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Recombinant laccase biosynthesis for efficient polydopamine coating

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ABSTRACT

Laccases are versatile biocatalysts with interest for various industrial applications. This study reports the expression of *Trametes versicolor* laccase in *Komagataella phaffii* X33. The cultivation parameters (methanol and $CuSO_4$ concentration, and temperature) for recombinant laccase production were studied in an orbital shaker. Enhanced laccase production was achieved by adding 1 % (v/v) methanol daily, supplementing 0.1 mM $CuSO_4$ and incubating at 25 °C. Under these conditions, laccase production was scaled-up in a 4 L stirred tank bioreactor. Subsequently, laccase was concentrated and purified using a combined protocol of ultrafiltration and acetone precipitation, achieving a purification factor of 3.02. The laccase produced exhibited robust stability within a pH range from 4.0 to 8.0 and thermal stability up to 30 °C. Michaelis Menten kinetic revealed Michaelis constant (K_M) and maximum rate of reaction (V_{max}) values of 44.5 μM and 110.9 μM/min, respectively. Finally, laccase was employed as a biocatalyst to assist the polymerization of dopamine to polydopamine, allowing the one-step coating of cellulose filter paper, as confirmed by diffuse reflectance spectroscopy (UV-Vis DRS) and scanning electron microscopy (SEM). This work represents an advance in the field of laccase production in both orbital shaker and bioreactor, while demonstrating, for the first time, the laccase-assisted polymerization of dopamine for the coating of filter paper with polydopamine.

1. Introduction

Laccases (EC 3.10.3.2) are multi-copper oxidoreductases with catalytic activity in the oxidation of a wide range of organic and inorganic compounds (*e.g.*, aromatic amines and phenolic compounds), by reducing molecular oxygen to water [1,2]. Nowadays, laccases are used as biocatalysts with multifaceted industrial applications, and are extensively known for their application in the paper and pulp industry and in wastewater treatment [3]. Due to their high specificity and efficiency, novel and promising laccase applications are envisioned, such as in biosensors, biofuels and polymer synthesis [1,4].

Under the framework of polymerization, particular interest has been given to the laccase-mediated synthesis of polydopamine (PDA) [5], a bioinspired biopolymer with unique adhesive and coating properties for (nano)materials (ceramics, glass, among others) [6]. These coatings exhibit exceptional properties such as biodegradation, biocompatibility, and antioxidant activity. The biocompatible nature of PDA and its enhanced ability to immobilize biomolecules renders it highly attractive for enzyme immobilization [7], to improve materials adhesion for tissue engineering [8], or to enhance the corrosion resistance of biosensors [9]. PDA provides a one-step solution for surface functionalization and is attractive as a coating material due to its chemical versatility and ease of processing [10]. However, the conventional PDA synthesis typically involves dopamine self-polymerization under alkaline conditions (pH > 8.5) [6]. In addition to being labor-intensive and time-consuming, requiring at least 24 h of reaction, it can compromise the coating of alkali-sensitive materials [11]. To overcome these issues, laccase-assisted polymerization appears as an efficient and controlled synthesis route for PDA coatings, enabling shorter reaction times and higher versatility in coating various materials, including those sensitive to alkaline conditions. To date, PDA coatings catalyzed by laccase have been studied for different materials, such as carbon nanotubes [12] and cotton [13]. Despite the promising potential of PDA coating in cellulose paper for low-cost diagnostic sensors or microfluidic devices [14], to the best of our knowledge, only non-enzymatic reactions have been studied to this end so far.

Laccases are naturally produced by several organisms, with white-rot fungi standing out as prominent laccase producers [1,2]. However, the

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Table 1
Culture media composition applied in different stages of this work for *K. phaffi* X33 manipulation, transformation and laccase production.

Culture medium	Application	Composition
Yeast peptone dextrose (YPD)	Growth and storage	1 % (w/v) yeast extract, 2 % (w/v) peptone and 2 % (w/v) glucose
Yeast peptone dextrose sorbitol (YPDS)	Selection of transformants	1 % (w/v) yeast extract, 2 % (w/v) peptone, 2 % (w/v) glucose, 2 % (w/v) agar and sorbitol (1 M)
Buffered Glycerol-complex Medium (BMGY)	Biomass production (inoculum preparation)	1 % (w/v) yeast extract, 2 % (w/v) peptone, 100 mM phosphate buffer (pH 6.0), 1.34 % (w/v) YNB, 4.00×10^{-5} % (w/v) biotin and 1 % (v/v) glycerol
Buffered Methanol-complex Medium (BMMY)	Inducing secreted expression (fermentation)	1 % (w/v) yeast extract, 2 % (w/v) peptone, 100 mM potassium phosphate buffer (pH 6.0), 1.34 % (w/v) YNB, 4.00×10^{-5} % (w/v) biotin and 1 % (v/v) methanol

use of native producers is usually associated with high production costs and low productivity. In addition, it yields a mixture of isoenzymes that is not desirable for large-scale applications and commercial purposes [15,16]. So, heterologous expression of laccase is a viable strategy to overcome these issues as it offers higher yields, better solvent tolerance, and higher stability and catalytic efficiency over native enzymes [16–18].

Among the hosts currently available, *Komagataella phaffii* (previously described as *Pichia pastoris*) has emerged as an efficient and versatile system for intra- and extracellular recombinant protein expression. With well-characterized genetics facilitating subsequent genetic manipulations [19], this yeast grows to high cell densities in minimal media, representing a cost-effective strategy compared to other expression systems. Adding to these advantages is the easy scalability of *K. phaffii* bioprocesses, its capacity to carry out essential post-translational modifications for protein function, as well as an enhanced ability to secrete proteins towards the extracellular medium [19,20]. The latter attracts special interest as fewer endogenous proteins are in the extracellular medium, streamlining the subsequent purification steps and saving time [21,22]. Due to the presence of the alcohol oxidase (AOX 1) gene, *K. phaffii* bioprocesses driven by the AOX promoter are strongly regulated by methanol, allowing inducible expression with high protein levels [19,20].

Over the years, *K. phaffii* has established itself as a productive industrial host capable to produce commercial proteins [22], including several laccases [23–25]. Nonetheless, the established methods are still limited by the low heterologous expression efficiency, inefficient secretion, and the lack of scalable methodologies. To address these challenges, the present study aims to achieve the heterologous production of *Trametes versicolor* laccase (gene lcc2) in *K. phaffii* X33 under the control of the AOX promoter. Firstly, the optimization of several variables (glycerol presence, methanol concentration, copper concentration and temperature) was carried out in an orbital shaker. Then, under optimized conditions, laccase production was performed in a 4 L stirred tank bioreactor. The recombinant laccase produced was applied for the first time to polymerize dopamine into PDA and to coat cellulose filter paper with PDA.

