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Laccases from *Pleurotus ostreatus* Applied to the Oxidation of Furfuryl Alcohol for the Synthesis of Key Compounds for Polymer Industry

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Laccases are oxidative enzymes with high synthetic potential. In this work, their value in biocatalysis is shown through the green and selective oxidation of furfuryl alcohol into furfural with the aid of mediators. The influence of different parameters, such as pH, enzyme/mediator composition, buffer type, cosolvent tolerance, and reaction times, is investigated. Under the optimal conditions, 20 mol% of TEMPO as mediator and 5.8 U mL^{-1} of laccases POXC and POXA1b from *Pleurotus ostreatus*, quantitative production of furfural is attained after 16 h. POXC laccase stands out for its ability to catalyze the reaction at pH 6.5, whereas POXA1b is notable for its high stability. Furfural conversions reach excellent values (95%) after 72 h using only 5 mol% of TEMPO at 100 mM. Furthermore, furfuryl alcohol bioamination is achieved by employing the amine transaminase from *Chromobacterium violaceum*, providing furfuryl amine, a key compound for the polymer industry, through a one-pot sequential approach.

Introduction

Traditional chemical oxidative reactions generally involve the use of stoichiometric amounts of often expensive inorganic oxidants, generating large quantities of wastes.[1] Indeed, chlorates, chromates, permanganates, and metal species from copper, gold, palladium, platinum, ruthenium, and manganese have been commonly employed.[2] However, owing to the poor chemoselectivity, harsh reaction conditions required and metal-containing waste generation, the implementation of these processes in industry is undesired. To address these drawbacks, enzymes offer several advantages for the synthetic industry towards the development of sustainable and selective oxidation and oxy-functionalization reactions. In this context, several oxidoreductase subclasses have a key role employing environmentally benign and inexpensive oxidants, such as oxygen or hydrogen peroxide.[3] Hence, bio-oxidants are able to provide selective greener oxidations of alcohols, aldehydes, ketones, and more complex molecules containing various oxidation-sensitive functional groups.[4] Oxidative enzymes have also been studied in combination with other bio- or chemo-catalysts for the synthesis of a wide variety of high-value chemicals.[5] By developing these reactions in cascade manner, unstable intermediates can be converted into the final products without any purification step, contributing to reduce the waste production and energy consumption through sustainable overall processes.[6]

Among oxidative enzymes, laccases (EC 1.10.3.2) have gained increasing attention regarding traditional chemical oxidations, owing to their action under mild reaction conditions and specificity towards phenolic compounds as natural substrates.[7] Indeed, laccases do not require any expensive cofactor and are not inhibited by secondary reaction products (such as H_2O_2 for peroxidases and flavin-dependent alcohol oxidases), since they generate only water as co-product in oxidative processes.[8] In addition, their versatility can be increased when combined with small molecules used as chemical mediators. Such oxidative methods (laccase-mediator systems, LMS) allow to perform the oxidation step by the oxidized form of the mediator that can be regenerated using an adequate laccase (Scheme 1a).[9]

Widely distributed in nature, laccases from different fungal and bacterial sources have been extensively studied and applied in biosensor technology, pulp and paper industry, organic synthesis, textile and cosmetic industries, exploiting their abilities either in synthetic or degradative processes.[10] Herein, we have exploited the potential of three commercially available laccases: two enzymes from *Pleurotus ostreatus* (POXA1b and POXC),[11] and one from *Trametes versicolor* (L*Tv*),[12] due to their properties in terms of stability[13] and redox potential profile for synthetic purposes.[14]

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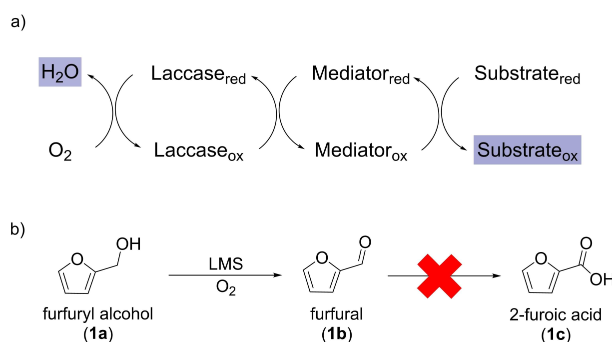

Scheme 1. a) Schematic representation of a laccase-mediator system (LMS). b) Selective bio-oxidation of furfuryl alcohol into furfural using a LMS under aerobic conditions.

