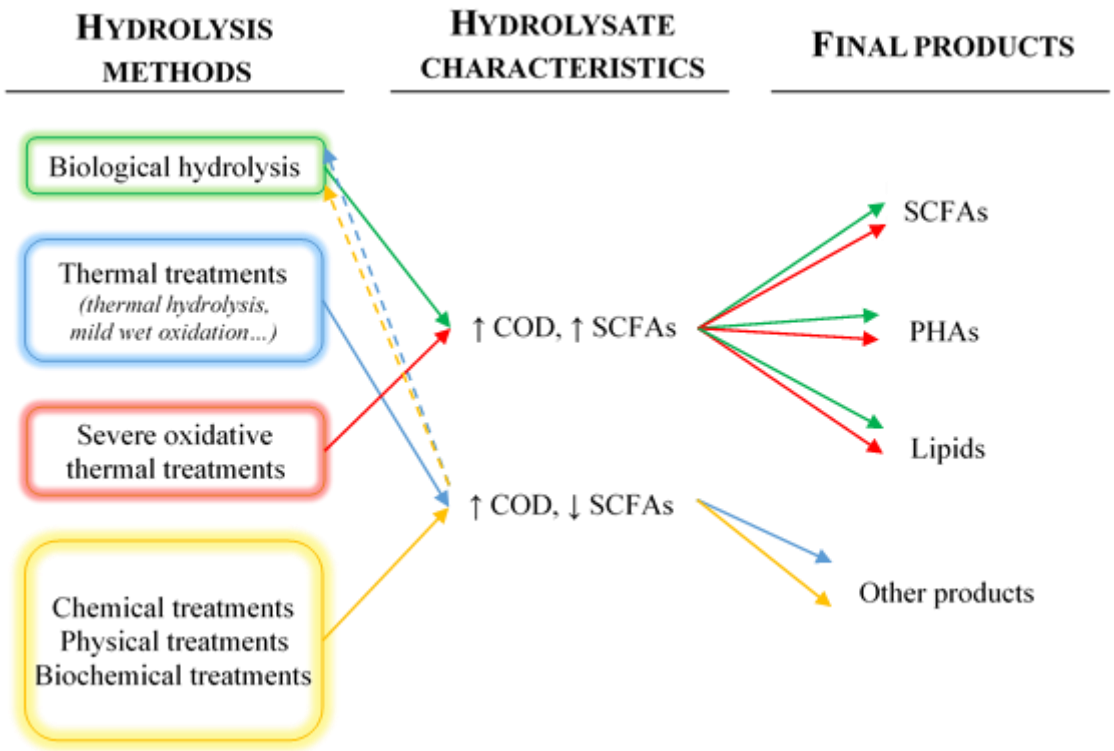


Figure 1. *Main hydrolysis methods for sludge, hydrolysate characteristics and potential final products. Dashed lines indicate the application of the previous hydrolysis method as pretreatment for the method they lead to.*

As explained, biological hydrolysis is carried out by microorganisms. However, these microorganisms hydrolyse the sludge to use it as substrate and in consequence, they transform the biomolecules present in the sludge into SCFAs. Since these hydrolysis and acidogenic processes are almost simultaneous, it is very difficult to separate them. This is the reason why biological fermentation of the sludge yields a high content of SCFAs, such as acetic acid, propionic acid, etc. than other hydrolysis processes [37,38]. Therefore, this kind of hydrolysates would be useful as substrate for fermentations in which the fermentative microorganisms need a readily assimilable carbon source, for instance, in polyhydroxyalkanoates (PHAs) or lipids production [39–41].

Moving on to the hydrolysates obtained from thermal treatments, these will be composed by proteins, lipids, carbohydrates and humic substances as well as cell debris and nutrients, such as ammonium or phosphorus [10,42,43]. Therefore, they will show high chemical oxygen demand (COD) contents due to solubilisation of organics like proteins and carbohydrates, but low SCFAs concentration [44]. This composition is suitable for its use as a fermentation medium for producing high molecular weight products such as enzymes. Obviously, hydrolysates obtained after a hydrothermal treatment can also be employed as substrate for a further biological fermentation to produce SCFAs, which can

be subsequently recovered or used as substrate for PHAs production. As mentioned, the extreme versions of the oxidative thermal treatments lead to high SCFAs production and would be adequate for sludges containing high proportions of humic substances or other molecules toxic or recalcitrant to biological degradation [45]. The hydrolysates thus obtained can be employed in the same processes as those generated by biological hydrolysis.

Separately, chemical, physical and biochemical hydrolysis methods will produce hydrolysates similar to those obtained by thermal methods, which can be employed in the same applications to those mentioned before [9,46]. At this point, it must be highlighted that the combination of different hydrolysis methods is feasible and can generate some positive synergies. Many examples of these combinations will be seen in the following sections by studying particular cases.

Therefore, the aim of this review is to recompile and discuss the most recent literature dealing with the use of sludge hydrolysates as fermentation substrates to obtain different bioproducts and the potential applications of these, paying special attention to the effect of the main operational conditions on yields. Previously, studies were divided into processes where hydrolysis and fermentation stage took place simultaneous or separately.

It should be stressed that, among the products that can be obtained by fermentation of sludge hydrolysates, the production of either biogas or biohydrogen has already been extensively studied in the bibliography, existing excellent reviews about these topics in the literature [5,47–51]. Because of this, the bioproduction of these metabolites has not been included in this work, which has been more focused in more innovative products.

2. Short chain fatty acids

2.1. General considerations

New approaches focused on the sludge anaerobic digestion as fermentative alternative to produce short chain fatty acids (SCFAs), and not biogas, have gained a heightened interest in the last few years. This strategy, based on stopping the anaerobic digestion at the end of the acidification step and before the acetogenic one, could balance the economics of a WWTP, due to the production and accumulation in the sludge hydrolysate of SCFAs such as acetic, propionic or butyric ones [38].

The worth of the SCFAs underlies in their commercial applications even if they are not in their pure forms. The potential uses of these molecules have already been extensively reviewed and they will be here cited very briefly [52–56]. These include their employment as substrates to obtain different products or electricity by fermentation as well as nutrient removal in the WWTP. After purification steps, their potential applications comprise food industry, as preservatives; cosmetic industry and pharmaceuticals one.

In recent times, SCFAs production from sludge hydrolysates has attracted some attention with several reviews [46,57–59]. In this review, only the most recently published articles were included, discussing the effect of the main operational conditions on the production and composition of SCFAs generated.

Table 1. *Summary of the main parameters of the studies dealing with SCFAs production. "–" indicates data not available or unable to be calculated. CSR stands for Continuously Stirred Reactor.*

Sludge	***Conditions for pretreatment***	***Pretreatment results***	***Conditions for biological hydrolysis*** *(anaerobic in all cases)*	***Fermentation results*** *(best results were those with higher SCFAs concentration)*	***Reference***
Settled secondary (VSS_o = 15.8 g/L) ($TCOD_o$ = 26.56 g/L) ($SCOD_o$ = 3.73 g/L)			Reactor: expanded granular sludge blanket reactor Acclimation at 35 °C and pH 10: two stages of 8 days each one, at 8th and 16th day 2/3 of sludge were replaced at pH 10 (after 16th day, steady state was reached) Semi-continuous operation at 35 °C and pH 10, 5 cycles of 20 days each. After each cycle, 2/3 of volume was replaced with fresh sludge	$[SCFAs_{MAX}] \approx$ 610 mg COD/g VSS (8 d of 2nd cycle) $SCFAs_{MAX}$ /SCOD = – $SCOD/SCOD_o \approx$ 2.1 for each cycle *Acid* (%): Ac. 84; Pr. 11.3; n-bu. 0.7; i-bu. 1.2; i-va. 2.7; n-va. 0.1	Li et al. 2018
Clarified secondary (VSS_o = 11.91 g/L) ($TCOD_o$ = 17.98 g/L) ($SCOD_o$ = 0.12 g/L)			Reactor: semicontinuous mixed at 37 °C for 80 days 5 pHs = uncontrolled, 7, 8, 9 and 10 No acclimation Equal parts of inoculum and feedstock 0.05 L replaced daily → HRT = 8 d, OLR = 1.49 g VS L^{-1} d^{-1}	Best results at pH 10 $[SCFAs_{MAX}]$ = 3762 mg COD/L (from 10 days) $SCFAs_{MAX}$ /SCOD = 0.58 (from 10 days) $SCOD/SCOD_o$ = 53.73 (from 10 days) *Acid* (%): Ac. 44.7; Pr. 17.1; n-bu. 9.2; i-bu. 10.5; i-va. 15.8; n-va. 2.6	Ma et al. 2019
Settled secondary with poly-aluminium chloride (VS_o = 12.01 g/L) ($TCOD_o$ = 13.29 g/L) ($SCOD_o$ = 0.05 g/L)			Reactor: semicontinuous mixed at 35 °C – 40 °C for 111 days pH (8 to 11) incremented in 1 unit after, at least, 2 SRTs No acclimation 0.1 L replaced daily → SRT = 8 d, OLR = 1.33 g COD L^{-1} d^{-1}	Best results at pH 11 $[SCFAs_{MAX}]$ = 4300 mg COD/L $SCFAs_{MAX}$ /SCOD = 0.68 $SCOD/SCOD_o$ = 127 *Acid* (%): Ac. 39.6; Pr. 13.9; n-bu. 11.1; i-bu. 10.4; i-va. 16; n-va. 9	Yan et al. 2021
Settled secondary (VS_o = 12.83 g/L) ($TCOD_o$ = 23.87 g/L) ($SCOD_o$ = 0.65 g/L)			Reactor: CSR batch at room temperature for 16 days 3 pH scenario: free pH, initial pH = 10, initial and daily pH adjustment to 10 No acclimation	Best results at initial and daily pH adjustment to 10 $[SCFAs_{MAX}]$ = 3080 mg COD/L (15 days) $SCFAs_{MAX}$ /SCOD = 0.5 (15 days) $SCOD/SCOD_o$ = 9.43 (15 days)	Chen et al. 2021
Settled secondary (VSS_o = 13.21 g/L) ($TCOD_o$ = 17.11 g/L) ($SCOD_o \approx$ 0.25 g/L)			Reactor: CSR batch at 35 °C, 12 days CER dosages of 0 (control), 1, 1.75, 3.5 g dry weight/g SS pH neither controlled nor monitored	Best results with 1.75 and 3.5 g CER/g SS (data for 1.75/data for 3.5) $[SCFAs_{OPT}]$ = 4415/4727 mg COD/L (4 days) $SCFAs_{OPT}$ /SCOD = 0.69/0.65 (4 days) $SCOD/SCOD_o$ = 25.6/29 (4 days) *Acid (%)*: Ac. / Pr. / n-bu. / i-bu. / i-va. / n-va. 1.75 g CER/ g SS: 37.5 / 20 / 15 / 7.5 / 15 / 5 3.5 g CER/ g SS: 40 / 17.5 / 14 / 8.5 / 15 / 5	Pang et al. 2020

Sludge	*Conditions for pretreatment*	*Pretreatment results*	*Conditions for biological hydrolysis* (*anaerobic in all cases*)	*Fermentation results* (*best results were those with higher SCFAs concentration*)	*Reference*
Settled secondary (VSS_o = 12.2 g/L) ($TCOD_o$ = 16.7 g/L) ($SCOD_o$ = 0.26 g/L)			Reactor: batch at 35 °C Different CER dosages, stirring intensities and fermentation times according to a CCD pH neither controlled nor monitored	Optimal results *CER (g/g SS)* / *Time (d)* / *Stirring (rpm)* / *DD_M (%)* / *SCFAs (mg COD/L)* 2.05 / 4.97 / 246.9 / 42.1 / 4351.5 1.78 / 6.46 / 261.2 / 42.8 / 4570.5	Pang et al. 2021
Waste activated (VSS_o = 9.96 g/L) ($TCOD_o$ = 13.45 g/L) ($SCOD_o$ = 0.15 g/L)			Reactor: CSR batch at 37 °C, 14 days Sand dosages to achieve VSS/TSS ratios of 45%, 50% and 55% (TSSo adjusted to 15 g/L by diluting with tap water) and control without sand (VSS/TSS ratio of 63.1%) Initial pH adjusted to 10	[$SCFAs_{MAX}$] = 3002 mg COD/L (6 days) $SCFAs_{MAX}$ /SCOD = – $SCOD/SCOD_o$ = – *Acid* (%): Ac. 58.1 / Pr. 13.3 / Bu. 10.2 / Val. 18.4	Jiang et al. 2021
Settled secondary (VSS_o = 8.63 g/L) ($TCOD_o$ = 17.33 g/L) ($SCOD_o$ = 0.26 g/L)			Reactor: CSR batch at 35 °C No acclimation Fermentation at 35 °C with Fe powder, persulfate (PS), Fe + PS and control for 12 days pH no controlled	Best results with Fe + PS [$SCFAs_{MAX}$] ≈ 2255 mg COD/L (8 days) $SCFAs_{MAX}$ /SCOD = – $SCOD/SCOD_o$ = – *Acid* (%): Ac. 76.9 / Pr. 9.7 / Bu. 5.3 / Val. 8.1	Luo et al. 2019
Settled secondary (VSS_o = 10.95 g/L) ($TCOD_o$ = 23.67 g/L) ($SCOD_o$ = –)			Reactor: CSR batch at 35 °C, 14 days No acclimation 4 conditions: control, PDS + Fe^{2+}, PDS + ZVI, PDS + NZVI pH neither controlled nor monitored	Best results with PDS + Fe^{2+} [$SCFAs_{MAX}$] = 5537 mg COD/L (10 days) $SCFAs_{MAX}$ /SCOD = – $SCOD/SCOD_o$ = – *Acid* (%): Ac. 59.6 / Pr. 7.6 / n-bu. 11.4 / i-bu. 6.3 / i-va. 11.4 / n-va. 3.7	Luo et al. 2020
Waste activated (VS_o = 7.97 g/L) ($TCOD_o$ = –) ($SCOD_o$ = 0.08 g/L)	CaO for 15 hours at different dosages: 0, 0.01, 0.03, 0.05 and 0.07 g/g TS (TS_o = 17.39 g/L)	Best solubilisation 0.07 g CaO/g TS $SCOD/SCOD_o$ = 15.14 SCOD/TCOD = – VS/VS_o = –	Reactor: semicontinuous at 35 °C, 12 days HRT = 16.7 by replacing 0.06 L daily Inoculum ratio 2:1 (sludge : inoculum) pH neither controlled nor monitored Feeding with sludge pretreated with 0.07 g CaO/g TS	[$SCFAs_{MAX}$] = 2611 mg COD/L (6 days) $SCFAs_{MAX}$ /SCOD = – $SCOD/SCOD_o$ = – *Acid* (%): Ac. 28 / Pr. 10 / n-bu. 32 / i-bu. 9 / i-va. 15 / n-va. 5	Xin et al. 2021

Sludge	***Conditions for pretreatment***	***Pretreatment results***	***Conditions for biological hydrolysis*** *(anaerobic in all cases)*	***Fermentation results*** *(best results were those with higher SCFAs concentration)*	***Reference***
Sewage sludge (VSS_o = 38.42 g/L) ($TCOD_o$ = 79.49 g/L) ($SCOD_o$ = 17.02 g/L)	Autoclaving at 120 °C, 2 bar, 15 min	$SCOD/SCOD_o$ = 1.16 SCOD/TCOD = 0.26 VSS/VSS_o = 1.1	Inoculum: anaerobic sludge (32 g VS/L) *Batch fermentation* Reactor: CSR at 30 °C, pH not controlled, 30 days 4 experiments with pretreated sludge and 4 without pretreatment, assaying in both cases substrate to inoculum ratios of 1, 2, 4 and 6 (g VS/g VS) *Semicontinuous fermentation* Reactor: 2 L semicontinuous stirred at 37 °C, with 0.4 L of inoculum and 1.6 L of untreated sludge. pH maintained at 5.6 by the feeding Acclimation of inoculum during first week with OLR of 0.9 g COD L^{-1} d^{-1} Operation of 65 days at OLR of 1.3 - 1.6 g COD L^{-1} d^{-1} and 2.4 - 3.5 g COD L^{-1} d^{-1}. HRT of 10 days by replacing 0.2 L with fresh sludge daily	*Batch fermentation* Best results at S/I = 6 with or without pretreatment $[SCFAs_{MAX}]$ ≈ 9500 mg COD/L (≈17 d) $SCFAs_{MAX}$ /SCOD = – $SCOD/SCOD_o$ = – *Acid* Ac. Pr. n-bu. i-bu. i-va. n-va. Untreat. (%) 48 18 15 5 7 7 Treat. (%) 39 23 19 3 9 7 *Semicontinuous fermentation* Best results at high OLR $SCFAs_{MAX}$ ≈ 650 mg COD/L (≈17 d at high OLR) $SCFAs_{MAX}$ /SCOD = – $SCOD/SCOD_o$ = – *Acid* (%) Ac. 36 Pr. 17 n-bu. 21 i-bu. 5 i-va. 11 n-va. 10	Iglesias-Iglesias et al. 2019
Settled secondary (VS_o = 8.06 g/L) ($TCOD_o$ = 11.67 g/L) ($SCOD_o$ = 0.08 g/L)	*Thermal pretreatment* 70 °C for 1 hour *Chemical pretreatment* SDBS dosage of 0.01 g/g TS for 1 hour at 20 °C *Chemical + thermal pretreatment* Different SDBS dosages of 0.005, 0.01 and 0.02 g/g TS for 1 hour at 70 °C TS_o = 12.41 g/L	$SCOD/SCOD_o$ = 24 SCOD/TCOD = – VSS/VSS_o = – $SCOD/SCOD_o$ = 3.3 SCOD/TCOD = – VSS/VSS_o = – (SDBS = 0.01 g/g TS) $SCOD/SCOD_o$ = 28.7 SCOD/TCOD = – VSS/VSS_o = –	Reactor: CSR batch at 37 °C, 7 days pH not controlled but monitored	Results for SDBS = 0.01 g/g TS $[SCFAs_{MAX}]$ = 187 mg COD/g VS (6 days) $SCFAs_{MAX}$ /SCOD = – $SCOD/SCOD_o$ = – *Acid* (%) Ac. 39.4 Pr. 18.6 n-bu. 7 i-bu. 10 i-va. 21 n-va. 4 Best results with 70 °C + SDBS = 0.01 g/g TS $[SCFAs_{MAX}]$ = 320 mg COD/g VS (4 days) $SCFAs_{MAX}$ /SCOD = – $SCOD/SCOD_o$ = – *Acid* (%) Ac. 56.4 Pr. 13.6 n-bu. 10 i-bu. 6 i-va. 9 n-va. 5	Wan et al. 2020

2.2. Simultaneous hydrolysis and fermentation

2.2.1. Biological hydrolysis without additives

2.2.1.1. Effect of pH

Several authors have concluded that pH plays a key role during the direct fermentation of sludge to produce SCFAs. In this sense, Li et al. [60] studied the alkaline fermentation, at pH 10 and 35ºC, of a settled secondary sludge in a semi continuous process.

Although results for sludge fermentation at the same conditions but without pH adjustment were not provided, this strategy at pH 10 allowed a high sludge solubilisation, with final soluble COD (SCOD) at the end of each cycle two times higher than the initial one. The production of SCFAs was also significant at pH 10, with a maximum value of approximately 610 mg COD/g VSS (VSS stands for volatile suspended solids) for a sludge retention time (SRT) of 8 days. However, for higher SRT, SCFAs concentration began to slowly decrease, probably due to the substrate depletion and the methanogenic activity. Focusing on the SCFAs composition, acetic acid was the overwhelming acid during the whole anaerobic fermentation time, accounted for about 84% of SCFAs, followed by propionic, which accounted for approximately 11%. Iso-butyric, n-butyric, iso-valeric, and n-valeric acids were also detected, but at very low concentrations: 1.2%, 0.7%, 2.7% and 0.1%, respectively. These authors also reported the predominance of *Clostridium, Bacillus, Amphibacillus* and *Peptostreptococcaceae* in the microbiota during the alkaline fermentation of sludge.