2. Materials and methods

2.1. Materials, plasmids and strains

K. phaffii X33 strain and the recombinant plasmid pPICZB containing the lcc2 gene from *T. versicolor* (GenBank Accession No. CAA77015.1, cf. Fig. S1 in the Supplementary Material (SM) for further details) were obtained from Invitrogen (Carlsbad, USA) and Genscript (Piscataway, New Jersey, USA), respectively. The reagents used in the preparation of the culture medium (shown in Table 1) were peptone and agar from Liofilchem (Teramo, Italy), yeast extract from Biokar (Cedex, France), biotin from Fagron and yeast nitrogen base (YNB) with ammonium sulfate (5 g/L) from Grisp (Porto, Portugal). Glycerol (analytical grade) and methanol (HPLC grade) were acquired from Merck (Darmstadt, Germany) and Fisher Scientific (Massachusetts, USA), respectively. Copper sulfate pentahydrate ($CuSO_4.5H_2O$) was obtained from Merck (Darmstadt, Germany), biotin from Fagron (Rotterdam, Netherlands), and zeocin was purchased from InvivoGen (California, USA). Glucose was purchased from VWR (Pennsylvania, USA), while agar was obtained from Liofilchem (Teramo, Italy). 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), dopamine hydrochloride and the laccase standard from *T. versicolor* (lot result 1.4 U/mg) were acquired from Sigma-Aldrich (St. Louis, USA). Phosphate buffer was prepared using dipotassium hydrogen phosphate trihydrate ($K_2HPO_4.3\,H_2O$, 98 % purity) and potassium phosphate monobasic (KH_2PO_4, 98 % purity) acquired from Scharlab (Barcelona, Spain). Total protein quantification was performed using the Pierce BCA protein assay from Thermo Fisher Scientific (Massachusetts, USA). NZYColour Protein Marker I was obtained from NZYTech (Lisbon, Portugal), and the tris-glycine pre-casted polyacrylamide gels and Tris-Glycine SDS running buffer were acquired from Invitrogen (Carlsbad, USA).

The culture media composition used for the cell growth and routine manipulation of *K. phaffii* X33, as well as those used during strain transformation and for laccase production, are shown in Table 1.

2.2. K. phaffii X33cultivation and laccase production

K. phaffii X33 was routinely cultivated in YPD medium and transformed by electroporation according to the manufacturer's instructions (*cf.* the SM for details). To confirm the expression of laccase in *K. phaffii* X33, the yeast was grown on YPD agar (2 % (w/v)) plates supplemented with 0.2 mM ABTS. Plates were incubated for 15 days at 30 °C.

2.2.1. Inoculum preparation

To prepare the *K. phaffii* X33 inoculum, yeast colonies were transferred from YPD agar plates (previously grown at 30 °C) to 250 mL Erlenmeyer flasks containing 50 mL of BMGY medium and incubated at 30 °C under stirring at 250 rpm. After 24 h, cell growth was determined by measuring the Optical Density at 600 nm (OD_{600}). Then, a determined volume was collected to achieve an initial OD_{600} of 1.0 Absorbance Unit (AU) in both fermentations. The sample was then centrifuged at 1400 × g for 5 min at 25 °C. The recovered cell pellet was directly resuspended in the induction medium (BMMY) and used as the inoculum for orbital shaker and bioreactor cultivations.

2.2.2. Laccase production in orbital shaker

Orbital shaker cultivations were performed in 250 mL Erlenmeyer flasks containing 50 mL of BMMY medium, incubated at 250 rpm for 13 days. The standard control condition at 30 °C was applied for starting the cultivation (addition of methanol 1 % (v/v) + supplementation of $CuSO_4$ 0.1 mM, both concentrations in the fermentation broth), as well as for the daily feeding with only methanol, 1 % (v/v) [26,27]. Control parameters were defined according to the yeast expression guide provided by the manufacturer for general yeast use [26] and the work of Balakumaran et al. [27] on the effect of copper supplementation in *K. phaffii* growth. Methanol feeding was performed on days 0, 1, 2, 3, 6, 7, 8, 9 and 10. Samples were collected to monitor cell growth, pH, protein concentration and enzymatic activity. In the initial stage, laccase production was assessed by measuring its bioactivity directly in the extracellular and intracellular fractions, following the application of a cell lysis and protein recovery protocol described in the SM. Since it was found that laccase was being secreted into the extracellular medium, this fraction was used throughout the entire work. Additional experiments to determine the optimal cultivation conditions for laccase expression encompassed the study of different concentrations of daily methanol

feedings (0–2.00 % (v/v)), $CuSO_4$ concentration (0–0.5 mM) and three temperatures (25.0, 30.0 and 32.5 °C). The relative laccase production was determined based on its bioactivity, according to Eq. 1:

$$Relative\ laccase\ production(\%) = \frac{Enzymatic\ activity\ in\ the\ sample\ (U/L)}{Enzymatic\ activity\ in\ the\ control\ (U/L)} \times 100 \quad (1)$$

where the enzymatic activity in control corresponds to experiments performed at 30 °C, with 1 % (v/v) methanol both in BMMY medium and for the daily feeding, and with $CuSO_4$ 0.1 mM.

The effect of pH (3.0–8.0) and temperature (4, 25, 30, 40 and 50 °C) on the laccase activity was also investigated according to the details provided in the SM.

2.2.3. Laccase production in stirred tank bioreactor

Laccase production was carried out in a 6 L stirred tank bioreactor Minifors II (Infors-HT) with a working volume of 4 L. This bioreactor is equipped with oxygen and pH electrodes, and two Rushton impellers. Culture conditions were 5 % (v/v) inoculum, 25 °C, 700 rpm, 1 vvm (air volume/medium volume/min) for 7 days. The BMMY medium was supplemented with 0.1 mM $CuSO_4$ and 1 % methanol daily. Antifoam was manually added when needed. Two strategies were investigated after the preparation of the inoculum (*cf.* Fig S2 in the SM for a schematic representation): I) the cultivation was initiated in a shake-flask (25 °C and 250 rpm) using BMMY medium for 5 days, daily supplemented with 0.1 mM $CuSO_4$ and 1 % (v/v) methanol, and later transferred to the bioreactor; II) the inoculum was directly transferred to the bioreactor. Cell growth, enzymatic activity, alcohol concentration (measured using an alcohol meter - Alcoholometer (Hydrometer) Gay Lussac and Cartier 0–100 %) and cell viability were measured for both strategies. The relative laccase production was determined according to Eq. 1, while the enzymatic activity in the control corresponds to the laccase activity detected on day 7 of strategy I.

2.2.4. Monitoring of cell growth and viability

Cell growth was determined by measuring the samples' optical density at 600 nm (OD_{600}) using a UV-Vis spectrophotometer (Shimadzu, UV-1280). In addition, cell viability was determined using the trypan blue dye exclusion method. Yeast cells were exposed to trypan blue dye (4 mg/mL) and subsequently counted using a Neubauer hemocytometer. Cell viability (%) was assessed through direct colony counting, and cell density was calculated based on colony-forming units (CFU/mL), according to Eq. 2.

$$Cell\ viability(\%) = \frac{number\ of\ viable\ cells}{total\ number\ of\ cells} \times 100 \quad (2)$$

2.3. Laccase purification and concentration by precipitation and ultrafiltration

Laccase purification and concentration from the fermentation broth were achieved by organic solvent precipitation and ultrafiltration. The fermentation broth was incubated at −20 °C with cold methanol (2:1 v/v solvent: sample) for 2 h and cold acetone (2:1 and 4:1 v/v solvent: sample) for 1, 2 or 4 h, under continuous orbital stirring. After incubation, the sample was centrifuged at 9480 × g for 15 min, and the resulting precipitate was dissolved in 400 µL of phosphate buffer (50 mM, pH 6.0). Ultrafiltration was investigated using VivaSpin® centrifugal concentrators (Sartorius, Gottingen, Germany, MWCO 20 kDa). Ultracentrifugation sample was used to perform protein precipitation with cold acetone (2:1 v/v solvent: sample) for 2 h, as described before.