This contribution aims to validate and explore a valuable bio-oxidative system for the oxidation of furfuryl alcohol (**1a**, Scheme 1b), a bio-based furan compound containing a primary alcohol moiety.[15] The aldehyde formation by traditional chemical methods is quite challenging due to its overoxidation towards 2-furoic acid (**1c**).[3c,16] Nowadays, furfural (**1b**) is commonly obtained by chemical acidic treatment of agricultural biomass containing xylan under harsh conditions.[17] Owing to their natural occurrence, furans are considered attractive building blocks for many synthetic processes.[18] For instance, furfural offers unique synthetic opportunities as chemical platform for high-added value compounds, such as furfuryl amine, furan dicarboxylic acid, tetrahydrofuran, succinic acid, and levulinic acid, among others.[19] Therefore, the design of selective and sustainable approaches for the production of this molecule under benign reaction conditions is highly appealing. The influence of key parameters in the oxidation of furfuryl alcohol has been studied to obtain an optimized biocatalytic process under mild conditions. As a further application of this laccase-mediated oxidative process, a one-pot sequential cascade was developed towards furfuryl amine, an interesting furan-based compound for polymer synthesis.[20]

Results and Discussion

Preliminary mediator screening using three laccases (L*Tv*, POXA1b and POXC) for the oxidation of furfuryl alcohol

Three laccases namely L*Tv*, POXC and POXA1b, endowed with different properties as thermal stability, specific activity, and redox potential (785 mV,[21] 690 mV,[13a] and 650 mV,[13a] respectively), were investigated for the oxidation of furfuryl alcohol (**1a**) into furfural (**1b**, Scheme 1b). A considerably high 100 mM substrate concentration was selected as starting point, and as expected, no direct oxidation of the substrate was observed for the three enzymes, even when extra oxygen was constantly supplied to the system. Hence, a panel of 26 possible laccase mediators (5 mol%),[22] both synthetic and natural derived, was investigated (see the Supporting Information, Table S1). Among all the different compounds tested, only few of them were efficient in catalyzing this oxidative reaction after 16 h at 30 °C, mostly (2,2,6,6-tetramethylpiperidin-1-yl)oxyl radical (TEMPO) and TEMPO derivatives such as 4-hydroxy-TEMPO and 4-acetamido-TEMPO. For the three tested laccases, TEMPO proved to be the most efficient mediator leading to moderate conversions into **1b** (31–58%). Therefore, TEMPO was selected for further optimization studies. The result obtained for the L*Tv*-TEMPO system (58% conversion) was not really surprising, since this is currently among the most explored laccase-mediated oxidative methods, and has already been applied to oxidize primary and secondary alcohols into the corresponding carbonyl compounds.[23] However, up to now, not very efficient processes have been set up for the oxidation of furfuryl alcohol using laccases. There are very few reports where TEMPO[24] or derivatives such as 4-acetamido-TEMPO[6a] or AZADO[25] have been used with L*Tv* for the oxidation of **1a** under oxygen atmosphere. Unfortunately, high enzyme loadings, low conversions or high quantities of the mediator had to be employed, respectively. Furthermore, Waghmode and co-workers reported the oxidation of several alcohols, including furfuryl alcohol, employing TEMPO in combination with *Tricholoma giganteum* laccase.[26] However, 75 mol% of TEMPO and 1,500 U mL^{-1} of enzyme at 37 °C had to be used to obtain quantitative furfural accumulation. These facts prompted us to study other laccases, such as the ones from *P. ostreatus*, to accomplish this transformation.

Study of the pH

The effect of the pH for the oxidation of **1a** (100 mM) into **1b** by the LMS was studied selecting TEMPO as mediator. In the case of L*Tv*, one of the most representative examples of the blue copper phenol oxidase family from fungi, it is well established that the optimum pH for this type of laccase is acidic.[27] In fact, from previous experiences using the L*Tv*-TEMPO system applied to the oxidation of different alcohol substrates,[28] pH values from 4.5 to 5 were known to be optimal. For our purpose, pH 5 was selected for further transformations. Therefore, we focused on the two laccases from *P. ostreatus*, studying the oxidations using 10 mol% of mediator loading (Figure 1). POXA1b resulted to be active in a pH range similar to that of L*Tv* (pH 4.5–5.5), finding 5.5 as the best value. On the contrary, LMS employing POXC was active in a wider pH range (pH 5.0–8.0), choosing 6.5 as the optimum one. Gratifyingly, after 16 h at 30 °C, conversions close to 70% and 90% could be obtained with POXA1b and POXC, respectively.