A deeper study about the pH effect during the sludge fermentation on SCFAs production was carried out by Ma et al. [61], who studied the acidogenic fermentation and characterised the dissolved organic matter (DOM) under different alkaline pH values. For this purpose, a secondary sludge was subjected to a set of fermentations in a semi continuous mode, at 37 °C for 80 days, with a hydraulic retention time (HRT) of 8 days and an organic load rate (OLR) of 1.49 g VS L^{-1} d^{-1}, under different controlled pH, ranging from 7 to 10 between experiments. Additionally, another fermentation was conducted at the same conditions but without pH control as reference.

Results revealed that the higher the pH, the higher the sludge solubilisation. As an example, SCOD for fermentation at pH 10 was 53.73 times higher than under uncontrolled pH, whereas this value was clearly lower for less alkaline media (11.69 times for pH = 7). In parallel, soluble protein concentration at alkaline pH values were

significantly higher than that at uncontrolled pH or 7, whereas soluble polysaccharide increased slightly with the increase in pH, but its content was always lower than the content of solubilised protein in all the reactors. This intense solubilisation at alkaline pH also led to a higher accumulation of SCFAs, increasing productions from 1315 mg COD/L, for uncontrolled pH, to 3762 mg COD/L at pH 10.

Nevertheless, pH had a low impact on the SCFAs/SCOD ratio, which slightly enhanced from 0.51 without pH adjustment to 0.58 at pH 10. To assess these findings, authors studied DOM evolution during the fermentations at each pH value, discovering that in spite of solubilising more intensely, alkaline pHs led to higher accumulation of refractory compounds, such as humic substances and lignin-like compounds.

Finally, although these authors reported an increase in the concentrations of acetic, propionic, butyric, iso-butyric, valeric and iso-valeric acids, the proportion of each acid did not change significantly with pH. Thus, the SCFAs profile at pH 10 was dominated by acetic acid (44.7%), followed by propionic acid (17.1%), iso-valeric acid (15.8%), iso-butyric acid (10.5%), n-butyric acid (9.2%) and, finally, n-valeric acid (2.6%). This SCFAs profile was slightly different to the obtained during the fermentation at uncontrolled pH, being the percentages for the same sequence of acids 46.2%, 26.9%, 9.6%, 7.7%, 3.8% and 5.8%. Therefore, it can be observed that an increase in the pH value during the fermentation led to a slight increase in iso-valeric and n-butyric acid proportions, at the expense of a reduction in the corresponding to propionic acid.

Continuing with the alkaline fermentation of sludge, Yan et al. [62] also investigated the performance of SCFAs production by anaerobic digestion for 111 days under mesophilic conditions (35 °C – 40 °C) with stepwise pH increases from 8 to 11, but employing a sludge previously dosed with poly-aluminium chloride. The results showed that either the SCOD concentration or SCFAs production increased gradually with increasing pH, with a maximum yield of SCFAs of 358.03 mg COD/g VS (volatile solid) at pH 11. Thus, $SCOD/SCOD_o$ and SCFAs/SCOD ratios ranged from 0.51 for pH 8 to 0.68 for pH 11, respectively.

As in the previous studies, acetic and propionic acids were the main SCFAs for all the pH tested, whereas butyric and valeric acids were detected and accumulated at pH of 10 and 11. Specifically, at pH 11, acetic acid predominated (39.6%) followed by iso-valeric acid (16%), propionic acid (13.9%), n-butyric acid (11.1%), iso-butyric acid (10.4%) and,

finally, n-valeric acid (9%). On the other hand, only acetic and propionic acid were present at pH 8, being their percentages of 30% and 70%, respectively. According to Yan et al. [62], these changes in the SCFAs distribution were related to the solubilisation degree, since when more proteins were present, a higher proportion of butyric and valeric acids (and their *iso* forms) were synthesized by the microorganisms.

Finally, an attempt to better understand the pH control on the acidogenic fermentation was made by Chen et al. [63], who evaluated sludge fermentations under different pH control strategies: without pH control, only fitting initial pH to 10 or keeping the pH constant at a value of 10 during the whole process. Their feedstock was a settled secondary sludge, carrying out batch fermentations at room temperature for 16 days without using especial inoculum or acclimation steps. Results revealed that fermentation at controlled pH 10 yielded the highest solubilisation ($SCOD/SCOD_o = 9.43$) and SCFAs accumulation, 3080 mg COD/L, which represented the 50.2% of the SCOD, after 15 days. On the other hand, when only initial pH was fitted to 10, the maximum SCFAs concentration was reduced to 1750 mg COD/L after 10 days, whereas SCFAs/SCOD was 0.58 and $SCOD/SCOD_o$ was 4.62. As can be seen, when the pH was only initially adjusted to pH 10 relative to when the pH was continuously maintained at 10, the purity of SCFAs in the fermented liquid was improved. In addition, initial pH 10 fermentation also improved the phosphorous removal efficiency, and fermented sludge dewaterability during waste activated sludge (WAS) fermentation.

Regrettably, no information about SCFAs composition was given in this work, although, as seen in the previous studies, it is expectable that the different strategies provided different SCFAs profiles.

As can be seen, all authors coincided in stating that a strongly alkaline biological hydrolysis led to higher solubilisation as well as the release of compounds refractory to biological transformation. Moreover, increasing the pH provoked a decrease in propionic acid proportion but favoured the butyric and valeric acids ones in the SCFAs profiles.

2.2.1.2. Effect of OLR

Although less studied than pH, the organic load rate (OLR) is another operational condition with a high impact on the acidogenic fermentation. Iglesias-Iglesias et al. [64] performed a continuous lab-scale experiment at 37ºC and without pH control to study the OLR effect, from 1300 to 3500 mg COD L^{-1} d^{-1}, on the SCFAs production. Authors

observed the greatest SCFAs accumulations occurred at the highest OLR, achieving a maximum value of 0.65 g $SCFAs_{COD}$ L^{-1} d^{-1} for an OLR of 3500 mg COD L^{-1} d^{-1}, but not the best SCFA/SCOD ratio (18%). This latter was found at a low OLR, 1600 g COD L^{-1} d^{-1}, with a value of 22%. Nevertheless, the SCFAs content at this OLR, 0.35 g SCFAs $_{COD}$ L^{-1} d^{-1}, was distinctly lower than for 3500 mg COD $L^{-1}d^{-1}$. Authors attributed this fact to the overload of the substrate as well as to the accumulation of heavy metals and other inhibitory compounds at high OLR. The SCFAs profile was dominated by acetic, propionic, butyric and *iso*-valeric acids for all the OLR tests, although it was reported that butyric and n-valeric acids proportion increased with the OLR.

2.2.1.3. Effect of inoculum size

Although an inoculum is not required to start the anaerobic digestion of sludge, its use can boost the process and, even, modify the SCFAs profile obtained. This finding was reported by Iglesias-Iglesias et al. [64], who carried out the acidogenic batch fermentation of sewage sludge at different substrate/inoculum ratios (S/I, from 1 to 6 g VS substrate/g VS inoculum). The inoculum used was collected from an anaerobic digester in a brewery WWTP. Authors reported that all the fermentations started immediately after the inoculation and, once the maximum SCFAs concentration was reached, its value remained almost constant. Results revealed that the higher the inoculum size, the lower the SCFAs accumulation, which ranged from 6450 mg COD_{SCFAs}/L for a S/I=6, to 2000 mg COD_{SCFAs}/L for the S/I=1 (results for non-inoculated sludge were not provided). Regardless the S/I ratio selected, acetic acid was the dominant product, followed by propionic, butyric and n-valeric acids. Nevertheless, the butyric acid concentration increased with the S/I ratio, meanwhile the proportion of acetic acid decreased. The authors proposed that higher S/I ratios involved higher organic loads, higher accumulation of SCFAs and, subsequently, a decrease of pH, favouring the long chain fatty acids production.

2.2.2. Biological hydrolysis with additives

2.2.2.1. Effect of cation exchange resins

The use of cation exchange resins (CER) is an interesting approach to promote the sludge solubilisation due to the recoverability and reusability of these. In this sense, Pang et al. [20] fermented a secondary sludge at 35 °C for 12 days and in presence of different CER dosages (from 0 to 3.5 g /g SS). This CER was able to exchange its Na^+ cations with

the multivalent ones present in the flocs, thus disrupting their structure and improving the cell breakage. As expected, higher dosages of resin led to higher solubilisation. As an example, the SCOD/$SCOD_o$ ratio at the end of the fermentation without CER was 8, but it raised up to 25.6 if 1.75 g CER/g SS were added. Nevertheless, as authors stated, increasing CER dosage beyond 1.75 g/g SS did not involve a significant improvement in terms of sludge solubilisation. The same trend was also found during the SCFAs production, where maximum SCFAs concentrations were 1279, 4415 or 4727 mg COD/L after 4 day-fermentation in absence or presence of 1.75 or 3.5 g CER/g SS, respectively, with SCFAs/SCOD ratio remaining almost constant in a value of around 0.67.

Putting the light on the SCFAs compositions, CER dosages higher than 1.75 g CER/g SS had a negligible effect in SCFAs profiles, with the following mean proportions: 40% acetic acid, 18.5% propionic acid, 15% n-butyric acid, 15% iso-valeric acid, 8% iso-butyric acid and, finally, 5% n-valeric acid. However, this SCFAs profile strongly differed to the found in presence of lower CER dosage, which favoured the propionic acid production in comparison to other acids.

These authors complemented this approach in a subsequent article [65], where a central composite design (CCD) evaluated by response surface methodology (RSM) was carried out to find optimal conditions of CER dosage (0.25- 3.25 g/g SS), fermentation time (1-8 days) and stirring strength (50-400 rpm) in terms of disintegration degree and of SCFAs production. Results showed that the optimal inputs were 1.78 g CER/g SS, 6.46 days and 261.2 rpm, which led to a disintegration degree of 42.8% and a SCFAs concentration of 4570.5 mg COD/L. As can be checked, these results are not far away from the best conditions obtained in the previous study [20]. Authors stated that when the input factors were lower than the corresponding optimal parameters, the removal efficiency of multivalent cations was increased as the increase of CER dosage, while the increased stirring strength was beneficial for the cation transfer via thorough sludge-CER mixing. However, the further increase in input factors did not improve sludge solubilization and SCFAs production because either CER dosage, stirring strength or treatment time were high enough to not involve a significant effect on sludge solubilisation.

2.2.2.2. Effect of sand

The use of inert additives as promoters for sludge acidogenic digestion was explored by Jiang et al. [66]. These authors studied the fermentation of waste activated

sludge in batch (37 °C, 14 days, initial pH 10) employing different superfine sand dosages (VSS/TSS ratios from 63%, for control without sand, to 45%). Sand acted as fermentation enhancer, which promoted the sludge hydrolysis by creating shear forces on the cells during the stirring. The effect of superfine sand on the solubilization stage was also characterized by measuring soluble protein and carbohydrate contents.

As expected, because of the presence of superfine sand, more shear force was generated, which accelerated the destruction of extracellular polymers, thus increasing the solubilisation at high sand dosages. However, results also revealed that superfine sand had no effect on the subsequent hydrolysis rate of soluble organic materials in sludge, from proteins and carbohydrates to amino acids and monosaccharides. Authors reported that SCFAs production during WAS anaerobic fermentation was positively affected by superfine sand, as indicated by an increase of 1.2 times after 4 days, from 2513 mg COD/L without superfine sand to 3002 mg COD/L with superfine sand (VSS/TSS = 50%). Nevertheless, higher sand dosages worsened SCFAs production, probably due to excessive shear forces that can also affect SCFA-producing microorganisms. Regarding the final distribution of SCFAs, acetic, propionic and valeric acid played dominant roles, although butyric acid was also present in significant proportions. When superfine sand was present in the WAS, the production of acetic and valeric acids was boosted.

2.2.2.3. Effect of oxidants

Another way to enhance the classical biological process is the incorporation of different chemicals to the fermentation medium, which act as oxidants and solubilise the sludge. This facilitates the hydrolysis step, which is considered the limiting one in the biological digestions, as previously explained.

This was the strategy followed by Luo et al., [67] who carried out classical acidogenic fermentations in presence of Fe(0) (1.25 mM/g TSS), persulfate (PS) (1.0 mM/g TSS), and the combination of both (Fe(0)/PS) (1.25 /1.0 mM)/g TSS). All the fermentations were conducted with sedimented secondary sludge in stirred batch reactors, at a temperature of 35 °C and without pH control. Authors reported that the solubilization and hydrolysis efficiencies were mildly improved with the sole Fe treatment, whereas the contributing effect was further enhanced remarkably with the involvement of PS. As a strong oxidant, PS was able to chemically disrupt the EPS structure and result in the release of substrates. The disruptive effects of PS were aggravated due to the generation of strong oxidizing sulphate radicals with the activation of Fe. Surprisingly, the maximal

concentration of soluble substrates was observed in PS reactor, and not in the PS/Fe one. Authors attributed this fact to the higher reactivity of sulphate radicals, compared with PS, thus causing a more severe inhibition on the hydrolases activity.

Moving on to the SCFAs profiles, acetic acid predominated when PS was present, individually or together with Fe (76.9% and 66.5% for Fe + PS and PS alone, respectively). In absence of PS, these percentages decreased drastically (37.1% and 21.9%, for Fe alone and control, respectively). It should be noticed that, although propionic acid was the main SCFAs at the end of sludge fermentations either without additives or with sole Fe (72.8% and 53%, respectively), the presence of PS in the medium highly inhibited its formation (9.7% and 4.9% for Fe + PS and PS alone, respectively). Moreover, PS favoured the presence of valeric acid (8.1% and 11.2% for Fe + PS and PS alone, respectively). Butyric acid was present in all cases, although its proportion did not follow a clear pattern.

Due to the promising results obtained, Luo et al. [68] also evaluated the roles of peroxydisulphates (PDS), and different iron forms as enhancers for acidogenic fermentations. To this purpose, they carried out batch fermentations at 35 °C for 14 days, adding three different iron forms (Fe^{2+}, ZVI (zero-valent iron) and NZVI (nano zero-valent iron)) to a settled secondary sludge already containing PDS.

Experiments revealed that the most efficient activation of PDS was achieved by Fe^{2+}, as well as promoting a best sludge dewatering and the highest VSS reduction. Thus, maximum SCFAs accumulation with PDS + Fe^{2+} was 5537 mg COD/L after 10 days, being this clearly higher than those achieved with other iron forms (3036 mg COD/L for PDS + ZVI, 3533 mg COD/L for PDS + NZVI -in this case after 8 days- and 702 mg COD/L for control). In any case, the SCFAs production was clearly diminished with no chemical addition (PS or activator), with the highest accumulation for the control reactor of only 702 mg COD/L after 10 days. Again, the synergistic effects of PDS and activators in contributing to SCFAs promotion were clearly demonstrated.

Regarding the effects of these activators on the SCFAs final composition, the addition of PDS and any iron form sharply increased the production of acetic acid, from 13.8% for the control to 59.6% with PDS + Fe^{2+}, 66% with PDS + NZVI or 72.2% with PDS + ZVI. On the contrary, these compounds also lowered propionic acid formation, from 61.1% for the control to 7.6% with PDS + Fe^{2+}, 20% with PDS + NZVI and 9.8% with PDS + ZVI. The changes in other SCFAs (for instance, n and iso-butyric and n and

iso-valeric acids) due to the presence of the different iron forms and PDS were less marked. As an example, n-butyric presence was not detected for control and PDS + NZVI fermentations but represented a 1.3% and an 11.4% of the total SCFAs for the PDS + ZVI and PDS+ Fe^{2+} fermentation.

2.3. Separated hydrolysis and fermentation

2.3.1. Chemical hydrolysis

2.3.1.1. Effect of CaO

The SCFAs production by acidogenic fermentation of sludge previously subjected to a chemical solubilisation by adding calcium oxide (CaO) was assayed by Xin et al. [69]. Focusing firstly on the chemical solubilisation, a waste activated sludge was subjected to a 15-hour treatment under different CaO dosages, from 0 to 0.07 g CaO/g TS. As expected, the higher the CaO dosage, the greater the sludge solubilisation, with the $SCOD/SCOD_o$ at the end of the pretreatment ranging from no variation, in absence of CaO, to 15.14 with a dosage of 0.07 g CaO/g TS. During the sludge solubilisation, CaO generated hydroxy radicals, which promoted the dissolution of flocs, and, at the same time, Ca^{2+} affected the sludge floc structure via bonding with functional groups, thus also improving the solubilisation.

After the CaO pretreament, Xin et al. [69] compared the acidogenic fermentations of the non-pretreated sludge (OLR = 0.005 g COD L^{-1} d^{-1}) and the hydrolysate obtained by treating the sludge with 0.07 g CaO/g TS (OLR = 0.073 g COD L^{-1} d^{-1}). The conditions of fermentation involved perfect mixing, a HRT of 16.7 days and a temperature of 35 °C. However, even though authors indicated that pH was measured, these data did not appear reported throughout the text. Maximum SCFAs concentration was achieved with the CaO pretreated hydrolysate tests after 6 days, being1.5-fold higher than for the control test at the same time (2611 mg COD/L and 1800 mg COD/L, respectively).

Additionally, the microbial diversity was shifted in CaO-pretreated sludge fermentation. However, SCFAs profiles at this point were quite similar, regardless the previous addition of CaO to sludge. Thus, the main acid was n-butyric (34% for control and 32% with pretreamtent), followed by acetic acid (25% and 28%), iso-valeric acid (12% and 15%), propionic (8% and 10%), iso-butyric (13% and 9%) and n-valeric acids (7% and 5% for pretreated sludge). As can be observed, there is a relatively high abundance of SCFAs with “long” chains (butyric and valeric acids), phenomenon that

authors linked to a high content of proteins in the hydrolysates, although this was not experimentally checked.

2.3.1.2. Effect of surfactants

Wan et al. [70] tested the effect of sodium dodecylbenzene sulfonate (SDBS) addition as pretreatment method on the SCFAs production. To this end, 0.01 g SDBS/ g TS were added to a secondary settled sludge, which after 1h at room temperature, was fermented comparing the results with the fermentation of the same sludge in absence of SDBS. After a week of fermentation at 35ºC, it was checked that SDBS significantly improved the production of SCFA, from 60 mg COD g VS^{-1} to 170 mg COD g VS^{-1}. In addition, the presence of SDBS also had impact on the SCFAs distribution, favouring the production of acetic and n-butyric acids in comparison to propionic acid. In fact, propionic acid was the main acid in the control fermentation (38%), but not in presence of SDBS, where acetic acid turned out to be the predominant one (37%), being lowered the content in propionic acid to 18.6%. The percentages for all the other acids did not changed when adding SDBS: iso-valeric acid (21%), iso-butyric acid (11%), n-butyric acid (6%) and n-valeric acid (3%).

2.3.2. Thermal hydrolysis

This section involves all the recent studies dealing with thermal hydrolysis as sludge pretreatment for the subsequent acidogenic fermentation of the hydrolysate obtained.

2.3.2.1. Effect of inoculum size

Iglesias-Iglesias et al., [64] evaluated the combined effects of using a thermal pre-treatment and inoculum size (120 °C and 2 bar for 15 min) on the SCFAs production. By means of the application of the thermal pretreatment, the SCOD increased a 16%, as well as the concentrations of $N\text{-}NH_4^+$ and $P\text{-}PO_4^{-3}$ (5% and 19%, respectively). Another effect of the thermal treatment was a 4% reduction in total COD (TCOD) due to the loss of volatile soluble compounds. The hydrolysate obtained was then inoculated with the microbiota of an anaerobic digestor at different S/I ratios, ranging from 1 to 6 g VS substrate/g VS inoculum, and fermented at 30 °C for 30 days without pH control.

It was found a higher SCFAs production with than without thermal pre-treatment, although this difference was less marked at high S/I, at which the maximum acidogenic potential was found (9500 mg COD_{SCFAs} L^{-1} after 10 days). As in the case of the raw

sludge fermentation (see section 2.2.1.3), the higher the S/I ratio, the higher the SCFAs accumulation after the fermentation of the thermally hydrolysed sludge. However, when authors calculated the degree of acidification, (the amount of acids generated per unit of initial TCOD), this was found to be higher when larger inocula were used for fermenting the thermally hydrolysed sludge, ranging from 45% for S/I=1 to 37% for S/I=6. Therefore, low inoculum sizes produced more concentrated but less pure streams of SCFAs at the end of the fermentation. In any case, higher degrees of acidification were observed when the sludge was thermally pretreated, especially at low S/I ratios. Finally, in comparison to the non-pretreated sludge, the thermal pre-treatment also modified the final SCFAs profile, enhancing the proportions of propionic, iso-butyric, n-butyric, *iso*-valeric and n-valeric acids at all the S/I ratios tested.