To evaluate the purity of laccase during the purification process, the specific activity was determined. Higher specific activity indicates a higher proportion of active enzyme in relation to other proteins present in the fermentation broth [28,29]. Specific enzymatic activity was calculated using Eq. 3, and the purification factor (fold) was determined resorting to Eq. 4.

$$Specific\ enzymatic\ activity(U/g) = \frac{Enzymatic\ activity(U/L)}{Total\ protein\ concentration(g/L)} \quad (3)$$

$$Purification\ factor\ (fold) = \frac{Final\ specific\ enzymatic\ activity(U/g)}{Initial\ specific\ enzymatic\ activity(U/g)} \quad (4)$$

2.4. Total protein profile and laccase enzymatic activity

2.4.1. Total protein

Total protein concentration was determined using the BCA (Bicinchoninic acid assay) protein assay kit (Thermo-Scientific, Waltham, USA) according to the manufacturer's instructions and using bovine serum albumin (BSA) as the protein standard (*cf.* Fig. S3 in the SM).

2.4.2. Polyacrylamide gel electrophoresis

The protein profile on the fermentation broth, *K. phaffii* lysates and laccase concentrated samples, was evaluated through reducing sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE). SDS-PAGE gels were stained with BlueSafe from NZYtech (Lisbon, Portugal). Semi-native PAGE was also applied to evaluate laccase enzymatic activity using ABTS as the staining reagent. For additional details on the experimental protocols for both techniques, please see the SM.

2.4.3. Laccase activity

To monitor the laccase activity, 300 µL of each sample was added to 700 µL of 1.7 mM ABTS dissolved in 150 mM citrate phosphate buffer pH 4.5. The increase in absorbance at 420 nm was measured using a UV-Vis spectrophotometer (Shimadzu, UV-1280) under kinetic mode. The enzymatic activity was estimated using Eq. 5:

$$Enzymatic\ \ activity\left(\frac{U}{L}\right) = \frac{abs/\min \times f_{dil} \times 10^6}{\varepsilon} \quad (5)$$

where ε is ABTS molar extinction coefficient (36000 L/(mol.cm) at 420 nm), abs/min is the increase in absorbance per minute, f_{dil} is the sample dilution factor, and 10^6 is the conversion factor from M to µM. One unit (U) of laccase activity is equivalent to the quantity of enzyme required to oxidize one µmol of substrate (ABTS) per minute. Each determination corresponds to the average of triplicates.

The Michaelis-Menten kinetic constants, K_M and V_{max}, were determined for the recombinant produced laccase (recovered and purified by Vivaspin® and acetone precipitation, diluted 1:10 v/v sample:water) as well as for standard laccase (0.67 mg/mL). ABTS was used as the substrate in concentrations ranging from 12 µM to 450 µM, prepared using citrate phosphate buffer (150 mM, pH 4.5). Each kinetic assay used 50 µL of laccase sample mixed with 950 µL of buffer with different ABTS concentrations at room temperature. All kinetic assays were conducted in triplicate. To determine the kinetic parameters (K_M and V_{max}), Curve Expert V1.3 software was used to fit the experimental data (Fig. S11 in the SM) to the Michaelis–Menten equation (Eq. 6):

$$v = \frac{V_{max} \times [S]}{K_M + [S]} \quad (6)$$

where v is the rate of the reaction, K_M is the Michaelis constant, V_{max} is the maximum reaction rate and [S] is the ABTS concentration.

2.5. Polydopamine coating of cellulose paper

The polymerization of dopamine for the coating of filter paper with PDA was performed using three different methods: without enzyme (as control), with commercial laccase, and with produced laccase

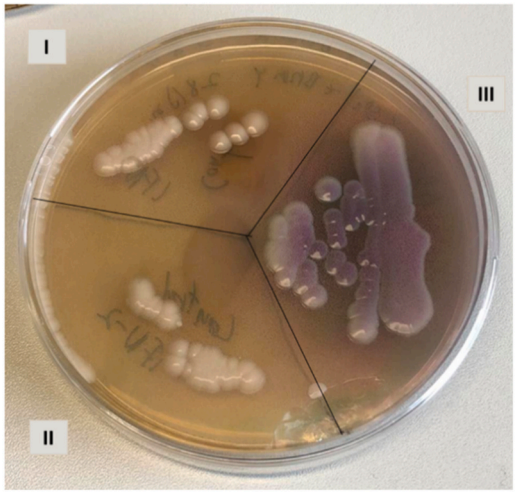

Fig. 1. Growth of the parental *K. phaffii* X33 strain (I and II) and the recombinant strain transformed with the plasmid containing the lcc2 gene (III) in YPD-agar plates supplemented with 0.2 mM ABTS.

(recovered and purified by Vivaspin® and acetone precipitation). Enzymatic polymerization reactions were carried out by mixing 5 mL of dopamine solution (2 mg/mL in phosphate buffer (50 mM, pH 7.0)) with 1 mL of each laccase solution (previously prepared to achieve an enzymatic activity of approximately 130 U/L), and 1.5 mL of distilled water. For the autooxidation reaction without enzyme, the volume of laccase was replaced by distilled water.

All dopamine polymerization reactions for the PDA coating were carried out in triplicates by immersing filter papers in the previous mixture reactions at room temperature under continuous magnetic agitation. After 18 h (overnight), the coated filter papers were gently immersed in water to remove excess PDA precipitates and then dried at room temperature.

The coated samples were analyzed by UV-Vis diffuse reflectance spectroscopy (UV-Vis DRS) and scanning electron microscope (SEM) to assess the polymerization efficiency and morphology of the PDA-coated surfaces. UV-Vis DRS spectra of uncoated and coated filter paper were recorded using a JASCO V-780 equipped with a JASCO ISN-901i integrating sphere (scanning speed of 100 nm/min), in the interval ranging between 200 and 800 nm. The Kubelka-Munk light absorption coefficient (k/s) was used to determine the quantity and type of light-absorbing materials at various wavelengths. SEM analysis resorted to Hitachi SU-70 microscope operating at 15 kV. Prior to analysis, all samples were coated with a carbon thin film.

3. Results and discussion

3.1. Development of the recombinant K. phaffii strain expressing laccase

A recombinant plasmid containing the lcc2 gene from *T. versicolor* was used to express a recombinant laccase by *K. phaffii* X33 using the native secretion signal (*cf.* the SM) and under the control of the AOX promoter. Initially, to confirm the successful transformation of *K. phaffii* X33 with the target plasmid, while qualitatively determining the presence of the target enzyme, both the recombinant and parental strain (control) *K. phaffii* X33 strains were grown in agar plates enriched with the laccase substrate ABTS. As expected, the parental *K. phaffii* X33 strain had a yellowish color (Fig. 1I and II), while a purple halo was observed in the recombinant *K. phaffii* X33 strain (Fig. 1III). While the usually green color is characteristic of the first oxidation state of ABTS ($ABTS^{1+}$), the purple color is characteristic of the second oxidation state of ABTS ($ABTS^{2+}$) [30]. The purple color in Fig. 1III indicates the presence of laccase production by the recombinant *K. phaffii* X33 strain. Overall, these results confirm the successful transformation of *K. phaffii* X33 with the target plasmid and that the recombinant laccase was expressed in a bioactive form since it was able to oxidize the ABTS substrate. Following these results, working cell banks were created with the isolated recombinant strain, and laccase expression was investigated first in shake-flask and then in bioreactor.