Most of the laccase-TEMPO applications are effective only at acidic pHs, thus reducing the possible combinations of these systems with other enzymes in cascade assets. Moreover, pH also influences the TEMPO efficiency in oxidizing the substrates: under acidic conditions, a bimolecular hydride transfer is reported as the main oxidative mechanism, while an alkoxide adduct is produced under basic conditions, with an oxo-ammonium species as intermediate.[29] Despite the full mechanism still remains incompletely understood, it has been shown that under basic conditions these reactions are considerably

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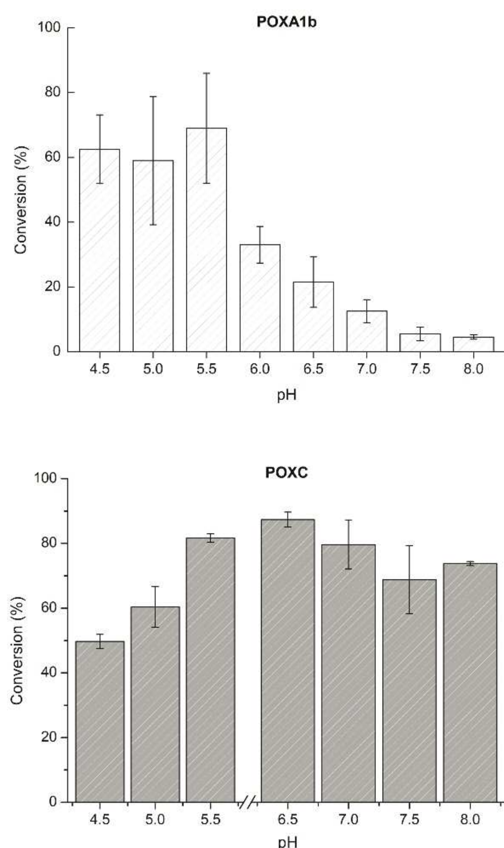

Figure 1. Study of the pH influence in the oxidation of furfuryl alcohol (100 mM) using TEMPO as mediator (10 mol%) at 30 °C after 16 h using POXC and POXA1b laccases (5.8 U mL^{-1}). Buffer Na-Citrate (50 mM) for pH 4.5–5.5; buffer KPi (50 mM) for pH 6–7.5; buffer Tris-HCl (50 mM) for pH 8. Conversions were calculated by GC analyses of the reaction crude mixtures (see the Supporting Information, Section 8).

faster than under acidic ones.[30] Nevertheless, basic pH has not been often applied for laccase-TEMPO mediated oxidations, since under these conditions most of the tested enzymes are not very active. Conversely, TEMPO in combination with POXC may efficiently be applied at pH up to 8.0, potentially overcoming these limits. Moreover, the three laccases were also compared in terms of stability at their optima pHs at 30 °C (Figure 2). Hence, POXA1b showed at pH 5.5 a considerable high half-life, preserving its complete enzymatic activity within 8 days of testing. This enzyme was reported as one of the most thermostable laccases, with a half-life of 3 h at 60 °C and pH 7, and 30 days at 25 °C and pH 9.[11b] POXC's half-life at pH 6.5 was around 20 h, which is in line with previous reports with the same enzyme,[11a] whereas L*Tv* at pH 5 was almost 70 h, similarly to other reports, where this enzyme has been shown stable for few days at this pH.[31]

Influence of TEMPO and laccase concentrations

The performance of the oxidative LMS with the three laccases, at their optimal pHs, was evaluated by varying TEMPO loading

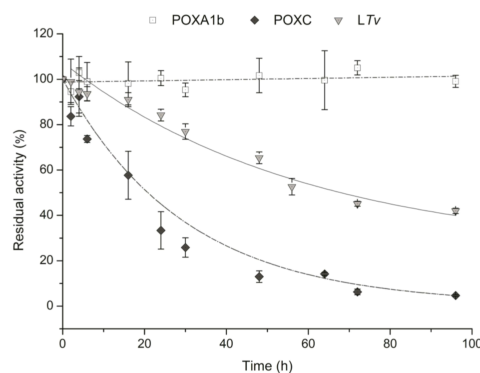

Figure 2. Residual activity of L*Tv* (▼), POXA1b (□) and POXC (♦) at pH 5, 5.5, and 6.5, respectively, at 30 °C (for details, see the Supporting Information, Section 5.2).