2.3.2.2. Effect of surfactants

The combination of pretreatments is also feasible. This is the case of Wan et al. [70], who tested the efficiency of combining SDBS and low-thermal pretreatment to improve the production of SCFAs from a secondary settled sludge. To this end, sewage sludge was firstly subjected to a thermal treatment (1 h, 70°C) in presence of different SDBS dosages (from 0 g/g TS to 0.02 g/g TS) and then fermented in batch reactors at 35 °C for a week, without employing an inoculum.

From the characteristics of hydrolysates obtained after the different pretreatments, it clearly emerged that the combination of SDBS and thermal treatment was the most effective option to solubilise the sludge. Thus, the SDBS addition (0.01 g/g TS) during the thermal pretreatment increased around a 20% the SCOD in the hydrolysate in comparison to the sole low thermal treatment. Meanwhile, the combination of temperature and SDBS also made possible to enhance a 768% the final SCOD with respect to the sole SDBS pretreatment. Comparatively, sole thermal pretreatment was clearly more effective than sole SDBS dosage solubilising the sludge.

The combined SDBS and thermal pretreament was also the most effective in terms of acidogenic potential of the hydrolysate obtained. The maximum SCFAs concentration was obtained after 4 days of fermentation of the sludge previously hydrolysed by a combination of thermal treatment and 0.01 g SDBS/g TS, with a value of 320 mg COD/g VS. This value was 1.8, 1.7 and 4.0 times higher than those from sole low-thermal, sole SDBS and the control test, respectively. It can be stressed that the dosage of more than 0.01 g SDBS/g TS resulted in a toxic effect during the acidogenic fermentation which not

only led to a reduction in the maximum SCFAs concentration, but also to a delay in the time at which it was reached.

Focusing on the SCFAs profiles, these differed depending on the pretreatment chosen. First of all, the SCFAs profile for the control test was: 21% of acetic acid, 38% of propionic acid, 12% of iso-butyric acid, 5% of n-butyric acid, 2% of n-valeric acid and 22% of iso-valeric acid. The employment of a low thermal treatment involved a clear increase in acetic acid (up to 62.6%) and an impoverishment in propionic, iso-butyric and iso-valeric acid percentages (down to 15.4%, 3% and 14%, respectively). The presence of SDBS (0.01 g/g TS) during the thermal pretreatment caused slight decreases in the acetic (from 62.6% to 56.4%) and iso-valeric acids percentages and, therefore, slight increases in the propionic, butyric and n-valeric acids.

3. Polyhydroxyalkanoates

3.1. General considerations

Although the methods described in the previous section generate added value products from sewage sludge, there are more alternatives comprising both sludge management and circular economy to be explored. In this regard, taking into account that society is currently more aware than ever of the problems generated by petrol-based plastics, the demand of new green materials that could replace them is opening up new opportunities for the sludge valorisation. Among these green materials, ones with a high potential are PHAs, which are biopolymers mainly composed by polyhydroxybutyrate (PHB) monomers, although polyhydroxyvalerate (PHV) is often found in their composition as well [71].

Chemically, PHAs are polyesters that microorganisms use as energy storage [72,73]. PHAs composition is similar to polypropylene, therefore they can substitute petrol-based plastics. In fact, their use has been explored in the packaging of cosmetics or feminine hygiene products, food coatings, formulation of wood composites and 3D printing inks, among others. Due to their biocompatibility, PHAs have also been employed in several fields of medicine, such as tissue engineering, repairing blood vessels, nerves conduits, artificial heart valves or implants or as drug carrier material. Other applications for PHAs included their use as encapsulation material for enzymes, fertilizers or insecticides or as materials to cover and protect the growing plants, the synthesis of chiral compounds or, even, their depolymerization to obtain platform molecules for further uses [73–77].

Although several types of species can synthesize PHAs, the ones here meaningful are bacteria, owing to its predominant proportion in sewage sludge and their faster growth abilities. The biochemical mechanism of PHAs accumulation by bacteria during WWTPs operation is well studied, so it is not required to be detailed here [78]. Suffice it to say that PHAs production naturally occurs in WWTPs simultaneously with phosphorus removal, since this biopolymer is naturally accumulated by bacteria under situations of anaerobiosis (and in absence of either nitrites and nitrates) by means of the uptake of low-complex carbon molecules, such as SCFAs, which can be easily obtained from sludge [78]. It is interesting to note that several studies have showed that some microorganisms were able to accumulate even more PHAs in aerobic conditions [78–80].

Given that SCFAs are the main substrate for PHA-producing microorganisms and their presence in raw sludge is low, a previous sludge acidogenic fermentation is required, although other non-biological pretreatments are also possible for this purpose, as will be explained ahead. In any case, the PHA-producing reactor feeding should have a significant content of SCFAs in its composition, since it is proved that the presence in a proportion higher than 30% of other carbon molecules, such as proteins or carbohydrates, largely hinders the PHAs accumulation [81,82]. Consequently, all those methods employed to enhance SCFAs production from sludge (see previous section) also represent an excellent pretreatment to improve the subsequent PHAs accumulation.

PHAs production not only depends on the total concentration of SCFAs, but also on the profile of these. Thus, the presence of a high proportion of acids with an even number of carbon atoms, such as acetic or butyric acids is a determining factor during the PHAs accumulation by microorganisms, although this phenomenon will be checked and discussed after the description of the studies [83]. Soluble nitrogen concentration plays other important role in the final PHAs production, since nitrogen is needed for cellular metabolism. Particularly, the highest the C/N ratio, the highest the PHAs accumulation, at least in ranges of C/N (in mass basis) lower or equal to 144 [84].

In the following lines, the most recent studies dealing with PHAs production from hydrolysed sludge obtained by different pretreatments will be discussed. It must be pointed out that only works where the feeding of the PHA-producing step is clearly detailed were included. A summary of these studies can be found in table 2.

Table 2. *Summary of the main parameters found in the studies dealing with PHAs production.*

Sludge	*Thermal treatment*		*Acidogenic fermentation*		*PHA fermentation*		*Reference*
	Conditions	**Results**	**Conditions**	**Results**	**Conditions**	**Results**	
Settled activated sludge			Spontaneous fermentation at 36 °C, 100 rpm for 1 day Different pHs: 10.0, 9.0, 8.0, 7.0, 6.0, 5.0 and 4.0	Better SCFAs/SCOD results at pH 10.0, 9.0 and 4.0: 61.8 %, 57.0 % and 58.0% respectively Mainly acetic acid, propionic acid and low valeric acid quantities	Seed sludge acclimated in ADF (10% of inoculum) at 25 °C in SBR for at least 3 cycles of 12 h Feeding of SCFAs liquid, nutrient solution of N and P, trace elements and seed sludge Five stages per cycle in a SBR at 25°C: influent (5'), anaerobic with mixing (25'), aerobic (660'), settling (25') and effluent (5')	0.6 g PHA/g VSS using pH 10 SCFAs liquid after 0.5 h Mainly composed of PHB (>90%)	(Liu et al., 2020)
Sludge from thickening tank (lab-scale experiments) Diluted dewatered sludge (pilot scale experiments)			Lab-scale: spontaneous fermentation at 40 °C, 1200 rpm without pH control Pilot scale: 50 °C	55.83% and 65.17% of SCFA/COD for lab-scale and pilot scale respectively. Mainly acetic acid, similar concentrations of propionic acid, butyric acid and iso-valeric acid	Seed sludge from secondary sedimentation tank acclimated in synthetic media at 30 °C and pH 7 in at least 3 cycles in feast-famine strategy Lab-scale: 30 °C, 150 rpm and pH 7 for 60 hours Pilot scale: feast-famine cycles (120 h) at 30 °C, 150 rpm without pH control	0.23 g PHA/g dry cell after 36 hours 0.59 g PHA/g dry cell after 72 hours and 4 batch at pilot scale	(Jia et al., 2014)
Thickened waste activated sludge			Spontaneous fermentation at 35 °C, 42 °C and 55 °C in 10 days batches. No pH control	90% of SCFA/SCOD at 42 °C and 4-5 days (the best condition) Mainly acetic acid (38%) followed by butyric acid (20%) and similar percentages of propionic, valeric and iso-valeric acids (≈ 10%)	Seed sludge from a SBR treating real wastewater in feast-famine (12 cycles a day) Production by feed-on-demand. Temperature and pH not controlled. Different accumulation times	Maximum of 0.38 g PHA/g VSS (22 °C – 25 °C, pH ranging from 7.5 to 8.1) after 15 h 70% of PHB, 30% of PHV (mean values)	(Morgan-Sagastume et al., 2015)

Sludge	*Thermal treatment*		*Acidogenic fermentation*		*PHA fermentation*		*Reference*
	Conditions	**Results**	**Conditions**	**Results**	**Conditions**	**Results**	
Secondary from sedimentation tank			Spontaneous fermentation in anaerobiosis Different temperatures and pHs: 21 °C, 35 °C, 60 °C; pH 8, 9, 10 and 11 Addition of 0.02 g/g VSS of SDBS	Optimal conditions: 60 °C and pH 11.0 2.56 g SCFA/L as TOC Mainly acetic acid (80.5%) and propionic acid (11.4%). Lower concentrations of butyric and valeric acids and their isomers	Sludge from aeration tank without acclimation Feeding in three pulses, when substrate exhausted 21 ºC, no pH control, 250 rpm	0.565 g PHA/g dry cell after 3 h 40’ 88.1% of PHB, 11.9% of PHV	(Mengmeng et al., 2009)
Chemically enhanced ($FeCl_3$) sedimentation primary sludge			Spontaneous fermentation at 37 ºC. No pH control Addition of different CaO_2 concentrations to enhance hydrolysis	Better results with 0.1 g CaO_2/g VSS: 93.5% SCFAs/SCOD after 16 days Mainly propionic and acetic acids (50% and 35% respectively) followed by a 10% of iso-valeric acid	SBR (feast-famine) with activated sludge as inoculum. 21 ºC with SRT of 10 days	Maximum of 0.223 g PHA/g VSS at day 35 Polymer made of equal parts of PHB and PHV	(Xu et al., 2018)
Municipal and primary + municipal waste activated sludge subjected to a CAMBI™ process			Semi-continuous spontaneous fermentation at 42 °C, 20 – 30 g/L d of organic load rate	In mix of sludges, 50% SCFAs/SCOD with predominance of acetic acid (≈ 27%) and butyric acid (≈ 23%), similar proportions of propionic and valeric acids (≈ 17%) and lower representation of valeric, caproic and isobutyric acids In only municipal sludge: 58% SCFAs/SCOD, being acetic acid the main one (≈ 32%) followed by butyric acid and propionic acid (≈ 18%), isovaleric acid (≈ 14%) and isobutyric and valeric acids (≈ 8%)	SBR reactor in feast-famine regime, with activated sludge as inoculum, controlled at 35 °C and 400 rpm. No pH control. Cycles of 4 hours	0.2 g PHA/g TSS Polymer made of a 74% of PHB and a 26% of PHV	(Morgan-Sagastume et al., 2010)

Sludge	*Thermal treatment*		*Acidogenic fermentation*		*PHA fermentation*		*Reference*
	Conditions	**Results**	**Conditions**	**Results**	**Conditions**	**Results**	
Primary + secondary sludge	155 °C – 175 °C, 6 bar for 30 minutes (CAMBI)	Increase of SCOD (from 2% to 27%), proteins (from 1% to 50%) and carbohydrates (from 1% to 69%) Little effect on SCFAs: only 0.3% more in TH sludge (1.1 g SCOD/L and 1.3 gSCOD/L in raw and TH sludge respectively)	Fermentation using anaerobic digested sludge as inoculum Fermentations at 35 °C with TH sludge and raw sludge; 55 °C with TH sludge and raw sludge. No pH control (ranging from 6.0 to 6.6). First at batch mode (10 days), then semi-continuous with SRT of 4 days.	71% and 62% SCFAs/SCOD for raw sludge at 35 °C and 55 °C, respectively 50% and 45% SCFAs/SCOD for raw sludge at 35 °C and 55 °C, respectively Mainly acetic acid (≈ 30%), followed by butyric (≈ 27%), valeric (≈ 24%) and propionic acids (≈ 17%)	Inoculum from aeration tank Feast-famine regime at 35 °C and 150 rpm Organic load at 5 g SCOD/L	Results from acidified TH-sludge at 35 °C after 15 hours in the eight cycle: 0.346 g PHA/g dry cell 70% of PHB and 30% of PHV	(Zhang et al., 2019)
Waste sludge from sedimentation tank	Thermal treatment at 60 °C, 80 °C, 100 °C and 120 °C for 2 hours	55.4% (the highest) SCFAs/COD at 60 °C with higher proportion of acetic acid than propionic, butyric or valeric ones			Seed sludge acclimated in synthetic wastewater, SBR with ADF at 25 °C, pH 7.5 in 3 cycles of 12 hours Feeding of thermally treated liquid, nutrient solution of N and P, trace elements and seed sludge SBR with ADF at 25 °C, pH 7.5 in cycles of 12 hour	0.241 g PHA/g VSS using 60 °C liquid after 1.5 h Mainly composed of PHB	(Liao et al., 2018)
Mix of primary and waste activated sludge with acidogenic fermentation	240 °C and 20 bar with O_2 (wet oxidation)	46% SCFAs/TOC Mainly acetic acid (74%) and lower proportions of propionic acid (12%) and butyric acid (8%)			Biomass conditioning in SBR in 12-hour cycle of feast-famine with effluent of WO at 800 mg C/L Temperature and pH controlled at 30 °C and 7.0 – 8.8 respectively	0.4 g PHA/g TSS Polymer made of 77% of PHB and 23% of PHV	(Wijeyekoon et al., 2018)

3.2. Biological hydrolysates for PHAs production

3.2.1. Non-assisted biological procedures

This section covers those studies dealing with the PHAs production from a sludge previously subjected to an acidogenic fermentation and without adding any reagent, as the simplest approach to obtain these biopolymers.

3.2.1.1. Effect of pH

In this light, Liu et al. [82] evaluated the effect of the initial pH on both SCFAs formation and subsequent PHAs production by fermentation, employing a settled activated sludge as feedstock. The initial pH values studied ranged from 4 to 10 and the acidogenic fermentations were carried out at 36 ºC for only 1 day.

As explained in section 2.2.1.1., results confirmed that fermenting waste sludge under initially alkaline conditions was more beneficial to solubilise and release organic matters than under initial acidic or neutral conditions.

Initial pH also had impact on the release of nitrogen from sludge during the acidogenic fermentation, observing the highest COD/NH_4^+ ratios at alkaline conditions, with values of 19.5 and 15.96 for pH 9 and pH 10, respectively. For lower initial pH values, this parameter ranged from 5.26 (pH = 7) to 9.57 (pH = 6). It was found that initial alkaline pH values enhanced the SCFAs production than acidic ones, obtaining hydrolysates with more than 50% of the soluble COD corresponding to SCFAs at initial pH values of 10 (61.8%), 9 (57%) and, surprisingly, also at 4 (58%). However, it should be notice that, a lower soluble COD value was reported at pH 4, with this being close to a half of that corresponding at initially alkaline pH values.

SCFAs profiles at the end of the acidogenic fermentations at different initial pH values were consistently dominated by acetic (45.0 – 74.2%), propionic (8.8 – 47.0%), butyric (3.9 – 20.0%) and little of valeric (2.2 – 6.2%) acid. It was observed that initial acidic pH during sludge fermentation favoured the production of acetic acid, whereas alkaline conditions promoted the generation of butyric acid and near neutral pH conditions speeded up the formation of propionic acid and valeric acid.

Once acidogenic fermentation was completed, the different sludge hydrolysates obtained were fermented to produce PHAs. It was found that the maximal PHAs content accounted for 60.3% of the dry cell and it was achieved employing the acidogenic liquid hydrolysate obtained from sludge at an initial pH of 10. Under these conditions, the

recovered polymer composition was 98.3% PHB and 1.7% PHV by mass. This PHAs accumulation was almost 3 times higher than the achieved using the hydrolysate from raw sludge at initial pH 7 as substrate.

The effect of initial pH of the raw sludge on the final production and composition of PHAs was linked to the composition of the acidogenic liquid obtained. Exploring the preferential consumption of the organic acids, Liu et al. [82] found that PHA-storing microorganisms preferred SCFAs with an even-number of carbon atoms as substrates. At the same time, the consumption of these even-number SCFAs also increased the proportion of PHB in the final polymer, whereas the bacterial uptake of odd-number SCFAs led to a higher production of PHV. Moreover, authors also delved into the consumption of other non-SCFAs substrates available in the sludge hydrolysate after the acidogenic fermentation, such as proteins or fulvic and humic acids. They reported that protein concentration sharply decreased, with more than 70% of the solubilised protein consumed in less than an hour. On the contrary, humic-like substances were poorly metabolised during the PHA-producing fermentation. This is an interesting fact, since sludge hydrolysates usually present an important content of humic acids [31,43].

3.2.1.2. Effect of temperature

Morgan-Sagastume et al., [86] subjected a thickened waste activated sludge to a spontaneous acidogenic fermentation at temperatures ranging from 30 to 55 ºC at pilot scale for 10 days. The centrates obtained were subsequently used to accumulate PHAs, thus discussing the effect of temperature on either the production or composition of these.

These experiments revealed that the optimal temperature for SCFAs production was 42 °C, temperature at which the SCFAs represented the 90% of the soluble COD in the centrate after 4-5 days of acidogenic fermentation. Nevertheless, WAS fermentation at 35 and 55 °C also produced SCFAs-rich centrates with lower but reasonably good SCFAs/SCOD ratios (> 70%), also amenable for use as substrate for PHAs production. It should be noticed that, although higher temperatures solubilised more solids and increased the SCFAs specific production rates per loaded waste sludge, also involved lower SCOD conversions into SCFAS. In fact, the maximum SCFAs volumetric concentrations achieved were not statistically significantly different among the three tested temperatures, with a mean value of around 8 g COD_{SCFA}/ L. Differences in SCFAs profiles due to temperature were slight, with these being dominated by acetic (28–38%, COD basis), butyric (15 – 26%), propionic (13 – 23%), iso-valeric (12 – 18%) and valeric

(4 – 11%) acids, with little of iso-butyric (6 – 9%) and trace amounts of caproic (< 1%) acids.

Regrettably, Morgan-Sagastume et al., [86] only tested sludge hydrolysate obtained by acidogenic fermentation at 42ºC as substrate for the PHA-producing fermentation. The maximum PHAs accumulation reported was 0.39 g PHA/g VSS, which were constituted by a mixture of PHB (70%) and PHV (30%).

Mengmeng et al. [87] continued studying the effect of fermenting sludge at different temperatures (from 21 to 60 °C) on the PHA-producing potential of the acidic hydrolysates generated. Nevertheless, when comparing to Morgan-Sagastume et al., [86], two clear differences in the experimental procedures can be distinguish. Firstly, Mengmeng et al. [87] added sodium dodecylbenzene sulfonate (SDBS) at the dosage of 0.02 g/g VSS in each batch experiments to improve sludge solubilisation [88]. Secondly, these authors also used $MgCl_2 \cdot 6H_2O$ after the sludge fermentation to recover ammonium and phosphorus by struvite deposition, and to control the carbon to nitrogen ratio in the PHA-producing fermentation. As expected, during the fermentations, SCFAs concentration value increased gradually with the rise of temperature at each pH tested (from 8 to 11), which indicated that more soluble substrates were produced from particulate organics in excess sludge under the alkaline conditions. It was also found that pH lower than 9 favoured the production of butyric and valeric acids. Under the best conditions (pH 11, 60ºC and 6 days), the maximum yield of SCFAs was 258.65 mg TOC/g VSS, with acetic and propionic acids being the main products. Specifically, after struvite recovery, sludge hydrolysate was mainly composed by acetic acid (80.5% - the highest found among all the revised studies), with an 11.4% of propionic acid and a low presence of other SCFAs, such as iso-valeric (2.2%), iso-butyric (1.3%), n-butyric (4.0%), and n-valeric acids (0.6%). The reason for the high ratio of acetic acid to other SCFAs was attributed to the high protein and carbohydrate content (55%) in the sludge of this study, which contributed to the generation of SCFAs with 2 – 5 carbon chain. Propionic, iso-butyric, *n*-butyric, iso-valeric, or *n*-valeric acids were easily biodegraded to form acetic acid and were not much accumulated in the anaerobic fermentation system.