3.2. Laccase production by K. phaffii X33 in shake-flask

The standard cultivation conditions were selected as a control to evaluate the performance of the recombinant laccase production by *K. phaffii* X33. These conditions comprise inoculum preparation in the BMGY medium, followed by cultivation in BMMY medium supplemented with 0.1 mM $CuSO_4$ and daily addition of 1 % (v/v) methanol at 30 °C [26,27]. During production time, cell viability was evaluated and, after 13 days, it had 96.1 ± 0.5 % of viable cells (Fig. S4 in the SM for details). After the cultivation process, cells were separated from the broth by centrifugation and cell lysis was accomplished using a mechanical method involving glass beads. The broth and the corresponding lysate were analyzed by SDS-PAGE and semi-native PAGE to evaluate if the target protein was intracellularly produced or secreted to the culture broth. Fig. 2 shows the total protein profile evaluated by SDS-PAGE (Fig. 2A) and the enzymatic activity pattern assessed by semi-native PAGE (Fig. 2B) of the fermented broth and the cell lysate.

The protein profile of the lysate sample (L) obtained by SDS-PAGE (Fig. 2A) shows a wide range of proteins with different molecular weights and in high quantities. On the other hand, fewer bands and less intense are seen in the fermentation broth (B), including a band close to the molecular weight of the laccase standard (circa 63 kDa), indicating

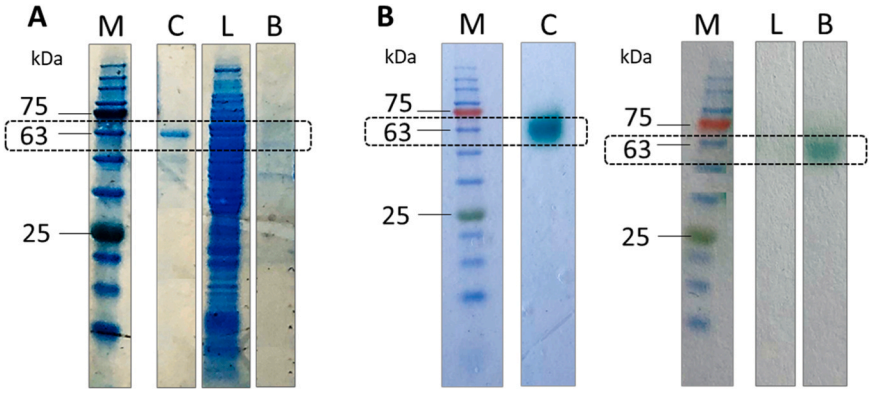

Fig. 2. SDS-PAGE (A) and semi-native PAGE (B) analysis of the fermentation broth and cell lysates obtained from recombinant *K. phaffii* X33 cultivated in standard conditions in orbital shaker. SDS-PAGE and semi-native PAGE gels were stained with BlueSafe and ABTS, respectively. Samples: M – Molecular weight marker; C – Commercial laccase standard; L – Lysate; B – Fermentation broth.

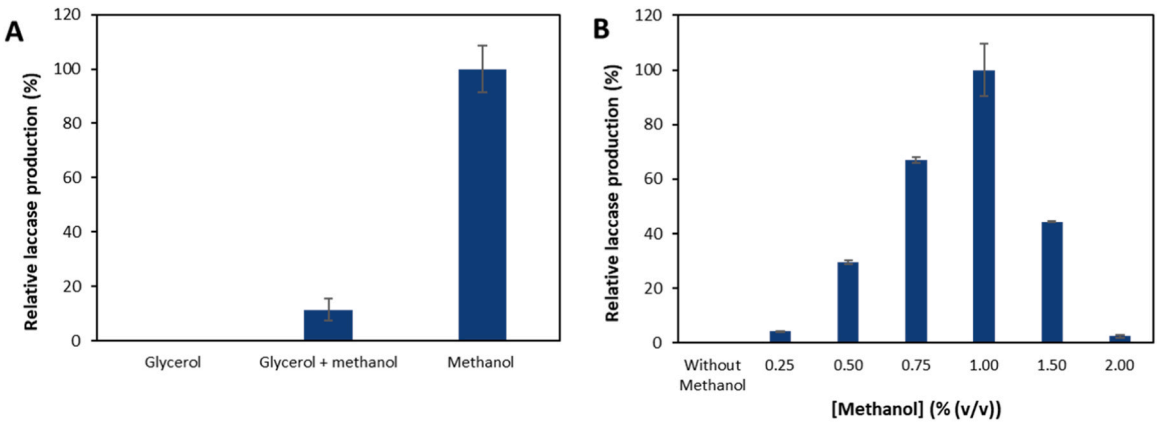

Fig. 3. Effect of the type and concentration of carbon source on relative laccase production levels, relative (%) to standard conditions: daily feeding of 1 % (v/v) methanol, supplementation of 0.1 mM $CuSO_4$ and 30 °C. A) Effect of carbon source: glycerol 1 % (v/v), methanol 1 % (v/v) (daily feeding), and glycerol 1 % (v/v) + daily feeding of methanol 1 % (v/v). B) Effect of the methanol daily feeding (0–2.00 % (v/v)) in cultures solely grown in methanol. Laccase activity was determined at the end of each experiment.

that the recombinant laccase was secreted to fermentation broth by recombinant *K. phaffii* X33 cells. This result was then confirmed by semi-native PAGE (Fig. 2B), where a green band near 60 kDa was identified. This band corresponds to the expected molecular weight of the laccase, and the green color indicates the oxidation of ABTS by laccase. This proves that laccase was successfully secreted into the fermented broth using its native secretion signal, and, most importantly, it is biologically active. Moreover, no enzymatic activity was detected in the lysate (L), further confirming that the active enzyme is being secreted to the culture medium. However, if some laccase is intracellular, it is biologically inactive, probably due to an unfolding state and/or without enzymatic activity. Overall, it was possible to target laccase toward the *K. phaffii* X33 secretory pathway with its native secretion signal. By rendering unnecessary a cell lysis step and due to the low amount of secreted endogenous proteins by the host, this approach contributes to reducing the number of required purification steps and, consequently, in laccase production costs.

3.2.1. *Improvement of laccase production*

After confirming that laccase was successfully secreted to the broth, several parameters were evaluated for enhancing laccase production levels. It is known that the medium composition and temperature are crucial factors affecting cell growth and heterologous protein expression

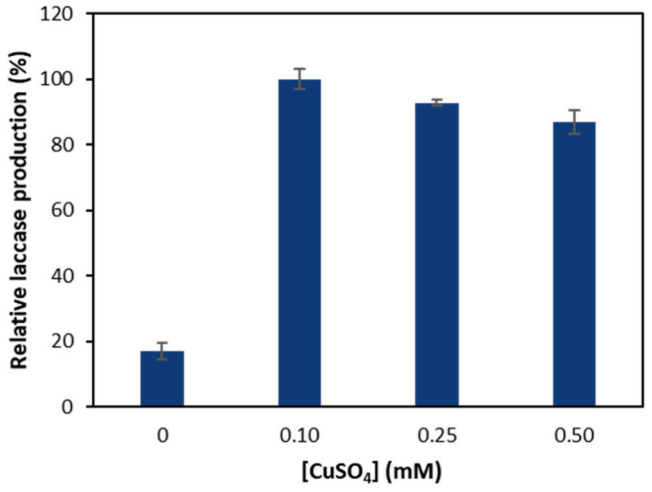

Fig. 4. Effect of $CuSO_4$ on relative laccase production (%). Control fermentation (daily feeding of 1 % (v/v) methanol, supplementation of 0.1 mM $CuSO_4$ and 30°C) was considered 100 %.