(10–33 mol%) and laccase concentration (2.9–5.8 U mL^{-1}). In the oxidation of **1 a** (100 mM) into **1 b**, the co-production of 2-furoic acid (**1 c**, produced by overoxidation of furfural) was observed in some cases, especially when higher amounts of TEMPO were used. The overall performances were compared, including the desired formation of **1 b** and the accumulation of **1 c** (Table 1). The formation of undesired carboxylic acid **1 c** in some extent (8–25%, entries 1, 2, 4 and 5) was only observed with the L*Tv*-TEMPO system, achieving selective oxidations of **1 a** into **1 b** when the lowest concentrations of the mediator were used (entries 3 and 6, 10 mol%). This can be due to the fact that the oxidation of **1 b** towards **1 c** proceeds through the hydrate form of the aldehyde. This reaction is catalyzed under acidic pH, and since this enzyme works better at low pH, the overoxidation process is also favored, as it has been previously described with the same system for other molecules containing primary alcohols.[28b] Conversely, owing to a lower reaction rate and the selection of higher pHs, no overoxidation of **1 b** was observed when using *P. ostreatus* laccases even at high mediator and enzyme concentrations (entry 1, 33 mol%). This fact can also be related to the lower redox potential of POXC (690 mV)[13a] and POXA1b (650 mV),[13a] with respect to the one from L*Tv* (785 mV).[21] Thus, using 5.8 U mL^{-1} of the laccase, comparable results were attained for L*Tv* and POXC at 10 mol% of TEMPO (entry 3), while higher mediator loadings (20–33 mol%) provided better results with both *P. ostreatus* laccases (entries 1 and 2), as they gave full conversion into **1 b**, not being detected the formation of co-product **1 c**.

A comprehensive analysis of the LMS performances, cost, and purity of the three laccases was performed, with the goal of selecting the best oxidative system to be coupled with a second reaction, particularly an amine transaminase (ATA)-catalyzed biotransamination. Thus, a cost analysis revealed the convenience of the process when integrating POXC and POXA1b; L*Tv* from Sigma–Aldrich, used in this contribution, has a cost of 0.21 € U^{-1} (excluding handling and shipping), while both *P. ostreatus* laccases cost from BioPox is 0.10 € U^{-1} (including handling and shipping). Furthermore, L*Tv* laccase has a specific activity for ABTS close to 100 U mg^{-1}, suggesting the presence

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Table 1. Comparison of furfural production applying the LMS at different TEMPO concentrations (mol%) and laccase loadings ($U\,mL^{-1}$) after 16 h at 30 °C.

1a, 100 mM → (Laccase, TEMPO, buffer, O_2) → **1b** → (Laccase, TEMPO, buffer, O_2) → **1c** (undesired)

Entry	Enzyme ($U\,mL^{-1}$)	TEMPO (mol%)[a]	L*Tv* [%][b]		POXC [%][b]		POXA1b [%][b]	
			1 b	**1 c**	**1 b**	**1 c**	**1 b**	**1 c**
1	5.8	33	80	20	99	< 1	99	< 1
2		20	89	11	99	< 1	99	< 1
3		10	99	< 1	97	< 1	48	< 1
4	2.9	33	75	25	74	< 1	80	< 1
5		20	92	8	56	< 1	60	< 1
6		10	98	< 1	55	< 1	58	< 1

[a] Referred to the amount of alcohol **1 a**. [b] Conversion values were calculated by GC analyses of the crude reaction mixtures using calibration curves (see the Supporting Information, Section 8).

of other contaminant proteins that could interfere with undesired secondary reactivity. Conversely, POXC and POXA1b have specific activities for ABTS of 670 and 550 $U\,mg^{-1}$, respectively, suggesting a higher degree of homogeneity for both enzymes. All these considerations led us to select *P. ostreatus* laccases for further experiments.

Effect of organic co-solvents

To prove the versatility and applicability of these *P. ostreatus* laccase-TEMPO (10 mol%) systems, they were tested in the presence of different co-solvents (10% v/v). The residual activity in water-miscible and immiscible organic solvents was evaluated (Figure 3). After 16 h at 30 °C, POXA1b remained partially stable (>60% of initial activity) in the presence of most of the solvents and its catalytic performance was preserved: in particular, in the biphasic systems with *tert*-butyl methyl ether (MTBE) and cyclohexane, much higher conversion values were measured (60–80%) in comparison with the reaction in plain buffer (40%). Conversely, POXC was more sensitive to all immiscible solvents, and its oxidative performances were negatively affected (usually <30% of initial activity) in almost all the tested solvents. For this enzyme, ethyl acetate (EtOAc), MTBE and cyclohexane provided the best results in terms of conversions into **1 b** (80–90%), similar to plain buffer. As a summary, most of the solvents were suitable for the POXA1b-TEMPO system, whereas few of them could be integrated in the POXC-TEMPO oxidative system. It was interesting to observe that both enzymes accepted immiscible organic cosolvents better than miscible ones, opening the door for their action with more lipophilic substrates.