As in the case of Morgan-Sagastume et al., [86], only the best centrate obtained (pH 11, 60 ºC) was employed as substrate for the PHAs-producing fermentation by Mengmeng et al. [87]. It was reported a maximum accumulation of 56.5% of PHAs in dry cell basis after 220 minutes of fermentation. As expected, acetic acid was again the

most easily assimilable by PHA-producing microorganisms. Finally, due to the high percentage of acids with an even number of carbon atoms (86%) in the hydrolysate, the PHA polymer was mainly composed by PHB (88.1%).

3.2.1.3. Effect of scaling up

Jia et al. [81] studied the PHAs generation from excess sludge fermentation liquid at lab and pilot scale. For the former, PHAs-producing fermentations were carried out using either a hydrolysate obtained by acidogenic fermentation of thickened sludge at 40 °C in batch experiments or a simulated acidic centrate with a similar composition, but prepared using commercial SCFAs, nitrogen and phosphorus sources. On the other hand, a hydrolysate obtained by acidogenic digestion at 50 °C of a dewatered sludge in a real WWTP was employed for the pilot scale fermentations.

Lab-scale PHAs fermentation in simulated sludge fermentation liquid produced a maximal PHAs content of 59.18% of the cell dry weight at 36 h. The specific uptake rate of acetic acid was the highest, secondly butyric acid and the last propionic acid, which confirmed that PHA-producing consortium preferred to utilize even-number acids. On the other hand, employing a real centrate for lab tests, fermentation led to a significantly lower PHAs accumulation, with only around a 25% of the dry cell weight after 36 hours. This lower value using the real centrate was attributed to a higher cell growth due to its lower SCFAs/SCOD ratio (56%), in comparison to simulated fermentation liquid (96%), almost completely composed by SCFAs. On the other hand, the highest PHAs production during the fermentation of the real acidogenic liquid at pilot scale was 59.47% of the dry cell weight, once the consortium was completely adapted to the environment and obtained a high ability of PHAs synthesis in this pilot-scale reactor. This value was significantly greater than at lab scale. These authors proposed that the adverse impact of excess nitrogen and non-SCFAs fraction in the real centrate on PHAs accumulation might be eliminated by pilot-scale domestication, which might result in community structure optimization and substrate selective ability improvement of the microbial population.

Anyway, these findings should be viewed with caution, due to the different characteristics of the real acidogenic liquids played for lab or pilot scale PHAs-producing fermentations. As an example, COD/NH_4^+ ratios were 19 for the hydrolysate employed in the laboratory experiments, 15.68 for the one used at pilot scale and 20.5 for the simulated acidogenic liquid. Additionally, no information is provided about the composition of the PHAs obtained. In any case, the authors proved that the integration of

anaerobic digestion and PHAs fermentation at pilot-scale was feasible to reduce excess sludge disposal and produce PHAs with low cost.

Continuing with the study of the scaling process, Morgan-Sagastume et al. [86] evaluated the integrated production of polyhydroxyalkanoates (PHAs) with municipal wastewater and sludge treatment by operating a pilot plant for 22 months. Although this study has already discussed in section 3.1.1.2, it should be recalled that the authors stated that producing a biomass with 0.5 g PHA/g VSS is considered to be realistically achievable within the typically available carbon flows at municipal waste management facilities.

3.2.2. Assisted biological procedures

Some authors have also opted to combine the biological hydrolysis of sludge with the addition of different chemicals in order to find potential synergistic effects between them on the PHAs production.

3.2.2.1. Effect of additives (peroxides)

Xu et al. [89] studied the feasibility of producing PHAs with calcium peroxide (CaO_2) treated chemically enhanced primary sedimentation sludge fermentation liquor. With this aim, different CaO_2 dosages, ranging from 0.02 to 0.1 g CaO_2/g SS, were added to a primary sludge previously thickened by $FeCl_3$ coagulation, observing a significant enhancement in the SCFAs generation during the subsequent acidogenic digestion at 37 °C and without pH control, in comparison to the control without CaO_2 addition. On the other hand, the release of P from sludge decreased with increasing CaO_2 dosage, which was probably attributed to the precipitation of calcium phosphate. CaO_2 also induced the microbial reduction of Fe(III) and helped to maintain the alkaline conditions, facilitating the sludge disintegration.

Numerically, a CaO_2 dosage of a 0.1 g/g SS increased a 35% the $SCOD_o$ solubilisation and a 44.7% the SCFAs yield, compared with the control sludge. It was also proved SCFAs yield with H_2O_2 treatment was not enhanced as much as CaO_2 did, due to a serious inhibition of the acidogenic bacteria by H_2O_2 at high dosages. CaO_2 addition not only changed the proportion of SCFAs, but also their distribution, by increasing the quantities of propionic acid (48% with CaO_2 and 40% for control) and lowering the acetic acid ones (36% with CaO_2 and 42% for control).

It should be noticed that CaO_2 addition also decreased Fe^{2+} concentration, which was toxic for the microbes. Therefore, CaO_2 dosage was beneficial to the further utilization of fermentation liquor as substrate for PHAs biosynthesis due to the reduced concentration of Fe^{2+}. Another advantage is the release of P from sludge decreased with the increasing CaO_2 dosage, due to the precipitation of calcium phosphate, which was also beneficial to utilise the fermentation liquor as substrate for PHAs production. Utilizing the fermentation liquor obtained with a CaO_2 dosage of a 0.1 g / g SS as medium, the PHAs content in activated sludge reached 22.3% after 35 days, which was comparable to those obtained using waste materials as carbon source. PHAs composition was 50% PHB and 50% PHV. This did not turn out to be surprising, taking into the high proportion of acids with an odd number of carbon atoms (57.4%) in the initial biological hydrolysate.

3.2.2.2. Effect of thermal pretreatment

Please, see section 3.3.2.

3.3. Thermal hydrolysates for PHAs production

The aforementioned studies make it clear that biological hydrolysis is a feasible sludge pretreatment to obtain suitable substrates for a subsequent PHAs bioproduction. However, there are other available pretreatments to improve the production of PHAs. In this sense, hydrothermal processes can enhance the solubilisation, hydrolysis and SCFAs content of the sludge. This is why, in the following lines, studies involving the hydrothermal pretreatment of sludge in order to enhance SCFAs production and, consequently, PHAs accumulation in a subsequent fermentation, will be revised.

3.3.1. Non-assisted thermal procedures

This section includes all those recent studies where thermally hydrolysed sludge is directly used as substrate for PHAs-producing fermentations, completely avoiding the acidogenic digestion of the sludge.

3.3.1.1. Effect of temperature

In this context, Liao et al. [90] assayed the effect of temperature during thermal pretreatrment of a waste sludge from a secondary sedimentation tank on the subsequent PHAs-producing fermentation. The range of temperature tested was 60 – 120ºC and the duration of the pretreatment, 2 h.

Predictably, as the increase of heat-pretreatment temperature, SCOD and concentrations of soluble proteins, carbohydrate, ammonia nitrogen and SCFAs

increased. The maximum solubilisation was reached at 100ºC, with final soluble COD and SCFAs concentrations 7.3 and 29 times higher than the initial ones. It is noticeable that temperatures higher than 100ºC involved a lower solubilisation, due to the proteins precipitation by conformational changes in their structure.

Nevertheless, the lowest SCFAs/SCOD ratio was also found at 100 ºC (25.5%), whereas it increased as temperature moved away from this value. In fact, 60 °C was the best value of the tested heat-pretreatment temperature range to improve the PHAs productivity, because the SCFA/SCOD was the highest (55.6%).

The pretreatment temperature did not only had an effect on the amount, but also on the distribution of SCFAs. Results showed that predominant SCFAs in the hydrolysis liquid at 60 °C was acetic acid, accounting for 47.1% of total SCFAs, while propionic acid was the major component in the hydrolysis liquid at 80 °C, 100 °C and 120 °C (46.0%, 53.4% and 42.2% for each)

During the PHAs-producing fermentation itself, microorganisms showed a quick adaptation to sludge carbon source at any pretreatment temperature. It was confirmed that 60 ºC was the optimal temperature for the sludge pretreament in terms of PHAs synthesis, with a maximal PHAs content of 24.1% of dry cell in less than 3 hours (≈14% for other temperatures). For all the pretreatment temperatures, PHA polymer was mainly composed by PHB, with the highest PHV percentage being observed using 100ºC hydrolysate (17.9%), temperature at which the highest percentage of odd-numbered SCFAs was found.

3.2.1.2. Effect of oxidising atmosphere

Although thermal hydrolysis is clearly the most commonly used non- biological technique to improve PHAs production (either in combination with a subsequent biological hydrolysis or alone), there is another hydrothermal treatment that has also been employed: wet oxidation. This is an oxidative treatment that shares hydrolytic characteristics with thermal hydrolysis, although it adds oxidation reactions due to the presence of pressurised oxygen during the treatment instead of an inert atmosphere, thus being more effective in terms of solubilisation [18]. The main advantage of this technique is that promote the generation of SCFAs as final products under appropriate conditions which, in turn, should improve the subsequent PHAs-producing fermentation.

On this basis, Wijeyekoon et al. [91] employed a wet oxidation pre-treatment at 240 °C and 20 bar for 2.5 hours to obtain a sludge hydrolysate from a mixed sludge. It is

interesting to note that prior to the wet oxidation treatment, this sludge was subjected to an acidogenic pre-fermentation, although the characteristics of either this pre-fermentation or of the initial sludge were not reported; it was only indicated that wet oxidation was able to remove almost 98% of the VSS. These authors not only tested the wet oxidation hydrolysate, but also same hydrolysate after being subjected to an ammonia stripping process with air, aiming to increase the C/N ratio from 4 to 12. Either the raw or ammonia-stripped hydrolysates showed SCFAs/TOC ratio of around 46.4% and were mainly composed of acetic acid (74.3%) and propionic acid (12.5%).

Results revealed that the maximum PHAs accumulation (40.1% of the TSS) was reached using ammonia-stripped hydrolysate as substrate and after 7 hours of fermentation at 30ºC. It is interesting to note that non-removal of ammonia slowed down the PHAs production (from 7 to 12 hours) but not the maximum accumulation (39%). Ammonia concentration in the hydrolysate did not have a significant effect on the PHAs composition for both hydrolysates, with 76.8% of the polymer weight being due to PHB and 23.2% remaining, to PHV. Authors also found that feeding regimes affected the rate of PHAs accumulation and composition. Equal doses of substrate fed hourly at 25% DO saturation was found to be the best conditions based on PHAs yield on substrate.

3.3.2. Assisted thermal procedures

This section collects all those recent studies where thermally hydrolysed sludge is subsequently subjected to an acidogenic digestion to increase its content in SCFAs before being fed to the PHA-producing fermenter.

The first study with this approach was published by Morgan-Sagastume et al. [92], who used a biologically fermented effluent from a CAMBI process as substrate for a subsequent PHAs-producing fermentation. CAMBI technology refers to a commercial thermal hydrolysis technology. During the CAMBI process, sludge is dewatered and preheated to 100 °C, then it is subjected to temperatures between 160 °C and 180 °C and pressures about 6 bars and finally directed to a flash tank where a sudden temperature and pressure decrease leads to a high cell breakage and solubilisation [93].

The CAMBI hydrolysate was later fermented at 42 °C for 2 days, reaching final SCFAs/SCOD and COD/NH_4^+ ratios of 0.5 and 10.53, respectively. This biological hydrolysate was rich in acetic acid (27.1%), butyric acid (23.1%), propionic acid (17.1%), iso-valeric acid (17.1%), caproic acid (7%), valeric acid (5%) and iso-butyric acid (3.5%).

Putting the light on the PHAs production results, the high proportion of non SCFAs compounds in the substrate (only 50% of the SCOD was due to SCFAs) tarnished the results of PHAs accumulation, only reaching a yield of 19.5% (0.195 g PHA/g TSS), although it is also true that this value was reached after 1.6 hours of cycle, resulting in a reasonable high specific PHAs production rate. PHA polymer was composed of PHB (74%) and PHV (26%). Again, following the previously addressed relationship between PHAs composition and SCFAs number of carbons, acids with an even-number of carbon atoms accounted for 61% of the total SCFAs, so it seems logical that PHA was mainly composed of PHB, as was experimentally observed, represented a higher percentage instead of PHV.

Zhang et al. [94] also proposed a PHAs-producing process from sludge, which was very similar to the studied one by Morgan-Sagastume et al. [92]. During their study, they compared the SCFAs yields during the acidogenic fermentation under mesophilic (35ºC) and thermophilic (55ºC) temperatures of raw or thermally hydrolysed sludges. The highest production of SCFAs was found to occur at mesophilic temperatures (35 °C) and with a thermally hydrolysed sludge, reaching a maximum of 9.63 $gCOD_{SCFAs}/L$. It is important to remark that, although the highest concentration of SCFAs was reached under these conditions, the highest SCFAs/SCOD ratio was observed using raw sludge at 35 °C (SCFAs/SCOD = 71.4%), and not with the hydrolysate from thermally pretreated sludge at 35 °C (SCFAs/SCOD = 50.2%).

Temperature did not have effect on the SCFAs profile when raw sludge was the substrate, with less than 3% variation in percentages between experiments. On the other hand, temperature did have impact on SCFAs distribution with thermally pretreated sludge as substrate for the acidogenic fermentation. In this case, higher fermentation temperatures led to a small increase in the proportions of acetic acid (28% and 33% for 35°C and 55 °C) and a slight decrease in valeric acid (26% and 21% for 35 °C and 55 °C).

Even though it is generally accepted that the higher SCFAs/SCOD ratio, the more favourable the PHAs accumulations, Zhang et al. [94] only selected the hydrolysate with the highest SCFAs concentration (raw sludge at 35 °C), but not with the highest SCFAs/SCOD ratio (thermally pretreated sludge at 35 °C), for assaying the PHAs bioproduction. This acidified hydrolysate, with a COD/NH_4^+ ratio of 14.3, surprisingly, showed butyric acid as the main acid present (30%), followed by acetic acid (28%),

valeric acid (26%) and propionic acid (16%). The PHAs fermentation was carried out at 35 ºC and led to a PHAs accumulation of 34.6% of the dry cell weight after 15 hours. PHA polymer was mainly composed of PHB (ranging from 71% to 76% of the total PHA mass, depending on the sampling time), whereas PHV only accounted for a maximum of 29% after 10 hours. This is an expected finding, taking into account the prevalence of acids with even-number of carbon atoms (56.3% of the total acids) in the medium after the acidogenic fermentation.

3.4. Other considerations

As it has been just seen, it exists a high variance in terms of PHAs yields and compositions, which is likely to be due to the wide range of operation conditions, treatments and raw sludges employed. Obviously, these facts greatly complicate the comparison of results between different authors. In order to try to facilitate this discussion, a table showing the most relevant parameters affecting the final PHAs content (SCFA/SCOD ratio, COD/NH_4^+ ratio, % of SCFAs with an even number of carbon atoms and acclimation of biomass) was included (table 3).

Table 3. *Summary of the key characteristics of the hydrolysates and other factors that influence final PHAs production in each work. - indicates data not available. *Specific PHA production velocity was selected at the half of the maximum %PHA. **authors measured TOC instead of COD.*

Sludge employed	***SCFA/SCOD ratio***	***% of acids with an even number of carbon atoms***	***COD: NH_4^+:P (COD/NH_4^+)***	***Acclimation of fermentative biomass***	***% PHA in dry cell basis (Δ%) (time)***	***Reference***
Settled activated sludge	62%	66%	1621.2:101.6:- (15.96)	Yes	60% (46%) (0.5 h)	(Liu et al., 2020)
Sludge from thickening tank (lab-scale)	56% (lab. scale)	63%	174.78:9.2:1 (19)	Yes, in both processes	23.2% (-) (36 h)	(Jia et al., 2014)
Diluted dewatered sludge (pilot scale)	65% (pilot scale)	66%	230.46:14.7:1 (15.68)		59% (34%) (72 h of the 4th batch)	
Thickened waste activated sludge	90%	65%	100:10:5 (10)	Yes	38% (36%) (15 h)	(Morgan-Sagastume et al., 2015)
Secondary from sedimentation tank	36%**	86%	661.3:6.9:1 (95.84)**	No	56.5% (52.5%) (3.7 h)	(Mengmeng et al., 2009)
Chemically enhanced ($FeCl_3$) sedimentation primary sludge	93.5%	42.6%	269:13.5:1 (19.93)	No	22.3% (after 32 d) 18% (13%) (0.375 h of cycle)	(Xu et al., 2018)
Thermally hydrolysed primary + municipal waste activated sludge	49.75%	61%	200:19:3 (10.53)	Yes	19.5% (after 75 d) 19.5% (4%) (1.6 h of the cycle)	(Morgan-Sagastume et al., 2010)
Primary + secondary sludge	50.2%	57.4%	418.1:29.3:1 (14.3)	No	34.6% (28.6%) (15 h)	(Zhang et al., 2019)
Secondary from sedimentation tank	55.4%	56.3%	977.5:66:- (14.72)	Yes	24.1% (11.6%) (1.5 h)	(Liao et al., 2018)
Mix of primary and waste activated sludge with acidogenic fermentation	46.3%**	85.1%	47:3.92:1 (12)**	Yes	40.1% (39.1%) (7 h)	(Wijeyekoon et al., 2018)

3.4.1. Sludge characteristics

It is interesting to notice from table 3 that many authors selected secondary activated sludges as starting materials [81,82,86,87,90]. These sludges, which were typically withdrawn from the sedimentation tank of a WWTP, showed TSS concentrations ranging from 4.5 g/L to 13.1 g/L [82,87,90]. Other authors opted for more dewatered sludges, with solid concentration between 30 and 43 g TSS/L [81,86].

The use of mixtures of primary and secondary sludges was less common, with solid contents ranging from 24 and 46 g VS/L [91,92,94]. Curiously, this combination was selected as the influent of thermal hydrolysis processes, whose hydrolysates were latterly the substrates of the PHAs production operations. Finally, only Xu et al. [89] used a primary sludge (5.7 gTSS/L) as starting material.

3.4.2. Hydrolysate characteristics

Focusing now on the hydrolysis method employed as pretreatment before the PHA-producing fermentation, anaerobic digestions were the predominant ones [81,82,86], followed by thermal hydrolysis [90,92,94]. Paying attention to biological hydrolysis, typical conditions to generate the SCFAs that will be later transformed into PHAs include temperatures ranging from 36 °C to 60 °C with or without pH adjustment. It should be noticed that, even when it was addressed that alkaline pH values yielded more SCFAs in the previous section (see section 2), it seems that this parameter did not have much relevance in comparison to SCFAs/COD ratios in terms of PHAs production. In this regard, SCFAs/COD ratios for sludge hydrolysates from acidogenic fermentation typically ranged from 55.83% to 65% [81,82]. It is surprising the high SCFAs/COD ratio (90%) reported by Morgan-Sagastume et al. [86], probably due to the lack of a primary waste sludge treatment in the WWTP, according to the authors.

For its part, the proportion of acids with an even number of carbon atoms in biological hydrolysates usually slightly oscillate among studies, from 63% to 66%, regardless of the conditions of treatment and the initial sludge characteristics. Results suggest that SCFAs/COD ratio can be increased by adding chemicals before the acidogenic fermentation, such as CaO_2 or SDBS.

Moving on to the hydrothermal treatments as pretreatment for biological acidification, the final hydrolysate had lower SCFAs/COD ratio than the one obtained by only employing biological treatments [92,94]. Nevertheless, the feeding in these cases

included primary sludges, whereas secondary sludges were employed in the studies with only biological acidification, so the effect of the thermal pretreatment could be compromised by the presence of the primary sludges. In any case, during the use of thermal treatments either as pretreatment or sole treatment to obtain the hydrolysate, the composition of the SCFAs showed little reduction on the proportion of acids with an even number of carbons, also if primary sludge was not present in the feeding (57.4% and 61% versus 56.3%). This values were lower to those obtained for acidogenic fermentations without thermal pretreatments.

