



# Integrated extractive fermentation of laccase using aqueous biphasic systems: From production to functional coatings

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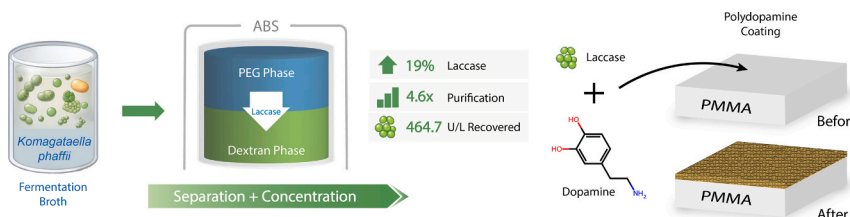
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## HIGHLIGHTS

- Extractive fermentation using ABS enabled integrated laccase production and recovery.
- Polymer-polymer ABS improved laccase production, extraction, and concentration.
- Laccase retains its catalytic efficiency and is recovered in a purified form (4.6 fold).
- Laccase was properly applied for polydopamine coating of poly(methyl methacrylate).

## GRAPHICAL ABSTRACT

Schematic representation of the integrated recovery and purification of laccase from *K. phaffii* fermentation broth using aqueous biphasic systems.



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## ABSTRACT

Extractive fermentation has increasing attention as intensified bioprocessing approaches by integrating enzyme production, recovery, and primary purification into a single step.

This work demonstrates the effective use of aqueous biphasic systems in extractive fermentation, integrating the separation, and concentration of *Trametes versicolor* laccase expressed in *Komagataella phaffii*. After assessing *K. phaffii* growth and laccase activity in various aqueous biphasic phase-forming compounds, two types of aqueous biphasic systems, namely polymer-salt (polyethylene glycol with a molecular weight of 8000 g/mol +  $K_2HPO_4/KH_2PO_4$ ) and polymer-polymer (polyethylene glycol with a molecular weight of 8000 g/mol + dextran with a molecular weight of 40000 g/mol) were selected for laccase separation, and concentration directly from the fermentation broth. The polymer-polymer aqueous biphasic systems (10.0 wt% polyethylene glycol with a molecular weight of 8000 g/mol and 5.0 wt% dextran with a molecular weight of 40000 g/mol) showed the most promising performance, leading to a 19% increase in laccase activity recovered in the dextran-rich phase, a 4.6-fold purification, and recovery of 464.7 U/L, compared to the buffer used as control. The recovered laccase retained its catalytic efficiency, and was successfully applied in the synthesis of polydopamine for functional coating of poly(methyl methacrylate). The coated material was further characterized using scanning electron microscopy, Fourier transform infrared spectroscopy, atomic force microscopy, and optical contact angle

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measurements. This work shows that aqueous biphasic systems can support a sustainable and integrated platform for enzyme separation and concentration and direct application in biocatalytic surface functionalization.

## 1. Introduction

Laccases (EC 3.10.3.2) are multi-copper oxidoreductases, which can catalyse the one-electron oxidation of a wide range of organic and inorganic substrates, including aromatic amines, organic pollutants, lignin-derived compounds and phenolics (Aghaee et al., 2024). They are ubiquitous enzymes naturally found in plants, bacteria and fungi, with white-rot fungi standing out as main laccase producers. Because of their remarkable versatility, laccases have found extensive application in diverse industries, from well-established applications in the paper and pulp industry and wastewater treatments (Sondhi et al., 2023), to emerging applications in food, biosensors, biofuels, and polymer synthesis (Isozaki et al., 2024; Li et al., 2015). Recent studies have emphasized the need for techno-economic evaluation tools to support the development of competitive fungal laccase production processes, highlighting that process design must consider not only enzyme yield but also cost and scalability (Gutierrez-Rangel et al., 2024). Among their various uses, notably, laccases are used as biocatalysts for synthesising polydopamine (PDA), a bioinspired biopolymer known for its unique adhesive properties (Ryu et al., 2018). PDA is valued for its ability to coat and functionalise a wide range of (nano)material surfaces, by a simple solution immersion and *in situ* deposition process, including ceramics, glass, metals, paper, and textiles (Ryu et al., 2018). This adhesive ability makes PDA highly attractive for various applications, such as in enzyme immobilisation, in developing antimicrobial and super-wetting textiles, and in tissue engineering (Popkov et al., 2023; Sarkari et al., 2022).