Furfural production at low TEMPO concentrations within the time

The effect of lowering the TEMPO concentration was also investigated in order to optimize a system with the lowest

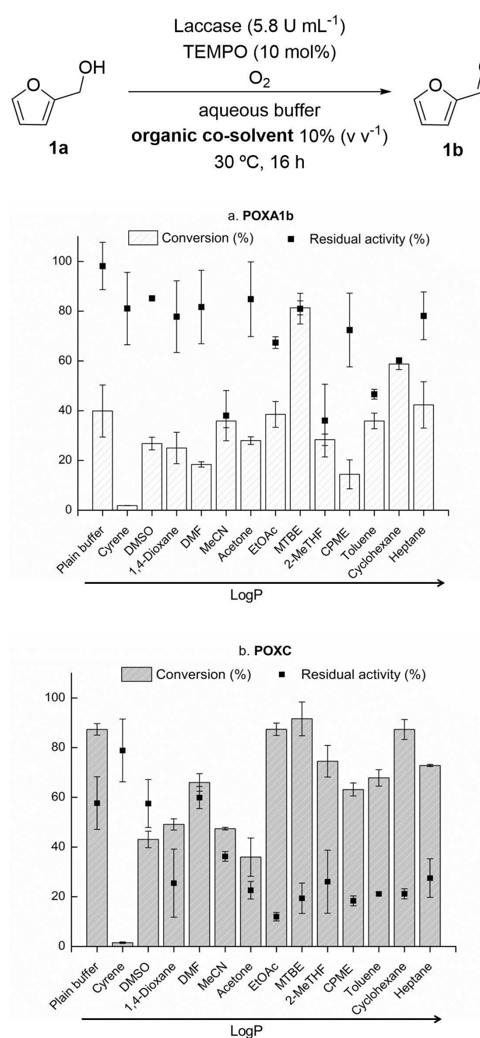

Figure 3. Bars: Conversion of **1 a** (100 mM) into **1 b** using POXA1b-TEMPO (a) and POXC-TEMPO systems (b) with 10 mol% of the mediator after 16 h at 30 °C in the presence of different organic solvents (10% v/v). The error is the result of two independent sets of reactions. *Dots* (■): Residual activity of the corresponding laccase in the analyzed solvent measured against ABTS (for details see the Experimental Section and the Supporting Information, Section 5.3).

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possible amount of the chemical oxidant (2.5–20 mol%, Figure 4). Interestingly, conversions up to 95% towards **1 b** were found even at 5 mol% of the mediator after 72 h or using 10 mol% TEMPO after 48 h, with the POXA1b system (Figure 4a). Similarly, POXC allowed to obtain a quantitative conversion of **1 b** at 20 mol% of the mediator after only 16 h or employing 10 mol% TEMPO after 24 h (Figure 4b). However, in the presence of only 2.5 mol% of the oxidant, low levels of conversion (<5%) were observed in the POXA1b-TEMPO system, whereas 60% furfural production was observed for POXC after 24 h.

It is important to remark that POXC quickly mediated this transformation with high conversions already after 16 h, while reactions catalyzed by POXA1b proceeded slowly, especially in presence of 5 and 10 mol% of mediator. However, in the first case no important variations were observed after that time, whereas POXA1b-catalyzed reactions showed increasing conversion values within the time. These results are in line with the higher stability observed for POXA1b compared to POXC (Figure 2), highlighting the potential of the first variant for possible oxidative transformations. Again, carboxylic acid **1 c** was not detected in these experiments.