The selection of hydrothermal treatments, either as sole hydrolysis method or as pretreatment for a subsequent acidogenic digestion, also reduced the final PHAs production, not only provoking a lower SCFAs/COD ratios in the hydrolysate, but also reducing the microbial growth due to the generation of inhibitory compounds during the treatment [18]. Nevertheless, these drawbacks can be avoided by means the presence of oxygen during the hydrothermal treatment. Thus, wet oxidation promoted the generation of SCFAs and reduced the formation of inhibitory products such as HMF or phenolic derivatives [45,95]. However, this statements need further research to be confirmed.

3.4.3. PHA effluent characteristics

Concerning the generation of PHAs itself using the hydrolysates obtained, initial inoculum usually consists on microorganisms from the activated sludge of the WWTP which are cultivated in a sequencing batch reactor (SBR) with a feast-famine strategy in cycles, using as feed real hydrolysates or, to a lesser extent, synthetic hydrolysates. However, some authors decided to use non-acclimated inocula directly [87,89,94].

Although it is extremely difficult to establish comparisons between different studies due to the high heterogeneity of conditions during both SCFAs and PHAs generations, results suggest that the prior acclimation of the PHA-producing inoculum is not a determining factor for increasing the final PHAs accumulation at the end of the fermentation. As an example, Mengmeng et al. [87] reported a 56.5% of PHAs accumulation in dry cell basis directly using raw sludge to inoculate the fermenter, whereas Jia et al. or Liao et al. [81,90] achieved lower yields, 23.2% and 24.1% of PHAs, respectively, even when they used a previously acclimated sludge.

It is also difficult to establish a pattern of the effects of the hydrolysate sludge composition on the final PHAs accumulation by microorganisms, again attending to the high heterogeneity in operating conditions and raw sludge characteristics. Anyway,

results from table 3 suggest that the PHAs production is favoured by sludge hydrolysates rich in SCFAs. However, when comparing the works of Liu et al. and Morgan-Sagastume et al. [82,86], both showing similarities in either the initial sludge or the hydrolysis method (biological hydrolysis), it can be deduced that an excessively high SCFAs/COD ratio can have a negative impact on PHAs bioproduction.

More specifically, Liu et al. [82] reported a SCFAs/COD ratio of 62% and the maximum PHAs accumulation of 60%. Meanwhile, Morgan-Sagastume et al. [86], with a SCFAs/COD ratio of 90%, observed a final PHAs content in the biomass of only 38%. In the same way, Xu et al. [89] also observed a low PHAs yield (18%) using a sludge hydrolysate with a high SCFAs/COD ratio (93.5%). Therefore, SCFAs/COD ratios higher than 90% hinder the PHAs production. At the same time, more than a 30% of non-SCFAs in the feeding reduce the final PHAs production. Therefore, it can be concluded that the highest PHAs accumulation can be obtained for SCFAs/COD ratios around 60 -70%. At higher ratios, SCFAs inhibited the bacterial growth, whereas low ratios caused an excessive cell proliferation and the no accumulation of PHAs, which only takes place under nutrient limiting conditions.

Additionally, nutrients as nitrogen also played an important role during the PHAs-producing fermentation According to table 3, too low or too high COD/NH_4^+ ratios in the sludge hydrolysates had a negative impact on PHAs production, observing maximum PHAs accumulations for ratios around 16.

Finally, the last but not the least parameter of the hydrolysate composition that played an important role on the final PHAs production is the percentage of acids with an even number of carbon atoms. This is in the line with the reported preferential uptake of theses SCFAs by microorganisms during PHAs production. As can be deduced from table 3, a higher percentage of these SCFAs in the sludge hydrolysate is usually linked to a higher PHAs accumulation by microorganisms, as long as the SCFAs/COD ratio was not higher than 90%, as previously discussed. For instance, the best PHAs yields corresponded to those hydrolysates with a higher proportion of acids with an even number of carbon atoms, which is the case of the works of Liu et al., Jia et al., Mengmeng et al. and Wijeyekoon et al. [81,82,87,91]. In these studies, where even numbered SCFAs predominate (66%, 66%, 86% and 85.1%, respectively), the final PHAs content was among the highest reported (60%, 59%, 56.5% and 40.1%).

4. Other metabolites

As seen in previous sections, sewage sludge hydrolysates are a powerful substrate to obtain added value products that could improve the sludge reuse during its management and introduce WWTPs in the circular economy. Despite having been the subject of many in-depth studies for last years, SCFAs and PHAs are not the one and only products that could be obtained from sludge hydrolysates by fermentation. Other metabolites with higher molecular weights, such as enzymes or lipids, can also be obtained from sludge by selecting adequate microorganisms and operation conditions during either its hydrolysis pre-treatment or the subsequent fermentation process. Using sludge hydrolysates for the production of these metabolites involve significant industrial savings, since typical fermentation media for this kind of processes account for 30% - 40% of the total production cost [96], whereas sludge is a cheap and ubiquitous waste.

However, as will be checked, these are still low explored alternatives, even though they represent a very attractive renewable source of high value products.

4.1. Enzymes

4.1.1. General considerations

As proteases and laccases are the enzymes which production will be here described, their applications will be cited. These have been extracted from the reviews of Razzaq et al. and Rodríguez Couto and Toca Herrera [97,98].

Proteases are commonly used enzymes in several fields. For example, they are widely spread in detergents. They are also used to facilitate chemical synthesis of organics from amino acids. Textile applications include the degumming of silk or the dehairing of leather. Their role in food industry is related to the production of bioactive peptides or hydrolysates with antioxidant activity or the improvement in dough properties by gluten breakage and in cheese manufacturing. Proteases have also been applied in decontamination of wastewaters which contain hair or feathers as well.

Regarding laccases uses, they have been employed in wood and paper industry for bleaching and pulping, as well as in decontamination of effluents. Their capability of degrading phenolic compounds makes them useful for decontamination of wastewaters from textile industries. Laccases can also be applied in food industry for the elimination of coloured phenols from beverages and to produce crosslinking for more resistant and

less elastic doughs. They also have cosmetic applications, for example in the manufacturing of hair dyes.

Putting firstly the focus in enzyme production, proteases are probably the most promising candidate to be obtained from sludge hydrolysates, attending to their composition. In this sense, proteins are largely the most abundant biopolymer in the sludge, rounding 60% [99]. Thus, sludge hydrolysates can be used as potential fermentation media for microorganisms with a significant proteolytic enzymatic activity. Proteases production is a matter of great interest, since these enzymes are responsible of almost 60% of the enzyme market [100]. Among the microorganisms available for this purpose, *Bacillus* species are known to be proteolytic and profusely producers of proteases such as subtilisin or alkaline proteases [100–102].

4.1.2. Production

The inoculation of *Bacillus* in sewage sludge hydrolysates with the aim of producing proteases was firstly studied by Drouin et al. [103], paying special attention to the effect of the sludge hydrolysis pretreatments on the final protease yields. The hydrolysis methods consisted in an alkaline thermal hydrolysis at pH 10 and at two different temperatures (121 and 140ºC) for 30 minutes. Authors also carried out fermentations using the raw secondary sludge and the hydrolysate obtained at 140ºC mixed with raw sludge (1:1) as substrates. In each case, *Bacillus licheniformis* was inoculated during its exponential growth phase, with the fermentation being carried out at 35 °C for 48 hours and keeping the pH controlled and fitted to 7.5, the optimal value for the microorganism development.

Results showed that mixing raw sludge and thermo-alkaline hydrolysate (140ºC) promoted the highest protease activity at the end of the fermentation, whereas the lowest protease activity was reported employing only thermo-alkaline treated sludge as fermentation medium. This fact was attributed by the authors to a high viscosity in the thermo-alkaline hydrolysate that difficulted oxygen transfer. Nevertheless, the difference in proteolytic activities was approximately of only 3 units (from 13.0 U/mL for the mixture to 9.8 U/mL for the thermo-alkaline hydrolysate). When *in situ* hydrolysed sludge or raw sludge were employed as growth media for *B. licheniformis*, similar proteolytic activities were reported, 11.5 U/mL or 10.4 U/mL, respectively.

These activity values seem very low, especially in the comparison of hydrolysates with raw sludge. As authors reported, viscosity could harm the oxygen transfer.

Moreover, as mentioned in the introduction section, hydrothermal treatments could generate inhibitors to microbial growth that also led to the poor results observed.

Recent research deals with enzymes production from sludge hydrolysates includes not only proteases but also laccases recovery. Simultaneous production of both protease and laccase enzymes was reported by García et al. [104] by means of the growth of *B. licheniformis* in thermally hydrolysed secondary sludge. In this study, it was compared the employment of thermal hydrolysates obtained in presence (wet oxidation) or absence (thermal hydrolysis) of an oxidising atmosphere as fermentation media for *B. licheniformis*. Both treatments were carried out at 160 °C and 40 bar of oxygen or nitrogen for 70 minutes, aiming not to completely destroy the biopolymers present in the sludge. Afterwards, *B. licheniformis* was inoculated in the hydrolysates, which were already sterilised after the thermal treatment. The fermentation was carried out at 37 °C, with an initial pH adjusted to 7.2. Moreover, authors also evaluated the *B. licheniformis* fermentation of either diluted hydrolysates (1:1) or in a synthetic hydrolysate, to investigate the potential generation of toxics during the hydrothermal treatment and to provide a basis of comparison, respectively.

Results showed that *B. licheniformis* was able to grow in both hydrolysates. In the case of the wet oxidation, it did it better in the diluted hydrolysate, probably due to the formation of toxics during the oxidising treatment, such as furfurals. Regarding biopolymers evolution in the thermal hydrolysis liquid, *B. licheniformis* firstly consumed carbohydrates, followed by proteins and, finally humic acids, which were only degraded when the stationary growth phase was reached (96 h). Similar trends were observed when the diluted hydrolysate obtained in absence of oxygen was employed as substrate. In the case of the wet oxidation hydrolysates, diluted or not, the sequential uptake behaviour was more marked. It is interesting to note that the initial carbohydrates uptake using the wet oxidation hydrolysates was higher than selecting the thermal hydrolysis hydrolysates, probably due to the greater solubilization provided by oxidising atmospheres. In turn, proteins from wet oxidation hydrolysate were less consumed and only metabolized during the stationary phase, being this phenomenon attributed to a higher damage caused by oxidation reactions. Humic acids behaviour was similar to that reported for protein.

Regarding activities of proteases and laccases, the production of the former was almost doubled during the fermentation of the thermal hydrolysis liquid, in comparison to the wet oxidation one (898 U/mL and 495 U/mL, respectively). For both hydrolysates,

proteases were mainly generated during the stationary growth phase, coinciding with the most intense protein uptake (72 hours in the case of the thermal hydrolysis and 120 in the case of the wet oxidation). The effect of dilution on the protease activity was in the line of the expectations, halving the maximum protease activity and demonstrating that the potential toxic compounds did not affect protease activity at the same time. On the other hand, focusing on laccase production, this was much lower for the thermal hydrolysis hydrolysate than for the wet oxidation one (28.8 U/OD at the end of the experiment and 55 U/OD after only 72 hours with final values in a rising trend), probably due to an easier assimilation of proteins from hydrolysates obtained under inert atmospheres, in contrast with the oxidising ones, thus not requiring to produce its laccases to start the metabolization of humic acids.

The dilution of hydrolysates strongly affected laccase production, but in different trends. For the fermentation carried out with thermal hydrolysis effluent previously diluted, final laccase activity was almost negligible, whereas using diluted wet oxidation hydrolysate, laccases production was strongly enhanced, reaching values higher than 50 U/OD in the first 24h, although it decreased until the end of the experiment.

Finally, authors also employed a synthetic hydrolysate with similar concentrations of biopolymers to those found in the real hydrolysates tested. In this medium, *B. licheniformis* was able to, at least, double its growth, in comparison to the previously reported for real hydrolysates. The consumption of biopolymers in synthetic media corroborated the preferential uptake of carbohydrates, especially the reducing ones, as was addressed for the real hydrolysates. Authors also reported a strong uptake on proteins when carbohydrate consumption was less intense.

Despite the high biopolymer uptake, enzymatic activity behaviour did not show significant differences to that found in the real hydrolysates when glucose was used as carbohydrate model. Nevertheless, the use of starch as reference highly increased the production of either proteases or laccases. According to the authors, this increase in enzymes production in presence of complex carbohydrates is attributed to lower bioassimilation of these by the bacteria, forcing them to use other carbon sources, such as proteins and carbohydrates. The authors concluded that, although results obtained from real hydrolysates were promising, there is still a lot of work to be done to improve the production of enzymes in sludge hydrolysates, for example, optimizing both hydrolysis

and cultivation conditions, as well as exploring the microorganisms that could produce high quantities of enzymes.

4.2. Lipids

4.2.1. General considerations

Moving now to the applications of lipids, these include mainly the production of oleochemicals. Examples of these are fatty alcohols, which have been used in detergents, biofuels or cosmetics. Moreover, they can be employed as substrates for the synthesis of rhamnolipids, sophorolipids and n-acetylethanolamines. Rhamnolipids and sophorolipids are biosurfactants, whereas n-acetylethanolamines are used in pharmaceutical synthesis. But the main application of lipids is the synthesis of biodiesel [40].

4.2.2. Production

Liu et al. [105] employed several pretreatments to obtain a final hydrolysate suitable to be used as growth medium for an oleaginous yeast, *Cryptococcus curvatus* ATCC 20509. Firstly, they subjected a dewatered secondary sludge (VSS = 5 g/L) to a thermal alkaline hydrolysis at 90 °C and pH 10 for 6 hours. Then, the hydrolysate obtained was anaerobically fermented at pH 8 for 10 days to produce a medium rich in SCFAs, especially in acetic (almost 3.5 g/L) and propionic acids (1.6 g/L), and with low concentrations of iso-butyric, n-butyric and valeric acids (0.77 g/L, 0.75 g/L and 0.85 g/L, respectively). Afterwards, the fermentation broth obtained was subjected to two different treatments: a struvite precipitation and a struvite precipitation plus an acid separation. Authors included these steps to increase C/N ratio, which initial value was 10. By means of the former, struvite precipitation, inorganic ammonia was removed raising C/N ratio to 60. With the second treatment, struvite precipitation plus acid separation, inorganic ammonia and proteins were removed, which resulted in rising C/N ratio to 111.

These effluents with different C/N ratios were subsequently fermented by *Cryptococcus curvatus* ATCC 20509, in a sequencing batch reactor at 30 °C. This reactor was periodically fed, replacing the medium by fresh hydrolysate when the yeast reached the stationary growth phase.

Lipid accumulation by the yeast showed results of 1.11 g/L (0.155 g lipid/g biomass) using the liquid obtained after struvite precipitation. This value, which was under the authors expectations, was attributed to a low C/N ratio. Nevertheless, the introduction of an acid precipitation step allowed to increase more the C/N ratio, but not

the production of lipids, which was around 0.98 g/L (0.31 g lipid/g biomass). In this case, authors suggested that the low lipid yield was yet caused by an excess of organics, such as proteins, carbohydrates or humic acids. This was the reason why authors decided added a third separation step, a coagulation step with poly-aluminium chloride, prior to struvite precipitation and acid separation.

The combination of these three treatments did not changed in a great extent the C/N ratio achieved with only struvite precipitation and acid separation with coagulation, but significantly increased lipid accumulation to 1.95 g/L (0.397 g lipid/g biomass). This yield was 2.56 times higher than the one achieved with only struvite precipitation and 1.28 times higher than the content reported with struvite precipitation and acid separation.

5. Conclusions

It has been shown that hydrolysed sewage sludge is a very promising fermentation medium for the bioproduction of added-value, non-energy molecules. The main limiting step of this approach is a proper solubilisation and hydrolysis of the sludge, in order to improve the bioassimilation of nutrients by the corresponding microorganism. To this end, biological and/or thermal pretreaments are the options more profusely employed by researchers.

In the literature, the bioproduction of SCFAs has been well studied due to their wide range of industrial applications. This SCFAs bioproduction from sludge consists mainly on conventional anaerobic digestions in which the methanogenic microorganisms are inhibited. Therefore, biological hydrolysis and SCFAs generation processes are coupled and an inoculation is not required. In this sense, pH plays a key role, with alkaline pHs speeding up the sludge solubilisation and, at the same time, inhibiting the methanogenic population. However, a strongly alkaline biological hydrolysis led to higher solubilisations as well as the release of compounds refractory to biological transformation, reducing the final SCFAs/SCOD ratio. The purity of SCFAs in the fermented liquid was also improved when pH was only initially adjusted to alkaline pH instead of keeping it continuously controlled during the whole fermentation. Moreover, increasing the pH provoked a decrease in propionic acid proportion but favoured the butyric and valeric acids ones in the SCFAs profiles.

SCFAs distribution was also related to solubilisation degree, since when more proteins were present, a higher proportion of butyric and valeric acids were synthesized by the

microorganisms. It was found that an excessive OLR caused the overload of the substrate as well as to the accumulation of heavy metals and other inhibitory compounds, decreasing the production of SCFAs. The initial inoculation of the sludge boosted the SCFAs generation and modified its profile. Results revealed that the higher the inoculum size, the lower either the SCFAs accumulation or the production of long chain fatty acids.

Biological hydrolysis can be also improved by adding CER, sand or oxidants during fermentation, although an excessive dosage of these had a negative impact on the SCFAs generation.

The initial stages of solubilisation and hydrolysis of sludge can be also carried out separately from the biological production of SCFAs by chemical or thermal or pretreatments. Focusing on the former, the dosage of CaO generated hydroxy radicals, which promoted the dissolution of flocs, and, at the same time, Ca^{2+} affected the sludge floc structure via bonding with functional groups, thus also improving the solubilisation and the generation of butyric and valeric acids. In a similar way, adding SDBS to sludge improved the production of SCFAs, favouring the production of acetic acid and n-butyric acid in comparison to the propionic acid.

Thermal pretreatments also increased the SCFAs bioproduction and the proportions of propionic, iso-butyric, n-butyric, *iso*-valeric and n-valeric acids, although a subsequent inoculation of the hydrolysate was always mandatory. In this regard, the higher the S/I ratio, the higher the SCFAs accumulation but lower their purity in the effluent after the fermentation of the thermally hydrolysed sludge. The combination of chemical (SDBS) and thermal pretreaments was highly effective in terms of acidogenic potential of the hydrolysate obtained.

The productions of SCFAs and PHAs are highly interconnected because the formers are the main substrate for PHAs-producing microorganisms. Taking into account that the SCFAs content in raw sludge is low, a previous sludge acidogenic fermentation is required to boost the PHAs production, although other non-biological pretreatments are also possible for this purpose. This also explains why findings previously explained for SCFAs production from sludge are also valid for PHAs. Nevertheless, it exists a high variance in terms of PHAs yields and compositions, which is likely to be due to the wide range of operation conditions, treatments and raw sludges employed. Obviously, these facts greatly complicate the comparison of results between different authors.

The proportion of acids with an even number of carbon atoms in biological hydrolysates usually slightly oscillate among studies, from 63% to 66%, regardless of the conditions of treatment and the initial sludge characteristics. Results suggest that SCFAs/COD ratio can be increased by adding chemicals before the acidogenic fermentation, such as CaO_2 or SDBS, or employing thermal pretreatments. The prior acclimation of the PHAs-producing inoculum was not a determining factor for increasing the final PHAs accumulation at the end of the fermentation. It can be concluded that the highest PHAs accumulation can be obtained for sludge hydrolysates with SCFAs/COD ratios around 60-70%, COD/NH_4^+ ratios around 16 and with even numbered SCFAs predominating.

Despite having been the subject of many in-depth studies for last years, SCFAs and PHAs are not the one and only products that could be obtained from sludge hydrolysates by fermentation. Other metabolites with higher molecular weights, such as enzymes or lipids, can also be obtained from sludge by selecting adequate microorganisms and operation conditions during either its hydrolysis pre-treatment or the subsequent fermentation process. In this regarding, *Bacillus licheniformis* was able to properly develop using sludge raw hydrolysates as substrate, with any kind of nutrient supplementation, sterilization, or previous dilution, to produce either proteases or laccases, although the presence of oxygen during the hydrothermal treatment promoted the formation of inhibitory substances. *Cryptococcus curvatus* can also use a thermally hydrolysed sludge as growth medium to produce lipids, although combined conditioning processes were required to increase productivities.