[31]. The *K. phaffii* bioprocess driven by the methanol-inducible AOX promoter can use both glycerol and methanol as carbon and energy sources; however, this strategy significantly impacts recombinant protein production levels [32,33]. Although mixed-feeding strategies using glycerol and methanol provide an opportunity to increase cell growth and protein expression, conflicting results have been reported, and such approaches can either increase [34] or decrease [35] the levels of recombinant proteins in AOX-driven *K. phaffii* X33 processes. To better understand the impact of glycerol and methanol on laccase production, experiments were performed evaluating solely glycerol as carbon source (glycerol 1 % (v/v)), mixed-feeding strategies with glycerol-methanol (glycerol 1 % (v/v) + daily feeding of methanol 1 % (v/v)) and solely methanol (daily feeding of methanol 1 % (v/v)). The obtained results are depicted in Fig. 3 A (Table S1 in the SM for details) and Fig. S4A in the SM for relative laccase production levels and cell growth, respectively. Fig. 3A depicts the effect of glycerol addition on the relative laccase production by *K. phaffii* X33 relative to control cultivation conditions (daily feeding of 1 %(v/v) methanol, initial supplementation of 0.1 mM $CuSO_4$ and 30 °C). In comparison with cultures solely grown in glycerol or methanol, the mixed-feeding strategy promoted an enhanced growth of the recombinant *K. phaffii* X33 strain. This trend was expected due to the higher access to additional carbon and energy sources. As shown in Fig. 3A, no enzymatic activity was observed in the culture medium supplemented solely with glycerol. Without methanol, the promotor AOX remain inactive, and recombinant protein production does not occur [36]. The highest laccase production levels were observed when methanol was applied as the sole carbon source, while promoter depression was partially observed in mixed-feeding strategies with relative laccase production levels close to 11 %. Thus, methanol was used as carbon source during the induction phase in the following experiments, which focused on determining the optimal methanol concentration for daily feeding.

Fig. 3B (Table S2 in the SM for details) shows the effect of the daily addition of different methanol concentrations (0 and 2.00 %(v/v)) on relative laccase production levels, while the effect on cell growth is displayed in Fig. S5 (SM). In general, the absence of methanol in daily feeding leads to poor growth of the recombinant yeast strain, while for methanol concentrations of 2.00 % (v/v), a decrease in cell growth was observed. This trend is in agreement with the literature, demonstrating that an excess of methanol can affect yeast metabolism and compromise the cell integrity [37]. In what concerns laccase production levels (Fig. 3B), the absence of methanol in daily feeding impairs laccase production. As the methanol concentrations increased from 0.25 % to 1.00 % (v/v), the relative laccase production levels increased from 4.24 % to 100 %, respectively, representing a 24-fold increase.

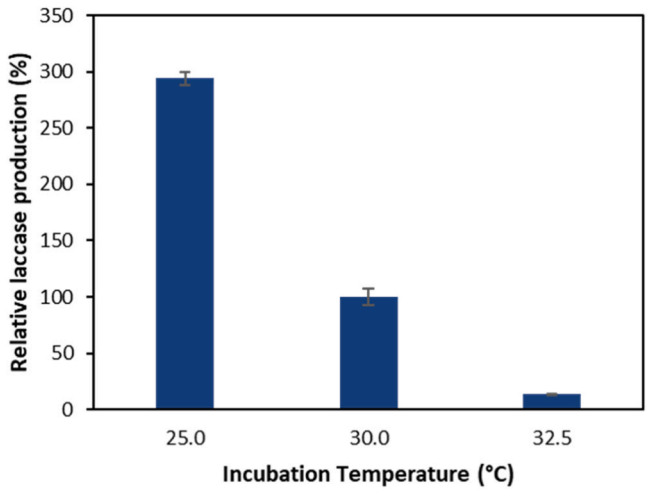

Fig. 5. Effect of incubation temperature on relative laccase production (%). Control fermentation (daily feeding of 1 % (v/v) methanol, supplementation of 0.1 mM $CuSO_4$ and 30 °C) was considered 100 %.

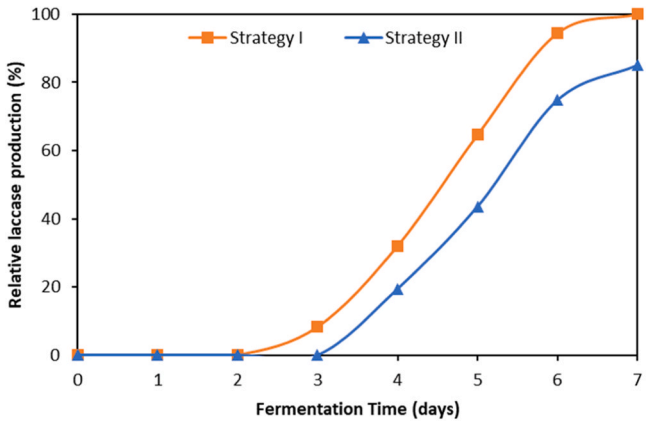

Fig. 6. Relative laccase activity (%) in relation to the highest activity (enzymatic activity detected in strategy I, day 7) by fermentation time (days) in the bioreactor in two different strategies: Strategy I) Start methanol adaptation in a shaker cultivation in bioreactor; Strategy II) Inoculate the bioreactor directly with inoculum growth in BMGY medium.

However, the addition of higher methanol concentrations (above 1.00 % (v/v)) exerts a negative effect on laccase production, probably due to methanol toxic effect on *K. phaffii* X33, as evidenced by the compromised cell growth (Fig. S5B in the SM). Tülek et al. [25] worked on process optimization for laccase production from *Madurella mycetomatis* in *K. phaffii* X33 and studied the effects of methanol concentration on laccase production (0.1–3 %). Similar to our results, laccase production increased with methanol concentration up to 1 % and decreased above this level, with no laccase detection with 3 % methanol in the medium. From these results, the daily addition of 1 % (v/v) methanol during fermentation was adopted throughout this work.

As multicopper oxidases, laccase relies on copper as an essential cofactor for its catalytic activity at the active site [2]. Therefore, an adequate copper content in the culture medium is vital for proper folding and activity of laccase, justifying further investigations of the optimal copper concentration in the culture medium. The effect of $CuSO_4$ concentrations, ranging from 0 to 0.5 mM, on recombinant *K. phaffii* X33 cell growth (Fig. S6 in the SM) and laccase relative activity (Fig. 4 and Table S3 in the SM) was investigated. The different concentrations of $CuSO_4$ had a negligible effect on cell growth, despite having a pronounced influence on laccase production levels. Although the YNB present in the BMMY medium already contains 0.25 μM $CuSO_4$, laccase was produced at low levels, requiring additional supplementation. In the culture medium supplemented with 0.10 mM of $CuSO_4$, the relative laccase production was 6 times higher than in the culture without supplementation, decreasing, however, for higher concentrations. The excess of copper can inhibit yeast metabolism, thereby reducing enzyme production, as reported by Balakumaran et al. [27], where a concentration above 0.2 mM was inhibitory for laccase production. A similar concentration of 0.1–0.2 mM was found to be best for laccase production from *M. mycetomatis* in *K. phaffii* [25], while the enzyme activity decreased above 0.4 mM. The results obtained in our work are in agreement with previously published data, as the best result for laccase production is 0.1 mM of $CuSO_4$ supplementation. In contrast, the work by Li et al. [38] showed that 0.5 mM of copper was the best concentration for laccase from *T. versicolor* in *K. phaffii* X33 production. According to our results, supplementation of 0.1 mM of $CuSO_4$ was selected for further experiments.