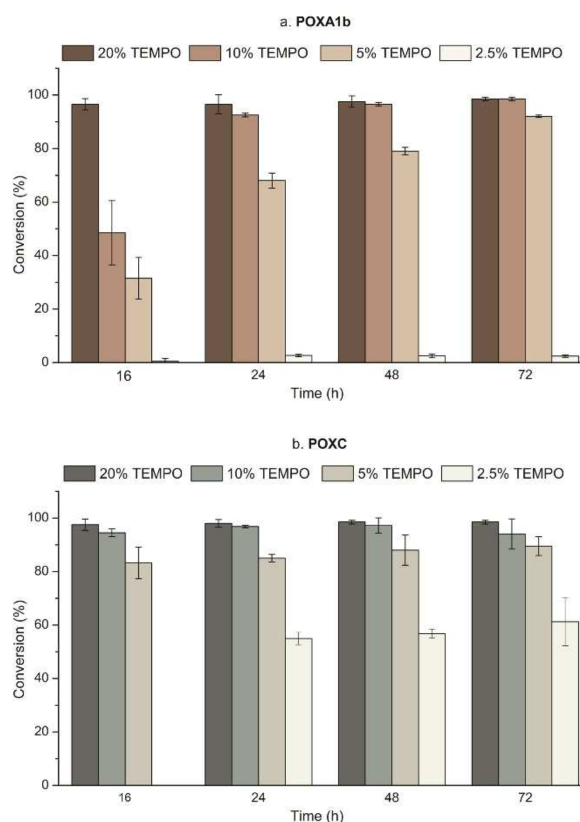
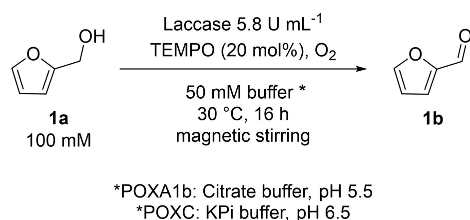


Figure 4. Conversion of **1 a** (100 mM) into **1 b** mediated by TEMPO (2.5–20 mol%) and employing: POXA1b (a) or POXC (b) at different reaction times (16–72 h).



Scheme 2. Optimized reaction conditions for TEMPO-mediated oxidation of furfuryl alcohol into furfural with POXA1b and POXC laccases.

Under the optimized conditions, both *P. ostreatus* laccase-TEMPO systems can quantitatively produce furfural, without formation of 2-furoic acid, up to a substrate concentration of 100 mM (Scheme 2). POXC-based system was also tested at 200 mM of substrate concentration, obtaining quantitative conversions into furfural after 16 h, although a higher loading of TEMPO (33 mol%) was necessary.

On one hand, we propose a laccase-TEMPO oxidation system able to provide quantitative conversions into furfural at less acidic pHs after 16 h. Alternatively, we offer similar performances at longer reaction times (72 h), but with an amount of mediator reduced up to 5 mol%, using a highly stable laccase. Furthermore, oxidation levels for both systems are differently affected by the presence of co-solvents; POXA1b activity is preserved in most of the common water-miscible and immiscible organic solvents at 10% v/v, which can increase the solubility of hydrophobic substrates.

LMS applied in a one-pot linear sequential fashion: Synthesis of furfuryl amine from furfuryl alcohol

L*Tv*-TEMPO system has been successfully applied for deracemization of racemic (hetero)aromatic alcohols[28a,32] or their amination into amines,[33] when combined with alcohol dehydrogenases or transaminases, respectively, and for isomerization of allylic alcohols into saturated carbonyl compounds in combination with ene-reductases.[34] In all these biotransformations, the oxidative step was performed at low substrate concentration (25–50 mM) and high TEMPO loading (33 mol%). In this study, furfuryl amine synthesis via TEMPO-mediated oxidation combined with laccases from *P. ostreatus* was explored, employing a higher initial substrate concentration (100 mM), a lower co-oxidant loading (20 mol% of TEMPO), and short reaction times (16 h), using cheaper and more active laccases.

In order to show the applicability of our laccase-TEMPO systems with POXA1b and POXC, the production of furfuryl amine (**1 d**) from furfuryl alcohol was investigated through a one-pot oxidation-transamination sequence. This amine is a key monomer for polymers manufacturing; however, the traditional synthetic chemical methods require harsh operative steps, which often generate imines as intermediates, that can later form other by-products, affecting therefore the final purity and yield of the desired compound.[35]

The sequential strategy was set-up using 50 mg of **1 a** selecting amine transaminase from *Chromobacterium violaceum* (*Cv*-TA)[36] after optimization of the transamination reaction conditions. The transaminase reaction was run with a molar

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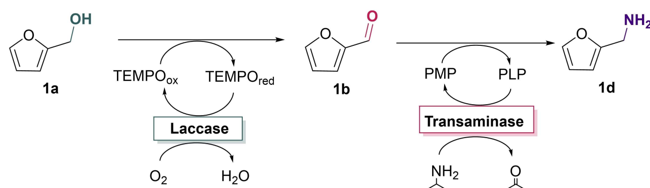
excess of isopropylamine ($^{i}PrNH_2$) as amine donor (1 M), which is usually required for shifting the equilibrium toward the amine synthesis.[37] Transamination of furfural intermediate was carried out after the oxidation step, in the same reaction pot after simple addition of the ATA, pyridoxal 5'-phosphate (PLP) as the cofactor and $^{i}PrNH_2$ as amine donor (Scheme 3). The concurrent cascade could not be set-up, owing to the already documented loss of activity shown by TEMPO in the presence of $^{i}PrNH_2$.[33] To adjust the pH for the second step, the amine donor was added as phosphate salt.