Future research about the use of sludge hydrolysates as fermentation media should look deeper into the development of new fermentative processes using pure cultures. This approach would allow the obtaining of non-energy metabolites with a higher added value than SCFAs, such as enzymes, lipids, proteins… The coupling of these processes with a previous thermal pretreatment of the sludge shows some positive synergies, such as higher capacities than biological or chemical pretreatments, the production of a stabilised sludge, the insolubilisation of heavy metals during the pretreatment or the direct inoculation of the hydrolysate, without requiring a previous sterilisation. A lot of work stills remain ahead to be done, such as testing different microorganisms or even by incorporating genetic engineering tools. Clearly, another factor to be taken into account

in future researches should be the downstream processing of the fermentation effluent to ensure either production volume or specification compliance for the product recovered.

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5. ANÁLISIS FINAL

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En este apartado se hará un resumen de los principales resultados de cada uno de los apartados, de modo que se pueda obtener una visión global de lo alcanzado en esta tesis.

5.1. TRATAMIENTOS HIDROTÉRMICOS DE LODOS Y LEVADURAS. RECUPERACIÓN DE PROTEÍNAS

La aplicación de HT y OH, tanto en lodos como en levaduras, es un método claramente efectivo en términos de minimización del sustrato. En este sentido, la OH se mostró como una técnica más eficaz, ya que la disminución de los SSV fue elevada, un 70% en el caso de lodos y un 97% para levaduras tras 180 minutos de tratamiento. En lo relativo a la solubilización, tanto OH como HT se mostraron como técnicas adecuadas para incrementar la DQOS, si bien la mayor agresividad de la OH condujo a mejores porcentajes de solubilización tanto en lodos como en levaduras, constituyendo la DQOS el 50% de la DQOT en lodos y el 78% en levaduras tras 100 minutos de OH, mientras que tras el mismo tiempo en HT estos porcentajes fueron 38% en lodos y 73% en levaduras. Sin embargo, la agresividad que favorece la solubilización durante la OH, también es responsable del decrecimiento observado en las etapas finales, donde la DQOS comienza a reducirse, indicando la capacidad de la OH para degradar y modificar compuestos orgánicos.

Tanto en la OH como en la HT de lodos y levaduras a 160 ºC y 40 bar, el tiempo más adecuado de tratamiento se encontró a los 80 minutos, debido a que es el punto de mayor concentración de proteínas solubilizadas.

En lo relativo a los biopolímeros, ambas técnicas produjeron una buena solubilización, en la línea de lo explicado anteriormente. En base a los datos de la bibliografía que indican el contenido en proteína de lodos y levaduras (Chen et al., 2007; Pacheco et al., 1997), se puede observar que la solubilización es prácticamente total. De nuevo, si el tiempo de tratamiento de OH se incrementa, las reacciones de oxidación son capaces de transformar los biopolímeros, haciendo que su concentración disminuya. Este aspecto es de gran importancia, ya que, si se busca recuperarlos, no es aconsejable emplear tiempos demasiado largos.

5.2. PRECIPITACIÓN DE PROTEÍNAS

5.2.1. Precipitación en hidrolizados de lodos

La precipitación de proteínas se mostró como un asunto complejo, obteniendo resultados poco claros especialmente en lodos. En esta materia prima, el efecto del tratamiento hidrotérmico no fue claro en cuanto a la capacidad de las proteínas para precipitar utilizando el mismo método. Tampoco jugó un papel clave en la selectividad de la separación, exceptuando el caso del *salting out*, donde la precipitación de proteínas fue más selectiva sobre el hidrolizado de HT. Estas diferencias podrían radicar en la agresividad de la OH, que modificaría las estructuras y grupos funcionales en mayor medida que la HT.

5.2.2. Precipitación en hidrolizados de levaduras

En cuanto a la precipitación de las proteínas presentes en los hidrolizados de levaduras, ésta dependió fuertemente de la atmósfera empleada en el tratamiento hidrotérmico y del método de precipitación. Seleccionando una atmósfera oxidante, el método más adecuado fue la precipitación a pH 3, logrando precipitar más del 80% de la proteína. Esto podría deberse a la creación de grupos funcionales nuevos que hagan que los puntos isoeléctricos de las proteínas converjan hacia ese valor. Además, la precipitación fue muy selectiva en ese caso.

Eligiendo la precipitación salina, la dependencia de la atmósfera de tratamiento fue menos evidente, especialmente a mayores concentraciones de sulfato de amonio. En cualquier caso, precipitó más del 50% de la proteína presente en el hidrolizado en todos los casos, salvo en el hidrolizado de OH y 50% de la concentración de saturación de sulfato de amonio. Empleando esa concentración de sal y el hidrolizado obtenido tras la HT, la selectividad fue muy alta. Todo ello sugiere que concentraciones altas de sal precipitan indiscriminadamente las biomoléculas presentes en los hidrolizados. Sin embargo, a menores concentraciones, la HT, por dañar menos las moléculas, parece una técnica más adecuada.

La menor modificación de las proteínas sufrida tras la HT también se manifestó en mayor capacidad de retención de proteína durante la IMAC (casi el triple que tras OH). Sin embargo, la separación es peor debido precisamente a esa baja agresividad, pues la OH podría ser capaz de romper las glucoproteínas, liberando los carbohidratos, lo que desemboca en mayores selectividades aplicando IMAC.

5.3. OXIDACIÓN HÚMEDA DE ÁCIDOS HÚMICOS

En este caso, se pudo comprobar que incrementar la temperatura tuvo efectos positivos en la degradación de los ácidos húmicos, así como en la eliminación de los intermediarios coloreados de reacción. Sin embargo, se favoreció la acumulación de productos refractarios, como el ácido acético. La modelización mostró que las reacciones principales de degradación de los ácidos húmicos fueron su conversión hacia intermediarios y CO_2 y la transformación de estos intermediarios en ácidos orgánicos.

La presión no mostró efectos significativos sobre ninguno de los parámetros.

El pH inicial se mostró como un factor clave en la degradación de los ácidos húmicos. Valores alcalinos de pH inicial favorecieron esta degradación, así como la acumulación de compuestos coloreados asociados a intermediarios de reacción y ácidos orgánicos. Por el contrario, valores ácidos de pH inicial favorecen la mineralización del ácido húmico, disminuyendo por tanto la acumulación de ácidos orgánicos. El modelo propuesto confirmó todos estos hallazgos.

5.4. FERMENTACIÓN DE HIDROLIZADOS DE LODOS

En lo relativo a los resultados de las fermentaciones de lodos, en primer lugar, se resumirán los resultados de la fermentación con *B. licheniformis* para producir enzimas, y finalmente, se hará un breve resumen de los aspectos más importantes encontrados en la revisión bibliográfica.

5.4.1. Obtención de productos no energéticos

En la revisión bibliográfica se pudo comprobar que el método más común de hidrólisis de lodos es la hidrólisis biológica. Ésta puede ser potenciada por diferentes aditivos, inertes o reactivos. La hidrólisis biológica siempre se encamina a la producción de AGCC, que se ve favorecida en medios alcalinos. El AGCC dominante en los hidrolizados es generalmente el ácido acético, teniendo una presencia inferior otros como los ácidos propiónico, butírico y valérico.

Los AGCC son el principal sustrato para obtener PHA a partir de los hidrolizados de lodos, por lo que todo aquel proceso que favorezca la producción de AGCC, también afectará positivamente a la de PHA. Esto es cierto en unos intervalos concretos, puesto que, en base a la bibliografía disponible, se pudo comprobar que la proporción óptima de AGCC en el hidrolizado es de un 63% aproximadamente. También juega un papel clave la relación entre la DQO y el amonio, siendo óptima esta relación en un valor de 16. La

implementación de tratamientos térmicos que mejoren la solubilización empeoró las producciones de PHA, si bien la OH, debido a su capacidad para generar AGCC sí mostró buenos resultados como método de hidrólisis para la posterior producción de PHA.

En lo relativo a otros productos, la revisión permitió comprobar que no existe mucha información disponible. El uso de hidrolizados térmicos de lodo para producir proteasas parece una alternativa prometedora, si bien debe ser más profundamente estudiada. En este sentido, el artículo incluido en esta tesis (apartados 4.5. y/o 5.4.2.) pretende ampliar la información disponible a este respecto.

5.4.2. Producción de enzimas

Se pudo comprobar que los hidrolizados obtenidos tras 80 minutos de tratamiento hidrotérmico (OH o HT) son medios de cultivo aptos para el crecimiento de *B. licheniformis*. Este microorganismo se alimentó inicialmente de los carbohidratos en ambos hidrolizados, utilizando las proteínas tras agotarlos y, en menor medida, los ácidos húmicos al agotar las proteínas. En cuanto a la producción de enzimas, la HT favoreció la producción de proteasas, doblando la actividad generada en el hidrolizado de OH. Sin embargo, la OH favoreció la actividad lacasa, posiblemente debido a la generación de intermediarios fenólicos tóxicos que el microorganismo tuvo que degradar para sobrevivir. Este hecho se confirmó con la dilución de los hidrolizados, observándose una mayor DO en el hidrolizado diluido de OH.

El uso de hidrolizados sintéticos mostró que las fuentes de carbohidratos complejas, como el almidón, condujeron a una mayor producción de proteasas, debido a la dificultad para asimilar dichas moléculas complejas. Sin embargo, no afectó a la producción de lacasas.

6. CONCLUSIONES

6. CONCLUSIONES

El trabajo realizado en esta tesis doctoral permitió obtener varias conclusiones:

- Los tratamientos hidrotérmicos son excelentes métodos de gestión de lodos, debido a su capacidad para minimizar su volumen y solubilizar sus componentes. Las proteínas, biomolécula mayoritaria del lodo, son solubilizadas casi en su totalidad.

- La oxidación húmeda es un tratamiento más eficaz para solubilizar las biomoléculas de lodos de depuradora. Sin embargo, es capaz de degradar los componentes del lodo en tiempos de reacción superiores a 100 minutos a 160 °C, debido a las reacciones de oxidación, mientras que la hidrólisis térmica es un proceso menos dañino.

- La oxidación húmeda es un proceso extremadamente complejo que conduce a la generación de diferentes intermediarios y ácidos orgánicos, especialmente a altas temperaturas y pH alcalinos y si los ácidos húmicos suponen una parte importante de la materia prima que se alimenta al reactor. Durante este proceso, se generan intermediarios de reacción coloreados que se asocian a derivados fenólicos.

- El tiempo óptimo de tratamiento a 160 °C y 40 bar para obtener la máxima concentración de proteínas solubilizadas a partir de lodos secundarios espesados por flotación es de 80 minutos, independientemente de si se emplea una atmósfera oxidante o inerte.

- La oxidación húmeda y la hidrólisis térmica son tratamientos fácilmente implementables en otros sustratos, como levaduras de cervecería. En esta materia prima, la solubilización de proteínas es completa a 160 °C, 40 bar y 80 minutos, tanto en atmósferas oxidantes como inertes.

- La recuperación de las biomoléculas presentes en los hidrolizados es un proceso complejo. La presencia en ellos de ácidos húmicos dificulta el proceso en gran medida. En el caso de los hidrolizados de levaduras, al carecer de ácidos húmicos, la precipitación a pH 3 en hidrolizados procedentes de oxidación húmeda es buena y selectiva, recuperando un 80% de la proteína. En el caso de levadura hidrolizada mediante hidrólisis térmica, la mejor técnica es la IMAC, logrando recuperar un 67% de la proteína inicial con una alta selectividad.

- La técnica IMAC no es una técnica adecuada para la recuperación de proteínas de hidrolizados de lodos de depuradora debido a la presencia de ácidos húmicos, que compiten por el cobre con las proteínas. Además, la presencia de cobre imposibilita la determinación de las concentraciones por el método de Lowry modificado.

- El lodo también puede ser revalorizado aprovechando sus hidrolizados de manera global, empleándolos como sustrato para fermentaciones con *Bacillus licheniformis* CECT 20.

- *Bacillus licheniformis* es capaz de crecer en los hidrolizados consumiendo los biopolímeros de forma secuencial, comenzando por los carbohidratos. Al agotarse éstos, utiliza las proteínas y finalmente los ácidos húmicos.

- Las proteasas son enzimas que *Bacillus licheniformis* produce en mayores concentraciones utilizando hidrolizados de hidrólisis térmica debido al menor daño que este tratamiento causa sobre las proteínas. En cambio, la oxidación húmeda es más adecuada para la producción de lacasas, debido a los derivados fenólicos que este proceso produce y que el microorganismo debe eliminar para sobrevivir.

- Las fuentes de carbohidratos complejas favorecen la producción de proteasas por *Bacillus licheniformis*, debido a la mayor dificultad que le supone asimilar este tipo de carbohidratos.

- Los principales productos que se pueden obtener por fermentación de hidrolizados de lodos de depuradora son los ácidos grasos de cadena corta y los polihidroxialcanoatos.

- El principal método de hidrólisis de lodos es la hidrólisis biológica. Ésta puede ser potenciada si se realiza en medios alcalinos. A partir de este proceso, se obtienen los ácidos grasos de cadena corta, que, a su vez, sirven como sustratos para la producción de polihidroxialcanoatos.

- Los métodos de hidrólisis no biológica han sido poco estudiados, aunque los hidrolizados obtenidos se han usado principalmente como medios de fermentación para la producción de enzimas.

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ANEXOS

ANEXO I

Sludge hydrothermal treatments. Oxidising atmosphere effects on biopolymers and physical properties

*José L. Urrea[a], Manuel García[a], Sergio Collado[a], Paula Oulego[a], Mario Díaz[a]**

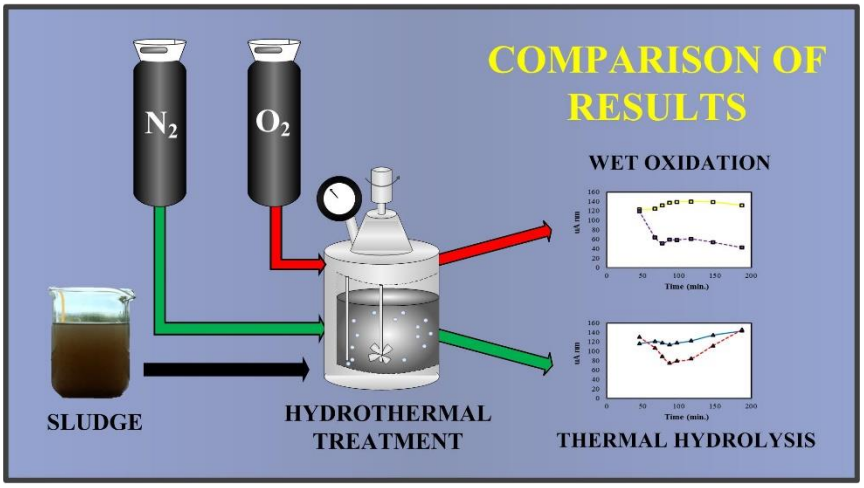
[a] Department of Chemical and Environmental Engineering. University of Oviedo.

C/ Julián Clavería s/n. E-33071 Oviedo, Spain. Tel.:34985103439; Fax: 34985103434

* Tel.: +34 985 10 34 39; Fax: +34 985 10 34 34

mariodiaz@uniovi.es

GRAPHICAL ABSTRACT



HIGHLIGHTS

An oxidising atmosphere caused a higher sludge solubilisation than an inert one

Increase of negative surface charge of the particles by solubilisation of biopolymers

An oxidising atmosphere improves settleability by effect of biopolymers degradation

Cellular lysis instead of hydrolysis of cellular components at the beginning of WO

Selective solubilisation or oxidation of specified cellular components were discarded

ABSTRACT

In this work, the role of an oxidising atmosphere during the hydrothermal treatment of an activated sludge at 160 ºC and 40 bar, was determined. The composition and molecular weight sizes of the soluble biopolymers generated during the sludge treatment in presence (wet oxidation "WO") or absence (thermal hydrolysis "TH") of oxygen were compared. Likewise, the characteristics of organic material, settleability, colour and pH of the treated sludge during both treatments were analysed. The thermal treatment in presence of oxygen provided better results in terms of solubilisation, settleability and mineralisation. WO initially favoured a more intense cellular lysis, causing a higher degree of solubilisation than that achieved by TH. Either in presence or absence of oxygen, thermal treatments caused a marked worsening of the settleability of the sludge. However, the degradation of biopolymers during WO led subsequently to an improvement of the settleability properties for longer reaction times. Both treatments caused a fast solubilisation of biopolymers at the beginning by effect of the release of extracellular and intracellular material from sludge. Subsequently, the presence of oxygen produced a significant decrease in the concentration of those biopolymers. In contrast, the proteins were the only one biopolymer that was degraded during TH.

KEYWORDS: Oxidation state; Proteins; Settleability; Size distribution; Thermal hydrolysis; Wet oxidation

1. Introduction

Sewage sludge is an inevitable by-product in wastewater treatment plants (WWTP), which management is very complicated due to the high volumes generated and its high water content. Current sewage sludge treatment and disposal methods, such as landfilling, incineration and gasification require drying the sludge, a costly pre-treatment step, and/or risk contaminating the environment (Silva et al., 2016).

In this regard, hydrothermal processing has progressively been recognised as an attractive alternative for the sludge management during the last years. Hydrothermal treatments refer to technologies involving reactions carried out in an aqueous solvent at elevated temperatures and pressures, under inert (thermal hydrolysis) or oxidising (wet oxidation) atmospheres. Either wet oxidation (WO) or thermal hydrolysis (TH) allow the breakage of floc structure by means of solubilisation and degradation of EPS and cellular lysis (Hii et al., 2014).

In sludge treatment, hydrothermal processing has traditionally had three main goals: the enhancement of a subsequent anaerobic digestion process, the reduction of the solid COD and/or the reduction of waste mass and volume (Barber, 2016; Chung et al., 2009; Genç et al., 2002; Khan et al., 1999). These approaches explain why, although the literature about sludge thermal treatments is abundant, the vast majority of these studies are only focused on the measurement of degrees of solubilisation and mineralisation, biodegradability indexes and/or biochemical methane potentials before and after treatment (Abe et al., 2011; Strong et al., 2011; Urrea et al., 2015). Nevertheless, information about the effects of thermal treatments on the composition or on other properties of the hydrolysed sludge is very scarce or does not even exist. As example, thermal treatments involve the release of high amounts of polymeric substances into the bulk medium during the cellular lysis, whose composition, concentration and molecular size will vary with the reaction time, especially under an oxidising atmosphere (Hii et al., 2014; Urrea et al., 2016). Evidently, information about the composition and properties of these biopolymers would be very interesting in order to design the following step of treatment of the hydrolysed sludge. For instance, fouling in membrane bioreactors is greatly affected by the protein and carbohydrates contents as well as their molecular sizes (Judd, 2011). In a similar way, the composition of soluble biopolymers in the medium also has effect on its viscosity, the settleability and wettability of the remaining solids or the biodegradability and chemical properties of the supernatant (Liu et al., 2013; Martins

et al., 2011; Ruiz-Hernando et al., 2015; Wang et al., 2014; Zhang et al., 2015). Moreover, taking into account that sewage sludge consists of 61% proteins and 11% carbohydrates (Chen et al., 2007), this gives rise to the following question: is it possible to obtain valuable products from the sludge by means of thermal treatments? Obviously, the first step to answer this question is to know the products obtained and the mechanisms involved in their formation.

Nevertheless, as it was previously mentioned, information about composition and properties of the products formed during thermal treatments (soluble biopolymers, mainly) are very scarce and deals only with TH processes. Thus, Ramirez et al. (2009) pointed out that soluble proteins, carbohydrates and lipids concentrations increased with increasing temperature for a fixed time and that proteins were released easier than carbohydrates from the VSS. However, Bougrier et al. (2008) and Li and Noike (1992) indicated that carbohydrates were more hydrolysable than proteins, and proteins more hydrolysable than lipids in turn. For temperatures ranging from 130ºC to 220ºC, soluble carbohydrates concentration decreased due to reactions between them or with soluble proteins (Bougrier et al., 2008; Ramirez et al., 2009). In the case of proteins, Xue et al. (2015) observed that the rise in their concentration with temperature is accompanied by an increase in the ammonia nitrogen concentration. Bougrier et al. (2008) and Donoso-Bravo et al. (2011) concluded that volatile fatty acids are produced by lipid degradation instead of by protein decomposition. Yin et al. (2015) studied the time and temperature dependence of soluble proteins and carbohydrates and ammoniacal nitrogen, and found that at 220 - 300ºC, soluble proteins and carbohydrates went through a maximum with the reaction time, and that the higher the temperature, the lower the time at which the maximum appear. At this point, it is important to stress that, although information about the composition of soluble biopolymer during the sludge TH is available, there is no studies dealing with the properties of these biopolymers, such as their molecular weight size.