Even though the ideal temperature for *K. phaffii* X33 growth and heterologous protein expression can vary, it is usually below 30 °C [21, 39,40]. Accordingly, we evaluated the effect of three different incubation temperatures: 25.0, 30.0 and 32.5 °C on recombinant *K. phaffii* X33 growth (Fig. S7 in the SM) and laccase relative production levels (Fig. 5 and Table S4 (SM)). While temperature changes did not affect yeast growth, a clear improvement in laccase production at 25.0°C (3-fold increase) was observed compared to 30 and 32.5 °C. At 32.5 °C, however, the relative laccase production drops from 100 % to 13 % (compared to 30.0 °C). These results are confirmed by SDS-PAGE gel (Fig. S8 in the SM), where a higher protein content can be observed, including a band close to 60 kDa (laccase molecular weight) in the 25 °C sample, compared with the other temperatures evaluated. These results agree with those previously reported by Tülek et al. [25], who investigated the effects of temperature on laccase production by *M. mycetomatis* in *K. phaffii* X33 and reached similar conclusions. Other works suggest that the improvement in protein production at temperatures below 30 °C is due to reduced protease activity, which reduces folding stress, improves cell viability [20,37], and increases oxygen solubility in the medium [41]. Temperatures above 30 °C, on the other hand, create an inhospitable environment for laccase production, as protein expression in the yeast is suppressed above 32 °C [39]. Following the optimization of several parameters, we found that laccase production levels are maximized using methanol as the sole carbon source, a methanol daily feeding of 1 % (v/v), supplementation with $CuSO_4$ 0.1 mM, and a temperature of 25 °C. These correspond to the operational parameters studied during the stirred tank bioreactor production addressed in the next section.

3.3. Production of laccase in bioreactor

Changing the production from orbital shaker to bioreactor is a complex process that requires a prior systematic optimization of various parameters, such as methanol addition and temperature. The laccase production in bioreactor was performed in a 6 L stirred tank bioreactor with a working volume of 4 L, using the conditions previously defined in shake-flash cultivations: 1 % (v/v) of methanol daily feeding, supplementation with 0.1 mM $CuSO_4$ and a temperature of 25 °C, as illustrated in Fig. S2 in the SM. Two cultivation strategies were applied: (I) After inoculum growth in BMGY medium, the yeast was adapted to methanol (present in BMMY medium) in shake-flask conditions for 5 days, and the fermentation mixture then transferred to the bioreactor; and (II) Inoculum grown in BMGY medium and directly transferred to the bioreactor.

The results for both approaches are presented in Fig. 6 and Fig. S9 (SM). In strategy I, where the yeast was adapted to the methanol before inoculation in the bioreactor, the enzyme was detected in the fermented broth one day earlier than in strategy II. At the end of the 7-day cultivation period, the levels of relative laccase production were higher in strategy I than in strategy II. The recombinant *K. phaffii* X33 growth

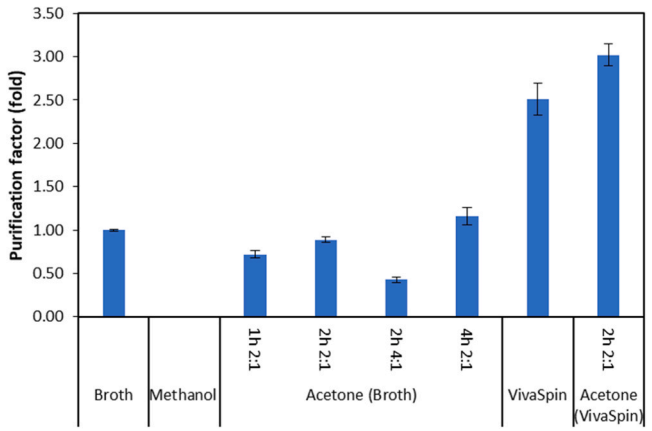

Fig. 7. Purification factor of samples after protein precipitation with methanol (using the crude supernatant as initial sample) and acetone (using the broth and Vivaspin® sample as initial samples). Precipitation with acetone was studied with time (1, 2 and 4 h) and concentration variation (2:1 and 4:1 v/v solvent: sample).

profile was similar in both strategies, with OD_{600} values reaching approximately 38.8 AU at the end of the experiments. In addition, the cell viability in the bioreactor after 7 days was 98.4 ± 1.0 % (Fig. S10 in the SM for details).

The addition of methanol increases the oxygen demand in *K. phaffii* [37]; this effect was detected in both strategies (Fig. S11A in the SM). The DO rate decreases abruptly when methanol was added, and it takes several hours for the rates to normalize to 100 %. For both strategies, the recovery time of DO levels in the medium decreased after each methanol feeding. However, the recovery was slightly faster with strategy I. This difference in the methanol metabolism of *K. phaffii* X33 could be one of the reasons for the higher laccase production when the yeast was previously adapted to a methanol-containing medium (strategy I). The presence of methanol in the medium was monitored each day before methanol feeding using an alcohol meter. No methanol was detected in the fermented medium, indicating that it is all consumed between the daily methanol feedings.

The pH value (Fig. S11B in the SM) remained stable throughout the cultivation in both strategies, with a value of 6.09 ± 0.29. This constant pH during cultivation is advantageous for the process, as it eliminates the need to add a buffer to maintain proper pH levels. Considering these results, adapting the yeast to the culture medium could be an asset to improve the fermentation process. This adaptation leads to faster and higher production compared to cultivation inoculated with an inoculum without prior adaptation to the culture medium and methanol. Moreover, our results show that the change of scale from shake-flasks to 6 L bioreactors (working volume 4 L) was successfully achieved without imparting cell viability.

3.4. Laccase recovery from fermentation broth

Laccase was extracted from the fermentated broth by precipitation with either methanol or acetone. The purification factors of the laccase obtained using both solvents are shown in Fig. 7. The values for the enzymatic activity, the protein concentration, the specific enzymatic activity and the purification factor are listed in Table S5 in the SM. While methanol precipitation was ineffective, laccase was extracted with detectable activity using acetone precipitation. So, the use of acetone for laccase precipitation was evaluated at three incubation times (1, 2 and 4 h) and two concentrations (2:1 and 4:1 v/v solvent: sample). An initial SDS-PAGE analysis (Fig. S12 in the SM) shows that acetone precipitation allows the recovery of the target laccase in the precipitate, but also some low molecular weight protein contaminants. The purification factors shown in Fig. 7 indicate that the purification factor increases with increasing incubation time. In particular, a purification factor of 1.16 was achieved after 4 h. The comparison of different acetone concentrations reveals that a higher acetone concentration negatively influences the laccase purification factor. On the other hand, a higher acetone concentration leads to a higher total protein concentration in the sample and to a lower specific activity. This indicates that a higher acetone concentration leads to the precipitation of other proteins, which has a negative effect on the laccase purity. Sondhi et al. [42] produced laccase from *Bacillus* sp. MSK-0 and purified it by precipitation with acetone at 70 % (v/v), achieving a 1.44-fold purification factor. Younes and Sayadi [43] produced laccase from *Scytalidium thermophilum* and employed multiple purification techniques, starting with acetone precipitation (2:1 v/v, 30 min), resulting in a purification factor of 1.1-fold. Sadhasivam et al. [44] produced laccase from *Trichoderma harzianum* and purified it by using as a first step acetone precipitation (66 % (v/v) for 6 h at −20 °C), yielding a purification factor of 1.51-fold. Therefore, our laccase purification factor of 1.15 obtained by resorting to acetone precipitation closely matches the values previously reported in the literature.