The one-pot/two-step system was satisfactorily developed applying *P. ostreatus* laccases and TEMPO obtaining, in a quantitative yield, the furfural intermediate which was then converted into furfuryl amine using *Cv*-TA and $^{i}PrNH_2$ (99% overall conversion). The final product **1 d** was isolated in excellent purity by liquid-liquid extraction, without need of further purification steps in 48% isolated yield.

Environmental impact

A quantification of the environmental impact of this sequential system was evaluated using the E-factor concept.[38] Particularly, evaluation was concentrated on the impact of the reaction conditions respect to the waste generated, media employed, as well as the work-up. For the reaction shown in Scheme 3, the downstream process affected by 89% the overall procedure. The organic solvent contribution for product extraction was more than 70% and the total contribution of water was 24% (Figure S2). In fact, excluding solvents, for the cascade transformation, an E-factor of 22.5 was obtained, which is in line with previous values attained for similar oxidative systems.[33a,39] As it can be envisaged, a more favorable impact of the laccase/TEMPO-ATA cascade protocol may be achieved implementing a continuous extraction work-up for reducing the solvent contribution, which could also increase the product extraction yield. Moreover, working with immobilized mediator and cofactors, the aqueous media could be reused for more than one cycle, and its impact could be further reduced.



Scheme 3. One-pot bienzymatic sequential approach for the synthesis of **1 d** using a laccase-TEMPO system and *Cv*-TA. Reaction conditions: Laccase POXC/POXA1b (58 U mmol^{-1} **1 a**), oxygenated phosphate/citrate buffer (50 mM, pH 6.5/5.5), TEMPO (20 mol%), 30 °C, 16 h, 250 rpm. Then, *Cv*-TA (10 mg DCW), KPi buffer (pH 6.5, 100 mM), PLP (1 mM), $(^{i}PrNH_3)_3PO_4$ (330 mM), 30 °C, 24 h, 250 rpm.

Conclusions

In the light of integrating sustainable processes in synthetic protocols for valorization of biorenewable compounds, mild oxidative systems based on laccase-TEMPO cooperation have been reported. The optimization of the reaction conditions employing three laccase-TEMPO systems (L*Tv*, POXC, and POXA1b), were investigated towards the synthesis of furfural in terms of mediator loading, enzyme amount, medium engineering, and reaction selectivity towards the aldehyde synthesis. Analysis of enzyme costs, stability, and versatility of the different LMS, led us to focus our attention on *P. ostreatus* laccases, which formed furfural in a very selective way, without overoxidation towards 2-furoic acid. POXC enzyme stood out for the wide pH range of action, whereas POXA1b was notable for its stability within the time, allowing reduction of the amount of the mediator up to 5 mol% while prolonging the reaction times up to 72 h. TEMPO oxidative systems with *P. ostreatus* laccases were also shown as versatile tools for lipophilic substrates, owing to their tolerance to different water immiscible cosolvents at high amounts (10% v/v). Satisfyingly, both oxidative systems were suitable in such application at 100 mM substrate concentration.

Both LMS were satisfactorily tested in a one-pot two-step cascade approach with a transaminase, which allowed the synthesis of furfuryl amine from furfuryl alcohol. Overall, this one-pot strategy is an effective example of a multienzymatic process involving laccases and transaminases for the production of a furan-based compound. This proof-of-concept paves the way for such LMS applications in the synthesis of further valuable bio-based derivatives.

Experimental Section

Materials

All chemicals were either purchased from commercial sources. Commercially available furfural and furfuryl amine were purchased from Sigma–Aldrich and used as standards. NMR spectra were recorded on a Bruker AV300 MHz spectrometer including ^{1}H and ^{13}C. All chemical shifts (δ) are reported in parts per million (ppm) and referenced to the residual solvent signal. Gas chromatography (GC) analyses were performed on an Agilent HP6890GC chromatograph equipped with an FID detector.

L*Tv* was obtained from Aldrich (103 U mg^{-1}). Recombinant POXA1b from *P. ostreatus*[40] expressed in the yeast *Pichia pastoris* under the control of the AOX1 promoter (induced by glycerol, 550 U mg^{-1}),[41] and POXC laccase produced from *P. ostreatus*[11a,42] (670 U mg^{-1}), were provided by BioPox srl.