Regarding the WO of sludge, the majority of the works about this technique are exclusively focused on volatile fatty acids, which are the main chemicals generated by WO of biomass (He et al., 2008; Hii et al., 2014), and specially, on acetic acid, whose concentrations usually exceed those of the other acids. In fact, no studies on the effects of the WO on the composition of soluble biopolymers have been found. Regarding the properties of these polymers, to the best of our knowledge, there is only a work,

corresponding to our research group, in which the molecular weight distribution of the solubilised matter during a WO treatment was determined by size exclusion chromatography (Urrea et al., 2016).

Therefore, based on the foregoing, the aim of this work is to study and compare, for the first time, the effects of hydrothermal treatments (TH and WO) on the composition and molecular weight sizes of the soluble biopolymers generated during the treatment as well as on the "classical" parameters of solubilisation, mineralisation and settleability.

2. Material and methods

2.1 Sludge samples

The activated sludge employed in the experiments was obtained from a municipal wastewater treatment plant of the region (Asturias-Spain). The sludge was extracted of a unit of thickening by flotation and stored at 4 ºC until its subsequent use. The characteristics of the sludge were as follow: total suspended solids (TSS): 31.9 g/L, volatile suspended solids (VSS): 26.5 g/L, sludge volume index (SVI): 31 mL/g, total chemical oxygen demand (TCOD): 37.2 g O_2/L, soluble chemical oxygen demand (SCOD): 0.2 g O_2/L, soluble total organic carbon (soluble TOC) 0.4 g C/L, initial pH: 6.5, soluble protein: 181 mg/L, soluble humic acids: 281 mg/L and soluble carbohydrates: 82 mg/L.

2.2 Experimental setup

The experiments of TH or WO were carried out in a PARR series 4520 reactor with a propeller stirrer (500 rpm). Oxygen (WO) or nitrogen (TH) previously conditioned in a humidifier, were fed since the beginning of the experiment to a constant flow rate of 1.2 L/min. Pressure was adjusted through a backpressure controller located at the end of the gas line, whilst the reactor temperature and the oxygen flow were regulated by means of PID controllers. The operating conditions stablished to carry out the reaction were 160 ºC and 40 bar. Eight samples were taken at different times of reaction. The first one was withdrawn when 100 ºC were reached in the reactor (45 minutes). The following samples were extracted periodically from minute 67, when the operating conditions were reached, to the end of the treatment (187 minutes).

2.3 Analytical Methods

The analysis corresponding to total suspended solids (TSS), volatile suspended solids (VSS), fixed suspended solids (FSS), chemical oxygen demand (COD), sludge volume index (SVI) and pH were performed according to Standard Methods (APHA, 1998).
In order to quantify the biopolymers solubilisation , their concentrations were measured by the following colorimetric methods: proteins and humic acids by the modified Lowry method (Frolund et al., 1995), using BSA and humic acids as standards, respectively, and carbohydrates by the Dubois method (Dubois et al., 1956), employing glucose as standard. UV-VIS scans from 190 to 900 nm were performed for supernatants employing a T80 UV/VIS spectrophotometer (PG Instruments Ltd). The colour of the soluble samples was determined by means of the colour number (CN), which is defined according to equation 1 (Tizaoui et al., 2007).

$$\mathrm{CN} = \frac{\mathrm{SAC}_{436}^2+\mathrm{SAC}_{525}^2+\mathrm{SAC}_{620}^2}{\mathrm{SAC}_{436}+\mathrm{SAC}_{525}+\mathrm{SAC}_{620}} \quad (1)$$

Where SAC_i corresponds to the spectral absorption coefficient at a wavelength of i nanometers.
Soluble total organic carbon (TOC) was measured using a TOC analyser (Shimadzu TOC-VCSH, Japan). The mean oxidation number of organic carbon (MOC) was calculated from the equation 2 (Vogel et al., 2000).

$$\mathrm{MOC} = 4 - 1.5\frac{\mathrm{COD}}{\mathrm{TOC}} \quad (2)$$

Where COD and TOC values are expressed in g O_2/L and g C/L, respectively, and MOC takes values from −4 (i.e. for methane) to +4 (i.e. for carbon dioxide).

2.4 Size exclusion chromatographic analysis (HP-SEC)

The size distribution of the solubilised biopolymers, commonly called “fingerprints”, was performed by High Performance Liquid Chromatography (Agilent 1200, Agilent Technologies Inc., California, USA), using a Yarra SEC-2000 (300 × 7.8 mm) column. A $NaNO_3$ solution was employed in order to determinate the total column volume (11.8 mL). The mobile phase consisted of a buffer solution of 9 mM NaCl, 0.9 mM Na_2HPO_4 and 0.005% NaN_3, adjusted to pH 7.0 (±0.1) with H_3PO_4, and with an ionic strength of 0.02. The flow rate employed was of 1 mL/min. All samples were filtered through 0.45 µm PVDF filters (Millipore) prior to injection (20 µl). The detection was carried out with a diode array UV detector at several wavelengths (210, 260 and 280 nm).

The calibration of the column was performed by means of a Protein Standard Mix (15-600 kDa) supplied by Sigma-Aldrich (69385), which was composed of four proteins: Ribonuclease A (13.7 kDa), Albumin (44.3 kDa), γ-Globulin (150 kDa) and Thyroglobulin (670 kDa), as well as a low molecular weight marker, ρ-aminobenzoic acid (0.14 kDa). The coefficient of determination (R^2) of the calibration curve was 0.93.

3. Results and discussion

3.1 Behaviour and characteristics of organic material during hydrothermal treatments

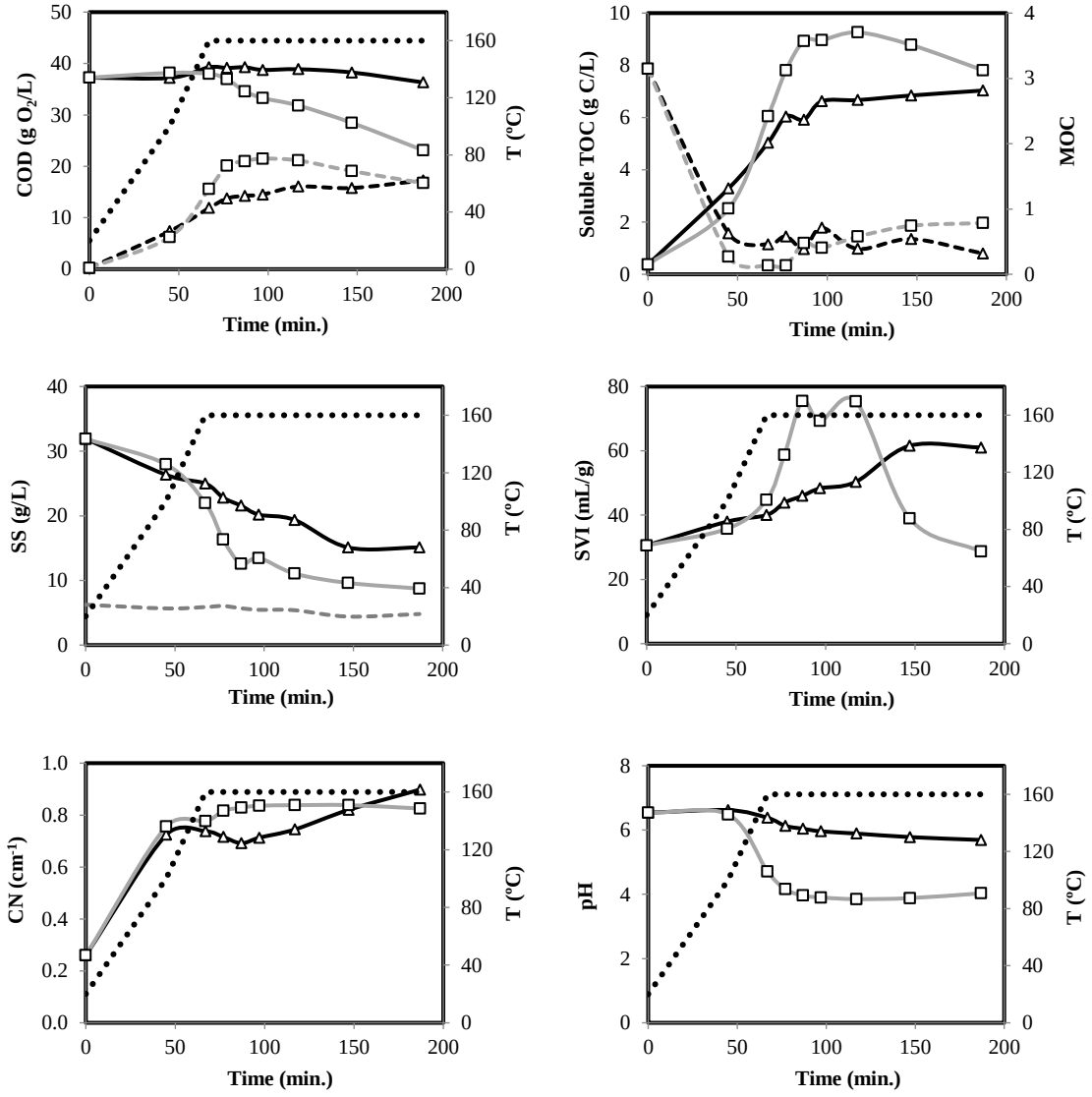


Figure 1. Effect of the presence (■) or absence (▲) of an oxidising atmosphere on sludge properties during the hydrothermal treatment at 160 ºC and 40 bar. (a) COD evolution (solid and dashed lines represent TCOD and SCOD, respectively), (b) Soluble

TOC (solid lines) and MOC (dashed lines) evolution, (c) TSS evolution (dashed line corresponds to FSS), (d) SVI evolution, (e) CN evolution and (f) pH evolution. Dotted lines represent the reactor temperature.

Figure 1a shows the evolution of total and soluble COD fractions during the TH or WO of an activated sludge. As has widely been commented in the literature, both treatments caused an increase in the soluble COD (SCOD), but WO also provoked a partial mineralization of the organic load of the sludge, thus observing a steady reduction of total COD (TCOD). In this case, TCOD decreased a 38% after 187 minutes of reaction in the case of WO, whereas it remained constant for TH.

It is interesting to point out that initially, when the temperature is not high enough (first 45 minutes, temperature lower than 160ºC) the degree of mineralization achieved with both techniques was null. Nevertheless, the presence of an oxidising atmosphere does have effect on the solubilisation of the sludge, even at these low temperatures. The solubilisation of organic compounds from sludge was faster when reactions of oxidation were involved. So, when the operating conditions were achieved (67 minutes), the SCOD for WO was a 30% higher than for TH, while TCOD remained constant in both experiments. After 187 minutes of treatment, the solubilisation degrees of solid COD (COD_S) were 83% and 49% for WO and TH, respectively, as Figure 1a shows. Thus, it seems reasonable to propose that free radicals initially formed tend to attack the solid COD instead of mineralising the soluble COD, thus favouring a higher solubilisation. Therefore, it can be concluded that an oxidising atmosphere offers a higher effectiveness in terms of sludge solubilisation than an inert one.

Obviously, as the CODs decreases, oxidation reactions of the soluble COD prevail, which explain the continuous TCOD and SCOD reductions observed after an hour and a half of treatment by WO, whereas SCOD increased over time for TH experiments. In fact, although the degree of solubilisation was higher for WO (83%) than for TH (49%), similar SCOD were achieved at the end of the both treatments (a 46% of the initial TCOD of the sludge).

Soluble TOC results also corroborate the dual effect of the oxidising atmosphere on the thermal treatment of the sludge, improving the solubilisation of solid matter and, in turn, oxidising the dissolved one (Figure 1b). The final soluble TOC reduction observed for WO also reveals that part of the COD removal was due to total oxidation of the soluble organic matter towards carbon dioxide and water.

Regarding the mean oxidation number of organic carbon (MOC), results showed that the oxidation state of soluble compounds decreased sharply with both treatments, from an initial value of 3.2 ± 0.1 to 0.45 ± 0.15 after only 45 minutes of reaction. This reduction of the MOC was attributed to the release of extracellular polymeric substances (EPS) and intracellular material, whose oxidation state is lower than that of soluble biopolymers of raw sludge (higher COD per unit of organic carbon) (Urrea et al., 2016). Likewise, it was also noted that the initial decrease in the MOC was more marked for WO than for TH, probably due to the higher solubilisation of the sludge in presence of an oxidising atmosphere. In a previous work (Urrea et al., 2016), the relationship between more hydrophobic characteristics of organic compounds and lower MOC was theoretically proved. Therefore, the lower MOC obtained in WO experiments is explained by the higher degree of cell destruction attained, as the biopolymers that integrate the cell membrane, such as phospholipids and proteins, have a hydrophobic character. Nevertheless, as the reaction progresses, WO caused a moderated increase in the MOC since minute 97, due to the oxidation of those biopolymers towards more hydrophilic products, such as volatile fatty acids. In contrast, the MOC in TH decreased slightly as a result of the solubilisation but no oxidation mechanism.

3.2 Solubilisation of SS and effect on settleability

The TSS disintegration and its effect on the settleability properties of the sludge for each treatment are showed in Figures 1c and 1d. As previously explained, WO caused a stronger impact on the solubilisation of the sludge than TH. In fact, the solubilisation degree of TSS achieved with WO after 77 minutes of treatment (49%) was almost the same obtained after 187 minutes of TH (53%). To the end of the reaction, TSS concentrations were 27% and 47% of the initial value for WO and TH, respectively. As expected, FSS concentrations do not change throughout both treatments.

It should be also pointed out that CODs/VSS ratio remained almost constant during the treatments, obtaining values of 1.5 ± 0.2 g O_2 g VSS^{-1} and 1.6 ± 0.2 g O_2 g VSS^{-1} for WO and TH, respectively. Burger and Parker (2013) reported a value of 1.44 for this ratio in an aerobic sludge without treatment, whereas 1.42 is commonly mentioned in the literature, which is obtained theoretically from the oxidation of the biomass ($C_5H_7NO_2$). No changes in the CODs/VSS ratio discard either the selective solubilisation of some compounds instead of others or the oxidation of the organic matter in the solid phase.

Regarding the effect of the thermal treatments on the settleability, both techniques initially lead to an increase in SVI, which means a worsening of its management during that stage (Figure 1d), as a result of an increase in the repulsion between particles by electrostatic forces. When 100 ºC were reached, the flocculate structure of the sludge was destroyed by both thermal treatments, causing the release of extracellular polymeric substances and the formation of smaller particles. At this time, an initial slight increase of SVI was detected. Afterwards, while the concentration of TSS continued to decrease, the amount of solubilised biopolymers increased in the medium, which goes hand in hand with an increase in the SVI. These changes suggest that a higher concentration of solubilised biopolymers led probably to an increase in the negative surface charge of remaining solid particles, which caused a worse compaction of the latter such, as clearly reflected in the SVI evolution. In fact, the SVI remained constant between minutes 147 and 187 during the TH, coinciding with the period in which the solubilisation reactions had concluded. Zhen et al. (2012) reported that the zeta potential of an activated sludge decreased from -12.6 to -18.0 mV after its treatment by TH at 80 ºC, being that result an evidence of the abovementioned.

In accordance with the previous explanation, the higher solids disintegration caused by the oxidant also involved a higher deterioration of the settleability, expressed as SVI. Nevertheless, as WO reactions progress, the SVI, after achieving a maximum in minute 117, began to drop. This is caused by the oxidation of the soluble biopolymers, thus reducing the negative surface charge of the solid matter and, therefore, the repulsion by electrostatic forces. Therefore, in the case of WO, it is noteworthy to stress that the evolution of SVI is strongly linked to the balance of reactions developed during the process. That is to say, SVI increased when the reactions of thermal hydrolysis were predominant. Subsequently, SVI remained stable when the magnitude of the reactions between thermal hydrolysis and oxidation were similar. At the end, SVI began to decrease from the time in which the oxidation reactions prevailed over the hydrolysis ones (see SCOD evolution in Figure 1a).

3.3 Effects on colour and pH

The effects of WO or TH on the colour number (CN) and on pH of the solubilised samples from sludge are showed in Figures 1e and 1f. For both treatments, the CN experienced a sharp increase in its value at the beginning of the reaction, then remaining approximately constant around 0.85 since minute 45. Comparing CN and SCOD, it can be concluded

that their trends are analogous, particularly for HT. In this case, the higher SCOD concentration, the higher CN was achieved, as expected. This trend is also observed for the first part of WO, where solubilisation reactions prevail. In fact, CNs between 45-147 minutes of treatment were higher for WO than for TH, since during this period the former caused a greater level of lysis cellular, achieving in turn higher SCOD concentration than the latter. Nevertheless, when oxidation reactions became dominant, reducing the SCOD, no significant decrease in the CN was observed. At the end of treatments (187 minutes), the CN obtained with TH was a bit higher (8% more) than that with WO, despite the SCOD concentrations were very similar between the final effluents of both treatments. The ability of WO to reduce the colour on different wastewater or sewage sludge has been widely proved (Fu and Kyzas, 2014; Oulego et al., 2016; Urrea et al., 2014) as well as the generation of high coloured intermediates during the oxidation of phenol-like compounds, such as quinhydrones (Collado et al., 2010). So, the no CN change observed for WO was probably due to a balance between the formation of highly coloured intermediates from the initially solubilised compounds and the oxidation of them to non-coloured products, such as low molecular weight acids (LMWA). The pH evolution also supports the higher formation of LMWA in presence of an oxidising atmosphere. While TH caused a minimum effect on the pH of sludge, WO produced a remarkable acidification of the medium (Figure 1f). During TH, the pH decreased slightly from 6.5 to 5.7, being this the result of a small formation of volatile fatty acids (VFA). Eskicioglu et al. (2006) reported the increase in the concentration of VFA from 281 to 936 mg/L for an activated sludge subjected to TH at 96ºC, which was withdrawn from the reactor exactly when this temperature was reached. Similarly, they observed that the pH slightly decreased with the treatment from 7.5 to 7.0.

In the case of WO, an abrupt drop in the pH values (4.7) was obtained when reaching the operating conditions (67 minutes). In that point of the reaction, no decrease in TCOD was noticed. Therefore, this indicates that the attack of the hydroxyl radicals during the initial solubilisation step of WO caused the formation of biopolymers with a stronger acid character than those formed only for TH. Afterwards, the oxidation reactions take place, causing a further decline in the pH value (4.0), which remained stable during the rest of the experiment. The acidification of the medium during WO is a common result of this process due to the production organic acids, which are considered as the main final product of reaction (Genç et al., 2002).

In addition to this, the changes in UV-VIS spectra obtained with each of the treatments (see Figure S1 in the supplementary material) allowed to stablish more detailed differences between the characteristics of the coloured compounds solubilised either by WO or TH. According to the evolution of the spectra and considering only the VIS zone (380-780 nm), it can be noted that the absorbance values for 530-780 nm decreased with both treatments after reaching the operating conditions, being this decrease more marked in presence of oxygen. Subsequently, the VIS spectrum for TH conserved its shape, being observed a continuous increase in the absorbance in the entire region with the advance of the reaction, that is to say, with the increase in the SCOD. On the other hand, the solubilised biopolymers by WO showed higher levels of absorbance to lower wavelengths (380-528 nm) than those by TH, specifically, during the first 147 minutes of reaction. Besides, the absorbance values increased up to be completed 117 minutes of WO, decreasing after this time. At the end of the treatments, the spectra area between 380-528 nm and 530-780 nm was of 143 and 146 uA nm for TH, and of 132 and 43 uA nm for WO, respectively.