Ultrafiltration (Vivaspin®, MWCO 20 kDa) was also applied to concentrate the enzyme from the fermented broth in order to improve the purification step. By removing some low-molecular weight contaminants, an increased purification factor of 2.51 was obtained. When acetone precipitation follows the ultrafiltration step, the purification factor increases to 3.02. In contrast, the direct precipitation from the fermented broth (under the same conditions: incubation time and solvent concentrations (2 h and 2:1 v/v solvent: sample) showed a significant difference (purification factor of 0.89). This result indicates that the direct acetone precipitation leads to the precipitation of many low molecular weight proteins from the broth sample. Thus, removing small proteins in the first step by ultrafiltration, followed by induced precipitation with acetone, allowed high laccase purification values.

An effective method to concentrate and purify laccase was achieved by combining an ultrafiltration step with acetone precipitation, achieving a purification factor of 3.02. Using the enzyme recovered by

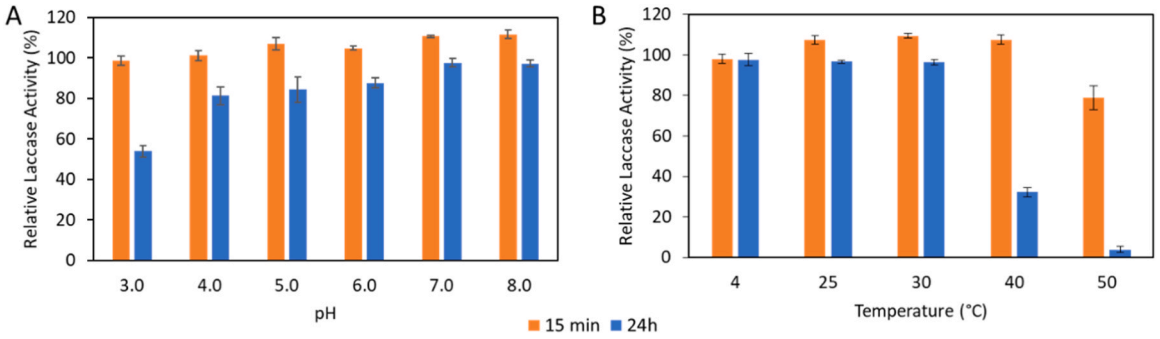

Fig. 8. Effect of pH (A) and temperature (B) on the laccase activity in two incubation times: 15 min (blue) and 24 h (orange). The initial enzymatic activity of the laccase solution in water was considered the control (100 %).

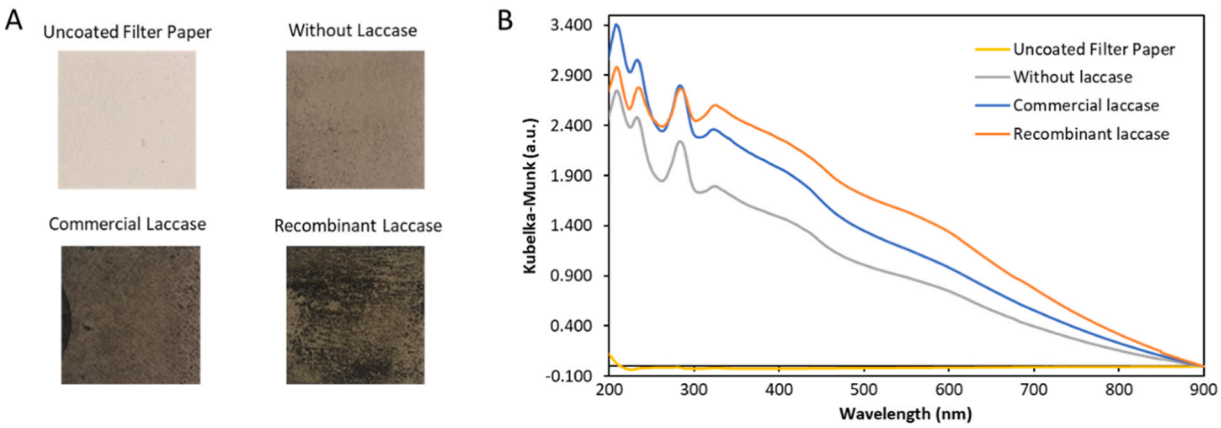

Fig. 9. PDA polymerization and coating of filter paper using the recombinant laccase, commercial laccase, and the auto-oxidation (without laccase) process. A blank consisting of uncoated filter paper is included for comparison purposes. A) Photographs; B) UV-Vis DRS spectra of all samples obtained from the average of three replicates.

this methodology, the effect of pH and temperature on the activity of the recombinant enzyme and their application for the PDA coating of filter paper was then addressed.

3.5. Effect of temperature and pH on laccase activity

The effect of the pH and temperature on the enzymatic activity of the enzyme produced and purified in this work using the optimized protocols was evaluated, being the results shown in Fig. 8 A and B, respectively.

The pH stability of the enzyme was analyzed between pH values of 3.0 and 8.0 (Fig. 8A). Except at pH 3.0, the relative laccase activity was above 100 at all pH values after 15 min. These results indicate that the laccase presents robust stability over a wide pH range. However, the enzyme incubation after 24 h at pH 3.0 decreased enzyme activity by about 30 %, which is the greatest loss of activity within the studied pH range. At pH values of 4.0–8.0, there was a slight loss of activity ranging from 6.8 % (at pH 4.0 and 6.0) to 13.4 % (at pH 5.0), showing good enzyme stability in this pH range. Among all pH values, the highest activity was obtained at pH 7.0 and 8.0. It is important to note that the optimal pH depends on the source of the genetic information. Laccases from *T. versicolor* typically exhibit optimal activity at acidic pH levels [45,46]. For example, laccases from *T. versicolor* ATCC20869 present the optimal pH of 4.0 [47]. Similarly, Li et al. [48] produced several laccase isozymes from *T. versicolor* in *K. phaffii,* and the highest enzymatic activity achieved was between pH 3.0 and 3.5, with a residual activity of more than 80 % after 24 h of incubation. Unlike other laccases from *T. versicolor*, the recombinant laccase produced in the present work has a wide pH stability range (4.0–8.0), keeping as much as 84 % of its activity after an incubation of 24 h at pH 7. This opens up a wide range of potential applications for this enzyme.

The temperature stability of the laccase was investigated between 4 and 50 °C for 24 h (Fig. 8B). Notably, laccase exhibited higher activity after 15 min when incubated at 30 °C. When incubated at 4, 25 and 30 °C, the enzyme retains 99.6, 90.0 and 88.0 % of its activity for 24 h, respectively, which means robust stability. However, an increase in temperature to 50 °C reduced the enzyme's activity, with only 5 % of the enzyme activity remaining after 24 h, probably due to enzyme denaturation. Like pH, the optimal temperature of the enzyme may also depend on the source of the genetic information. For example, laccases produced by *T. versicolor* are more active at 30–40 °C. In a study in which laccase was produced from *T. versicolor* ATCC20869 and two mutant variants, the optimal temperature was 40 °C [47]. Li et al. [48] produced several laccase isoenzymes from *T. versicolor* in *K. phaffii* and determined the optimal temperature. The optimum temperature for all isozymes was between 50 and 60 °C [48]. In the present work, the produced enzyme shows good stability under milder conditions, which is consistent with some previous studies. The thermostability of laccase under milder conditions (25 – 30 °C) is essential for some enzyme applications (e.g., the ideal temperature for PDA synthesis using laccase is 30 °C [5]).