ABTS assay

ABTS assay was used for measuring laccase apparent units. ABTS (ε_{420} = 36,000 M^{-1} cm^{-1}) test was performed at room temperature in 100 mM citrate buffer pH 3.0 with 2 mM final concentration of the substrate, and a suitable amount of enzyme necessary to obtain an absorbance of 0.5–1 after approximately 1 min. The resulted increasing coloured radical cation (ABTS$^{+\bullet}$) was tracked using a

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UV-Vis spectrophotometer at 420 nm. One unit of laccase activity was defined as the enzyme amount able to oxidize 1 μmol of the substrate per min.[43]

General procedure for the laccase-mediated oxidation of furfuryl alcohol (1a)

In a test tube open to air, TEMPO was added to a solution of **1 a** (0.1 mmol, 100 mM) in an oxygen-saturated buffer (50 mM, at the proper pH). The reaction mixture was magnetically stirred for a few minutes to dissolve all the reagents, and a buffered solution with laccases from L*Tv* or *P. ostreatus* (5.8 U mL^{-1}) was then added. The mixture was magnetically stirred (250 rpm) for an additional 16 h at controlled temperature (30 °C). After this time, the product was extracted with EtOAc (2×2 mL), the organic phases were combined, dried over anhydrous Na_2SO_4, and filtered. An aliquot was taken for the determination of the degree of conversion by GC analysis (see the Supporting Information, Section 8).

Study of the solvent influence for the laccase/TEMPO-mediated oxidation of 1a in the mono- or biphasic buffer/organic solvent system

In a test tube open to air, TEMPO (10 mol %) was added to a solution of **1 a** (0.1 mmol, 100 mM) in a monophasic or biphasic mixture of an oxygen-saturated buffer (50 mM, Na-Citrate buffer, pH 5.5 for POXA1b; KPi buffer, pH 6.5 for POXC), and the organic solvent (10 % v/v, for a total volume of 1 mL). The reaction mixture was magnetically stirred for few minutes to dissolve all the reagents, then the corresponding laccase (5.8 U mL^{-1}) was added, and the mixture was magnetically stirred (250 rpm) for additional 16 h at 30 °C. After this time, the product was extracted with EtOAc (2×2 mL), the organic phases were combined, dried over anhydrous Na_2SO_4, and filtered. Finally, an aliquot was taken for the determination of the degree of conversion by GC analysis (see the Supporting Information, Section 8).

Preparative scale for the one-pot synthesis of furfuryl amine (1d) from furfuryl alcohol (1a)

In a test tube open to air, TEMPO (20 mol %) was added to a solution of **1 a** (9.8 mg, 100 mM) in an oxygen-saturated buffer (5 mL, KPi buffer pH 6.5 for POXC, citrate buffer pH 5.5 for POXA1b). The reaction mixture was magnetically stirred for a few minutes to dissolve all the reagents, then the laccase (5.8 U mL^{-1}) was added, and the mixture was stirred for additional time at 30 °C. After 16 h, PLP (1 mM), and $(^iPrNH_2)_3PO_4$ (330 mM) were added to the mixture containing furfural (**1 b**) as intermediate. The addition of this concentrated salt provided $^iPrNH_2$ to the reaction medium and increased the pH from the initial value to approximately 6.5. No further pH adjustment was required for the biotransamination reaction. Finally, whole cells expressing *Cv*-TA (10 mg) were added. No pH changes were observed in the KPi buffer after residual POXC action. The tube was closed and the reaction mixture was vigorously shaken for 24 h, at 250 rpm and 35 °C in an orbital shaker. After this time, the reaction was stopped by addition of 6 M aqueous HCl solution (2 mL, pH 2). Degree of conversion into amine **1 d** was determined by GC analysis (see the Supporting Information, Section 8). The acidified solution was extracted with EtOAc (4× 5 mL). The resulting aqueous layer was basified by adding aqueous 10 M NaOH solution until pH 13 and extracted with EtOAc (4× 5 mL). The organic layers were combined, washed with brine (5 mL), dried over anhydrous Na_2SO_4, and filtered. After evaporation of the solvent under reduced pressure, the corresponding amine was obtained pure as yellow oil (24 mg, 48 % isolated yield), confirmed by NMR analysis.

Sample preparation for GC analysis

In a 2.0 mL Eppendorf vial, the reaction mixture (1 mL) was taken and extracted with EtOAc (3×0.5 mL), and the combined organic layers were next washed by brine (1 mL), and dried over anhydrous Na_2SO_4, filtered and analyzed by GC.

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Conflict of Interests

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available in the supplementary material of this article.

Keywords: Biocatalysis · Furfural · Furfuryl amine · Laccase-mediator system · Oxidative processes

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