Despite its relevance, conventional PDA synthesis involves the self-polymerisation of dopamine under alkaline conditions ( $\text{pH} > 8.5$ ) (Ryu et al., 2018). This method is labour-intensive and time-consuming (often requiring at least 24 h of reaction) and is unsuitable for alkali-sensitive materials (Li et al., 2018). To overcome these limitations, laccase-assisted polymerisation has emerged as a more effective, fast and controlled approach for PDA synthesis under mild conditions, while enhancing material coatings' versatility (Li et al., 2018).

To fully leverage the advantages of laccase catalysed PDA, an efficient and sustainable laccase production is essential. Usually, laccase native producers lead to low productivity and high production costs (Preethi et al., 2020; Yang et al., 2017). *Komagataella phaffii* (formerly known as *Pichia pastoris*) has emerged as a promising host for the heterologous laccase expression due to various advantages, such as high expression levels, easy scalability, and the ability to perform crucial post-translational modifications. Additionally, *K. phaffii* efficiently secretes recombinant proteins with minimal endogenous proteins, simplifying the further downstream purification steps (Pan et al., 2022). Despite these advantages, the entire enzyme production process remains challenged by the large fermentation volumes and the complex downstream operations, which involve multiple steps and is time-consuming, significantly increasing the cost of the enzyme (Straathof, 2011). Integrating the upstream and downstream steps through fermentative extraction could address these issues, while reducing costs and time, allowing the design for a more competitive and sustainable enzyme manufacturing platform.

Extractive fermentation is an advanced bioprocessing strategy that integrates traditional fermentation with *in-situ* product extraction, effectively combining upstream and downstream into a single step (Badhwar et al., 2019). This integration allows maintaining the optimal fermentation conditions for long periods, while enhancing product yield and process efficiency and intensification (Badhwar et al., 2019; Combes et al., 2021; Leong et al., 2019). One of the most promising approaches in extractive fermentation involves the use of aqueous biphasic systems

(ABS), which are liquid-liquid extraction systems formed by mixing water and at least two water-soluble components, such as salts and polymers. When these compounds are mixed above specific concentrations, the mixture separates into two coexisting aqueous phases. If ABS are well designed, water could be the most abundant component, and biocompatible ABS components can be selected as phase-forming agents, making them suitable for bioprocesses. ABS have been applied in downstream processes for the recovery and purification of biomolecules, considering their ability to selectively partition the target molecule into one of the phases (Pereira et al., 2020). Moreover, ABS enable the integration of multiple production steps, including fermentation and clarification (Ferreira-faria et al., 2020; Kee et al., 2020; Pereira et al., 2020), and can be tailored to concentrate the target product by adjusting the volume of the ABS phases. Overall, this ABS flexibility can allow the reduction of process steps while boosting the target bioproduct's production, extraction and purification.

ABS-based extractive fermentation has been successfully applied in producing various proteins from different microorganisms, enabling simultaneous production and recovery, and facilitating the recycling of phase-forming compounds. For instance, Santos et al. (2022) demonstrated the use of extractive fermentation with ABS to produce lipase and citric acid by *Yarrowia lipolytica*, achieving high citric acid recovery and lipase production (634.32 U/L) in opposite phases of ABS composed of polyethylene glycol (PEG) 2,000 and salt. Similarly, Kee et al. (2022) used ABS, composed of PEG 2,000 and tri-sodium citrate, to produce and recover keratinase produced by *Kytococcus sedentarius* TWHKC01, achieving a maximum yield of 161.33 U/mL. However, no evidence currently indicates that the extractive fermentation for laccase production and recovery using *K. phaffii* as a host has not yet been reported. The limited number of recent reports directly addressing this specific ABS-based extractive fermentation configuration suggests that the approach remains underexplored, particularly when combined with recombinant laccase production and downstream applications.

The present work addresses, for the first time, the use of ABS for extractive fermentation of *T. versicolor* laccase expressed in *K. phaffii*, integrating the separation and concentration steps. The effect of various ABS phase-forming compounds on *K. phaffii* growth and the produced laccase activity were evaluated to design an optimal extractive fermentation process. Finally, the produced and recovered laccase was applied in the polymerisation of dopamine into PDA for polymethyl methacrylate (PMMA) coating, demonstrating the systems versatility and potential for sustainable bioprocessing.