3.4 Solubilised biopolymers. Yield and size distribution

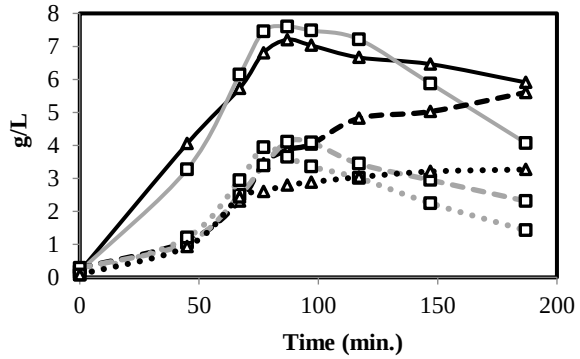


Figure 2. Solubilised biopolymers by effect of the presence (■) or absence (▲) of an oxidising atmosphere during the hydrothermal treatment of the sludge at 160 ºC and 40 bar. Solid, dashed and dotted lines represent proteins, humic acids and carbohydrates, respectively.

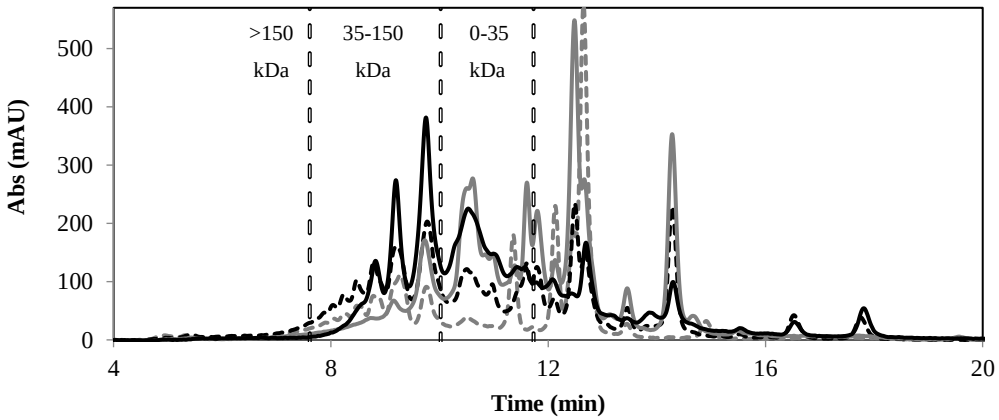


Figure 3. Changes on the fingerprints of supernatants by effect of the presence (black lines) or absence (grey lines) of an oxidising atmosphere during the hydrothermal treatment of the sludge at 160 ºC and 40 bar. Dashed and continue lines correspond to time sampling of 67 and 187 minutes, respectively.

The solubilisation of biopolymers from sludge and their size distribution during WO or TH treatments are collected in Figures 2 and 3, respectively. Both treatments caused a considerable increase in the concentration of soluble biopolymers, with the proteins being identified as the main components. Initially, a fast increase in the soluble biopolymers concentrations was observed for both TH and WO during the first 67 minutes of reaction. This preliminary solubilisation was the result of the destruction of the flocculate structure of the sludge, which caused the release of extracellular polymeric substances towards the supernatant, as well as of intracellular material from of the cell lysis. In the following minutes, the rate of solubilisation of biopolymers decreased for both treatments, being this due to the lower VSS concentration, as well as to oxidation reactions in the case of WO. Nevertheless, the biopolymers evolution was different for both treatments since minute 87. On one hand, when 87 minutes of WO was reached, the concentration of biopolymers achieved a maximum with values of 291, 164, 141 mg/g VSS for proteins, humic acids and carbohydrates respectively, but afterwards, their concentrations began to continuously decrease. On the other hand, although proteins concentration also achieved a maximum during TH in minute 87 (272 mg/g VSS), their removal after this minute was significantly slower than in the case of WO. As expected, protein decomposition is faster in presence of an oxidising atmosphere (oxidative plus thermal decomposition) than in its absence (only thermal decomposition). Another difference between both treatments is that humic acids and carbohydrates concentrations increased throughout the reaction of TH, meaning that these biopolymers were less sensitive to thermal degradation than

proteins. At the end of TH (187 minutes), the concentrations of proteins, humic acids and carbohydrates was of 223, 212 and 123 mg/g VSS, respectively. The values obtained by WO for the same time were 158, 97 and 57 mg/g VSS for proteins, humic acids and carbohydrates, respectively. These values correspond to 54, 59 and 40% of the maximum soluble proteins, humic acids and carbohydrates concentrations, that is to say, those achieved in the minute 87 of WO.

In order to compare the changes in the size distribution of the solubilised biopolymers caused by each of the treatments, the evolution of their fingerprints was analysed (Figure 3). To carry out a more exhaustive analysis, the fingerprints area was divided into four categories as follows: three of them were stablished in the size exclusion zone for low (0–35 kDa), medium (35–150 kDa) and high (>150 kDa) molecular weights, whilst the fourth one corresponded to the area out of the column total volume. The presence of peaks out of the column total volume has been related to molecules with hydrophobic characteristics that interact with the filling material of the column (Görner et al., 2003). After an hour of treatment, the areas for low (0–35 kDa), medium (35–150 kDa) and high (>150 kDa) molecular weight biopolymers were 4, 8 and 1.2 AU s for TH and of 9, 14 and 1.5 AU s for WO, respectively. These results indicate that WO produced a higher solubilisation of low and medium size polymers than TH. Regarding to hydrophobic biopolymers, areas corresponding to times higher than that for total elution were 13.4 and 14 AU s for TH and WO, respectively. Taking into account that the polymers eluted in the zone of size exclusion should be those with hydrophilic characteristics, and that this kind of polymers is mainly located in the cell cytoplasm, it seems reasonable to suggest that the presence of an oxidising atmosphere favours a higher degree of cellular lysis in the initial stage of thermal treatment of sludge, rather than the complete hydrolysis of the cellular components, that is to say, the solubilisation of the cellular wall and membrane.

Finally, when both treatments were concluded, the peaks located at lower elution times in the fingerprints disappeared, whilst peaks corresponding to medium and low size biopolymers increase their height. These changes demonstrated that both reactions, thermal hydrolysis and oxidation, caused the hydrolysis of the larger polymers, which were initially solubilised from sludge, to form other of lower molecular weight. According to the stablished categorisation, the areas of the fingerprints for final effluents were of 15, 7, 0.7 and 24 AU s for TH and 15, 15, 0.4 and 15 AU s for WO. As it can be

observed, a higher proportion of hydrophobic polymers was obtained by TH than by WO. This is because oxidation reactions make the oxidised compounds more hydrophilic.

4. Conclusions

TH and WO showed a high efficiency to reduce the volume of activated sludge, causing a high solubilisation degree of the extracellular and cellular components of the sludge. However, the use of an oxidising atmosphere offered a more complete solution of treatment, providing better results of solubilisation, settleability and mineralisation of the sludge than an inert one.

The free radicals initially formed by WO favoured a higher solubilisation of the sludge instead of its mineralising. Likewise, these radicals showed particularly high affinity to cause the cellular lysis at the beginning of the reaction, rather than the complete hydrolysis of the cellular components such as cellular wall and membrane.

Despite of that WO caused a higher degree of solids solubilisation than TH, the yield of solubilisation of the main component of the sludge, the protein, was similar between both techniques. This result was a factor of the degradation of proteins caused by free radicals formed under an oxidising atmosphere.

The presence of oxygen during the hydrothermal treatment of sludge favoured a higher solubilisation of low and medium size polymers, as well as a higher hydrophilic character in the composition of the effluent. In addition, an oxidising atmosphere caused the degradation of each solubilised biopolymers from sludge, whilst an inert one only caused the degradation of proteins, demonstrating thus that proteins were more sensitive a thermal decomposition.

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Supporting Information.

Additional figure including the evolution of the UV-VIS absorption spectrum during the treatment by wet oxidation or thermal hydrolysis of the sludge. (PDF)

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Supplementary Information for

‘Sludge hydrothermal treatments. Oxidising atmosphere effects on biopolymers and physical properties’

*José L. Urrea[a], Manuel García[a], Sergio Collado[a], Paula Oulego[a], Mario Díaz[a]**

[a] Department of Chemical and Environmental Engineering,
University of Oviedo, c/Julián Clavería s/n, E-33071, Oviedo, Spain

(2 Pages, 1 Figure)

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*Corresponding author’s e-mail: mariodiaz@uniovi.es
Phone: +34 985 10 34 39; Fax: +34 985 10 34 34

1. UV-VIS absorption spectrum during wet oxidation or thermal hydrolysis of sludge.

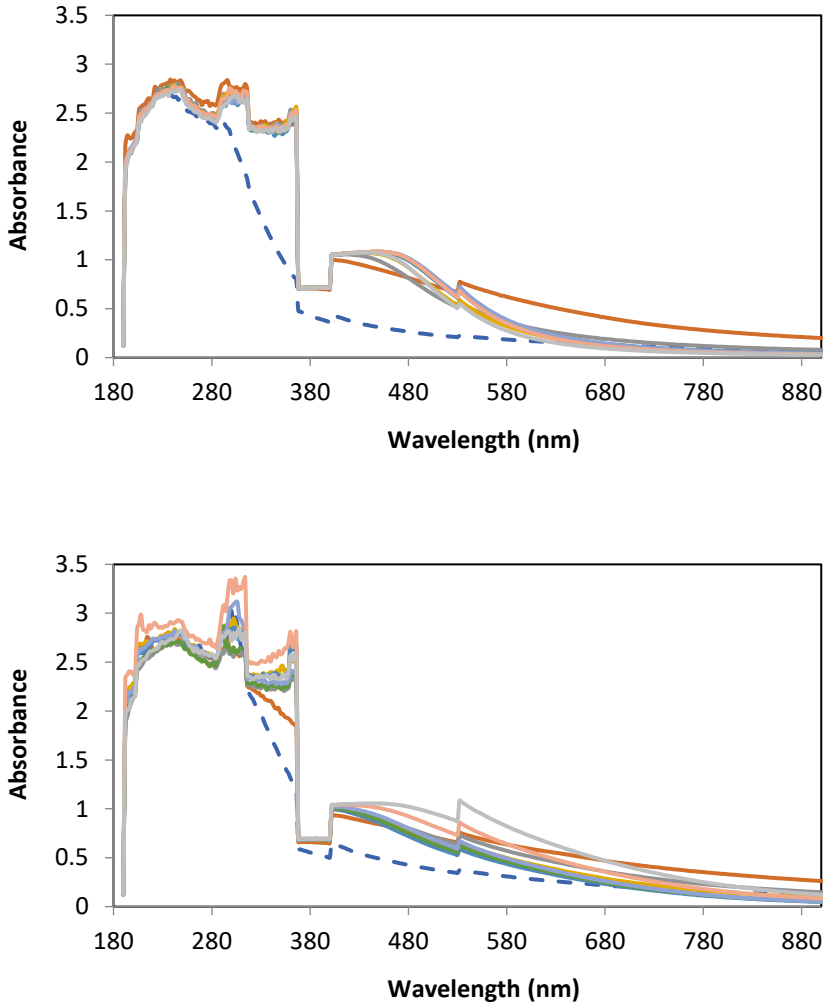

Figure S1. Evolution of UV-VIS spectrum of supernatants from sludge during wet oxidation (a) or thermal hydrolysis (b) at 160 ºC and 40 bar. Time sampling: initial, 45 min, 67 min, 77 min, 87 min, 97 min, 117 min, 147 min, 187 min.

ANEXO II

Comunicación tipo póster en las XXXV Jornadas Nacionales de Ingeniería Química, celebradas en Salamanca en Junio del 2018

Recuperación de proteínas de lodos mediante tratamientos hidrotérmicos a bajas temperaturas

M. García, S. Collado, P. Oulego y M. Díaz
Dpto. Ingeniería Química y Tecnología del Medio Ambiente. Universidad de Oviedo. C/ Julián Clavería, s/n. 33006 – Oviedo. Asturias.
mariodiaz@uniovi.es

Introducción

La gestión de los lodos generados en una planta depuradora supone un elevado coste económico y material para la misma debido a la dificultad de tratamiento, resultando necesario el desarrollo de nuevos procesos. Una de las alternativas son los tratamientos hidrotérmicos, como la hidrólisis térmica y la oxidación húmeda, sobre las que comienzan a aparecer estudios acerca de sus efectos en los componentes del lodo (1).

Los lodos están compuestos principalmente por proteínas y carbohidratos (2), así como por ácidos húmicos. Estas moléculas pueden tener un alto valor añadido, sobretodo las proteínas, por lo que su purificación puede ser de gran interés. Por ello, los tratamientos hidrotérmicos suponen una prometedora alternativa, al ser capaces de solubilizar estas moléculas, haciendo posible una separación posterior.

El objetivo del presente trabajo es estudiar la posibilidad de recuperar biomoléculas, principalmente proteínas, de lodos tratados mediante oxidación húmeda e hidrólisis térmica en condiciones suaves (60 °C y 10 bar) mediante métodos sencillos y fácilmente extrapolables a escala industrial, como son la modificación de pH y el *salting out* con sulfato de amonio.

Materiales y métodos

El lodo usado en los experimentos (imagen 1) fue un lodo secundario espesado con las características de la tabla 1.

Imagen 1. Lodo usado en los experimentos.

*Tabla 1. Parámetros del lodo usado en los experimentos. * indica concentraciones solubles.*

Parámetro	Unidades	Valor
pH		6.54
DQOt	mg O2/l	33700
DQOs	mg O2/l	550
SST	g/l	35,60
SSV	g/l	29,65
IVL	ml/g	28,09
Proteínas*	mg/l	62,50
Ácidos húmicos*	mg/l	308,53
Carbohidratos*	mg/l	72,90

El diseño experimental se muestra en la imagen 2 (1). Las condiciones experimentales fueron 60 °C y 10 bar, en presencia de oxígeno (OH) o de nitrógeno (HT). Se midieron los parámetros físico-químicos y las concentraciones de biopolímeros solubles antes y después de aplicar los métodos de separación.

Imagen 2. Diseño experimental: (1) reactor, (2) humidificador, (3) botella de O_2 o N_2, (4) controlador de flujo, (5) válvula de control, (6) válvula de control de presión, (7) puerto de toma de muestra y (8) controlador PID.

Para cada método se evaluó el porcentaje precipitado de cada biopolímero (% precipitado) y la selectividad (α_{ij}) para proteínas frente a ácidos húmicos o carbohidratos, utilizando las siguientes ecuaciones:

$$\%_{precipitado} = \left(1 - \frac{\text{concentración en el sobrenadante después de la precipitación}}{\text{concentración en el sobrenadante antes de la precipitación}}\right) 100 \qquad \alpha_{ij} = \left(\frac{\frac{(C_i)\ antes\ de\ la\ precipitación} - \frac{(C_i)\ sobrenadante\ antes\ de\ la\ precipitación}{(C_j)\ antes\ de\ la\ precipitación} - \frac{(C_j)\ sobrenadante\ antes\ de\ la\ precipitación}{(C_j)\ sobrenadante\ después\ de\ precipitación}}{(C_j)\ sobrenadante\ después\ de\ la\ precipitación}\right)$$

Resultados y discusión

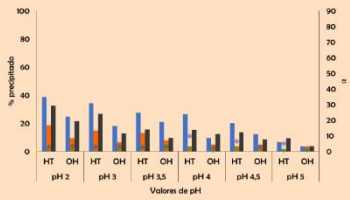

Imagen 6. Porcentaje precipitado para cada biopolímero e hidrolizado (OH o HT) tras añadir $(NH_4)_2SO_4$. ■ proteínas, ■ ácidos húmicos, ■ carbohidratos, ♦ α prot/a.h., ♦ α prot/carb.

- El ajuste de pH causó porcentajes de precipitación inferiores al 50% para cualquier valor e hidrolizado (imagen 5).
- La adición de sulfato de amonio (imagen 6) llevó a altos porcentajes de precipitación de proteínas (superiores al 50% en todos los casos), si bien fueron mayores para HT (superiores al 80%).
- En ambas técnicas los porcentajes son mayores para HT, debido a la ausencia de oxidación, lo que preserva las propiedades de las biomoléculas.
- La adición de sulfato de amonio de HT conduce a mejor selectividad en la separación de proteínas y ácidos húmicos que ajustando el pH. Este efecto es más notable en el caso de los hidrolizados de HT, por lo comentado anteriormente.
- Tanto los porcentajes de precipitación de proteínas como la selectividad fueron superiores a los obtenidos en estudios previos (3), por el menor daño sobre las biomoléculas debido a la suavidad de las condiciones de operación.

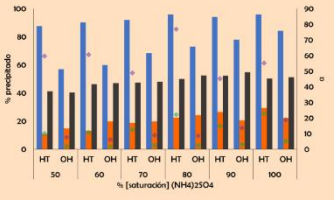

Imagen 6. Porcentaje precipitado para cada biopolímero e hidrolizado (OH o HT) tras añadir $(NH_4)_2SO_4$. ■ proteínas, ■ ácidos húmicos, ■ carbohidratos, ♦ α prot/a.h., ♦ α prot/carb.

Conclusiones

- ✓ Los tratamientos hidrotérmicos (OH e HT) son una alternativa interesante para la gestión del lodo.
- ✓ Los porcentajes de precipitación de proteínas más altos se obtienen con sulfato de amonio, tanto para OH como para HT.
- ✓ La adición de sulfato de amonio ofrece mejor selectividad para la separación de proteínas.
- ✓ Condiciones de tratamiento más suaves (60 °C frente a 160 °C) conducen a menor daño en las biomoléculas y mejoran los porcentajes de precipitación y la selectividad.

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1. *Urrea et al., 2016. Water Research 105, 282–290.*
2. *Chen et al. 2007. Water Research, 41(3), 683-689.*
3. *García et al., 2017. Waste Management 67, 278-287.*

Agradecimientos

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Abstract de la comunicación tipo póster en las XXXV Jornadas Nacionales de Ingeniería Química, celebradas en Salamanca en Junio del 2018

Título: Recuperación de proteínas de lodos mediante tratamientos hidrotérmicos a bajas temperaturas

Autores: Collado Alonso S., García San Miguel M., Oulego Blanco P., Díaz Fernández M.

Centro de Trabajo: Departamento de Ingeniería Química y Tecnología del Medio Ambiente

Palabras Clave: lodo; oxidación húmeda; proteína; tratamiento hidrotérmico;

Comunicación: La gestión de los lodos generados en una planta depuradora supone un elevado coste económico y material para la misma, debido a la dificultad de tratamiento, resultando necesario el desarrollo de nuevos procesos. Una de las alternativas son los tratamientos hidrotérmicos, como la hidrólisis térmica y la oxidación húmeda . Estos métodos consisten en someter al lodo a elevadas presiones y temperaturas, a fin de solubilizarlo y degradarlo, reduciendo así el volumen y la carga orgánica. Hasta ahora, los estudios existentes sobre estos tratamientos se centraban en los efectos de los mismos sobre las características físicas del lodo (1), si bien recientemente se está profundizando más en los mecanismos de reacción y los efectos sobre los componentes del lodo (2). Los lodos, debido a su naturaleza biológica, están compuestos principalmente por proteínas y carbohidratos así como ácidos húmicos. Estas moléculas son de alto valor añadido, sobre todo las proteínas, por lo que su purificación puede ser de gran interés a la hora de abaratar los procesos. Por ello, los tratamientos hidrotérmicos suponen una prometedora alternativa al ser capaces de solubilizar estas moléculas, haciendo posible una separación posterior. Anteriormente se ha trabajado recuperando las biomoléculas en condiciones de temperatura media (unos 160 °C y 40 bar) (3). El objetivo del presente trabajo es estudiar la posibilidad de recuperar biomoléculas, principalmente proteínas, de lodos tratados mediante oxidación húmeda e hidrólisis térmica en condiciones suaves (60 °C y 10 bar). Se han utilizado métodos sencillos, como son la modificación de pH y el salting out con sulfato de amonio. Los resultados muestran selectividad en la separación de proteínas, siendo el sulfato de amonio el que mejores porcentajes de recuperación de proteínas proporciona.

1. Urrea, J.L., Collado, S., Laca, A., Díaz, M., 2014. Journal of Environmental Management 146, 251–259.

2. Urrea, J.L., Collado, S., Oulego, P., Díaz, M., 2016. Water Research 105, 282–290.

3. García, M., Urrea, J.L., Collado, S., Oulego, P., Díaz, M., 2017. Waste Management 67, 278-287.

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Trabajo, Artículo 3

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Coautor4 x Doctor □ No doctor . Indique nombre y apellidos	**Jose Mario Díaz Fernández**