3.6. Kinetic parameters

To determine the kinetic parameters of laccase, different ABTS concentrations were used to measure laccase activity. The experimental data were fitted to the Michaelis-Menten equation, from which K_M and V_{max} were calculated (Fig. S13 in the SM for details). The K_M values for the laccase produced in this work and the commercial laccases were 44.5 and 30.0 μM, respectively. This lower K_M value indicates that our produced laccase has a lower affinity for ABTS compared to the commercial laccase. The V_{max} values for the recombinant laccase produced in this work and the commercial (standard) laccase were 110.9 and 125.20 μM/min, respectively. Overall, the enzyme produced in this work has catalytic activity profiles similar to the investigated commercial laccase, with affinity constants for substrate in the same order of magnitude as other laccases previously reported in the literature [49, 50].

3.7. Cellulose filter paper coating with polydopamine

The laccase-assisted PDA polymerization for coating filter paper was investigated with the laccase produced in this work as well as with commercial laccase, used for comparison purposes. The filter paper was brought into contact overnight with a dopamine solution and laccase at pH 7.0, selected based on previous results for which the enzymatic activity is maximized. As a control, the filter paper was immersed in a dopamine solution without laccase under the same conditions. Fig. 9A shows pictures of the uncoated filter paper (blank) and filter paper coated with PDA. Fig. 9B presents the UV-Vis DRS spectra of the uncoated filter paper and filter paper coated with PDA without laccase, with commercial laccase and the laccase produced in the present work.

The photographs depicted in Fig. 9A demonstrate that the color of filter papers coated with PDA change from white to brown/black, being more pronounced as dopamine polymerization becomes more efficient. The higher absorbance across the investigated wavelengths indicates the successful modification of the material with PDA (Fig. 9B). Three peaks can be observed in the spectrum of the three samples with PDA that were not found in the uncoated filter paper spectrum: 208, 233 and 290 nm, similar to other works in the literature [51,52]. Notably, the PDA produced using the recombinant laccase displayed higher signal intensity, demonstrating the recombinant laccase has a high potential to assist the dopamine polymerization process. This result was probably due to the

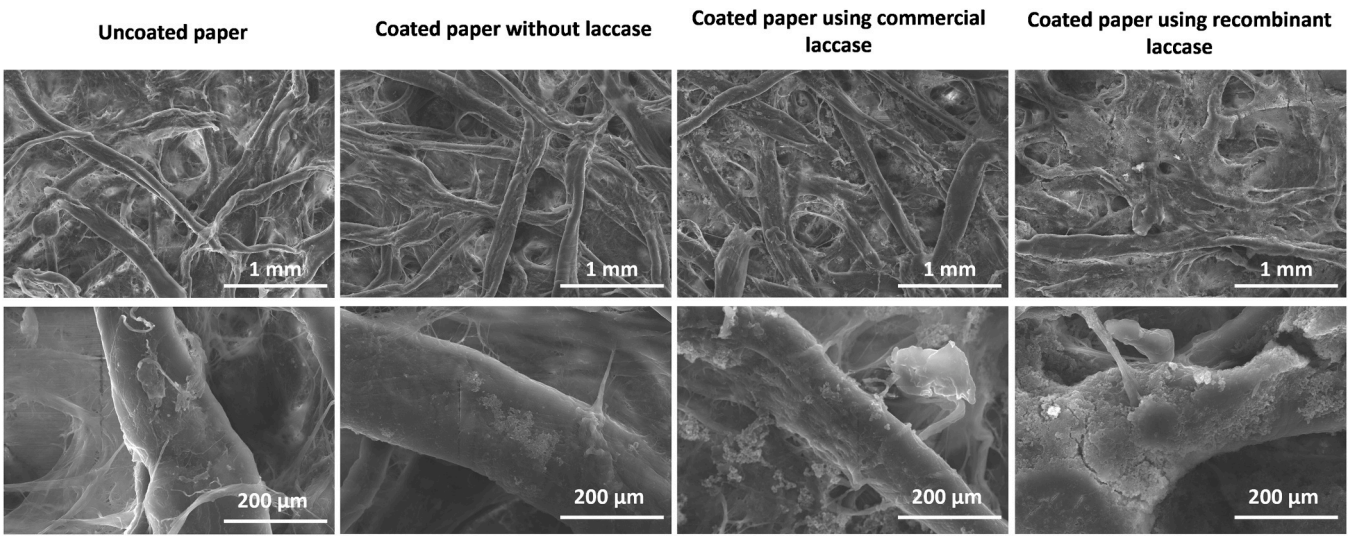

Fig. 10. SEM images of the surface morphologies of the uncoated filter paper (blank), filter paper coated with PDA without laccase, and coated filter paper with PDA (assisted by commercial and recombinant laccase).

use of optimal pH 7.0 for recombinant enzyme in this assay, whereas the optimal pH for the commercial laccase is usually around 5.0 [45,46].

Surface morphologies of the uncoated filter paper, filter paper coated with PDA without laccase, filter paper coated with PDA using the recombinant laccase and with commercial laccase were observed using SEM (Fig. 10). SEM images show a smoothed surface before polydopamine coating, while a rough morphology appearance can be observed after recombinant laccase-assisted coating. In conclusion, UV–VIS DRS and SEM results showed that laccase assists the PDA coating of filter papers, supporting this enzyme potential in polymerization. For the first time we show the enzyme-assisted PDA coating of paper, while using a recombinant laccase produced by *K. phaffii* X33.

4. Conclusion

This study demonstrated the successful expression of *T. versicolor* laccase in *K. phaffii* X33 cells using the AOX promoter. The enzyme was secreted into the fermentation broth, purified with biological activity, and used for coating filter papers with PDA. After optimizing several parameters, laccase production was enhanced with 1 % (v/v) daily methanol feeding, 0.1 mM $CuSO_4$ media supplementation, and cultivation at 25 °C. These laccase production conditions were scalable to stirred tank bioreactor with minimal loss of enzyme activity. A purification protocol combining acetone precipitation and ultrafiltration yielded a purification factor of 3.02. The produced laccase exhibited stability across pH 4.0–8.0 and up to 30 °C. Kinetic constants were determined, showing affinity values for ABTS comparable to previous reports. For the first time, recombinant laccase successfully polymerized dopamine, allowing to efficiently coat filter papers. This work contributes to advances in recombinant laccase production and its application in material coatings.

CRediT authorship contribution statement

Valéria Santos-Ebinuma: Writing – review & editing, Validation, Supervision, Resources, Methodology, Conceptualization. **Ana P. M. Tavares:** Writing – review & editing, Validation, Supervision, Resources, Methodology, Funding acquisition, Conceptualization. **Augusto Q. Pedro:** Writing – review & editing, Validation, Supervision, Methodology, Investigation, Funding acquisition, Conceptualization. **Mara G. Freire:** Writing – review & editing, Conceptualization, Validation, Funding acquisition, Supervision, Resources. **Flávia F. Magalhães:** Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Márcia C. Neves:** Writing – review & editing, Resources, Funding acquisition, Formal analysis.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgements

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.bej.2024.109483.

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