## 2. Experimental materials and methods

### 2.1. Materials, plasmids and strains

The recombinant plasmid pPICZB, which contains the *T. versicolor* *lcc2* gene (GenBank Accession No. CAA77015.1) and the *K. phaffii* X-33 strain, were acquired from Invitrogen (Carlsbad, USA) and Genscript (Piscataway, New Jersey, USA), respectively (Magalhães et al., 2024) for details on the construction of the recombinant strain. The following reagents were used to prepare the culture media: yeast extract from Biokar (Cedex, France); YNB with ammonium sulphate from Grisp (Porto, Portugal); peptone and agar from Liofilchem (Teramo, Italy); biotin from Fagron; copper sulphate pentahydrate ( $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ ) and glycerol (analytical grade) from Merck (Darmstadt, Germany); zeocin from InvivoGen (California, USA); methanol (HPLC grade) from Fisher Scientific (Massachusetts, USA); and glucose from VWR (Pennsylvania, USA). The culture media composition (Yeast extract peptone dextrose (YPD), the buffered complex glycerol medium (BMGY), and the buffered

complex methanol medium (BMMY)) used for the growth and routine manipulation of *K. phaffii* is presented in the [Supplementary Materials](#).

For ABS preparation, polypropylene glycol (PPG) with a molecular weight of 400 g/mol (PPG 400), dextran with a molecular weight of 450,000–550,000 g/mol (dextran 500), polyethylene glycol (PEG) with a molecular weight of 2,000 and 8,000 g/mol (PEG 2000 and PEG 8000, respectively), and sodium sulphate ( $\text{Na}_2\text{SO}_4$ , 99 wt% purity) were acquired from Sigma-Aldrich (St. Louis, USA). PEG with a molecular weight of 400 and 1,500 g/mol (PEG 400 and PEG 1500, respectively) and the ionic liquid (IL) cholinium chloride ( $[\text{Ch}]\text{Cl}$ , 98 wt% purity) were purchased from Acros Organics (Antwerpen, Belgium). Furthermore, dextran with a molecular weight of 40,000 g/mol (dextran 40) was acquired from Panreac (Barcelona, Spain). 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic 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 ( $\text{K}_2\text{HPO}_4 \cdot 3\text{H}_2\text{O}$ , 98 wt% purity) and potassium phosphate monobasic ( $\text{KH}_2\text{PO}_4$ , 98 wt% purity) bought from Scharlab (Barcelona, Spain), as well as ammonium sulphate ( $(\text{NH}_4)_2\text{SO}_4$ , 99 wt% purity). NZYColour Protein Marker I was obtained in NZYTech (Lisbon, Portugal), and the tris-glycine pre-casted polyacrylamide gels and Tris-Glycine SDS running buffer were acquired from Invitrogen (Carlsbad, USA). For HPLC analysis, sulfuric acid was purchased from Honeywell Research Chemicals (New Jersey, USA).

## 2.2. *K. phaffii* cultivation and fermentation conditions for laccase production

*K. phaffii* was grown in yeast extract peptone dextrose (YPD) agar plates at 30 °C for 72 h. The yeast colonies were transferred to 250 mL Erlenmeyer flasks that contained 50 mL of BMGY medium. The flasks were then incubated at 30 °C and 250 rpm to prepare the *K. phaffii* inoculum. Cell growth was assessed after 24 h by measuring the optical density at 600 nm ( $\text{OD}_{600}$ ). After that, a specific volume set to achieve initial  $\text{OD}_{600}$  values of 1.0 in the fermentation was aliquoted and centrifuged for 5 min at 25 °C at 1,500 rpm. The recovered cell pellets were immediately resuspended in the BMMY induction medium and used as the inoculum for fermentation. Standard fermentations were performed in 250 mL Erlenmeyer flasks containing 50 mL of BMMY, incubated on an orbital shaker at 250 rpm for 13 days at 25 °C. BMMY was supplemented with 0.1 mM  $\text{CuSO}_4$ . For induction, 1.00% (v/v) methanol was added daily. The temperature, culture volume, and methanol feeding strategy were selected based on the established fermentation conditions for heterologous expression in *K. phaffii* (Magalhães et al., 2024).

## 2.3. Design of the extractive fermentation process for laccase production

### 2.3.1. Effect of ABS phase-forming compounds on yeast growth

To design the extractive fermentation process, it is crucial to evaluate the effects of ABS phase-forming compounds on the *K. phaffii* growth. Cell growth was performed in six-well plates containing 5 g of medium composed of 90 wt% of BMMY and 10 wt% of each ABS compound. Several compounds were tested, namely salts ( $\text{Na}_2\text{SO}_4$ ,  $(\text{NH}_4)_2\text{SO}_4$  and  $\text{K}_2\text{HPO}_4/\text{KH}_2\text{PO}_4$ ), one IL ( $[\text{Ch}]\text{Cl}$ ), and different polymers (dextran 40 and 500, PPG 400 and PEG 400, 1500, 2000 and 8000). A fixed concentration of 10 wt% was set for each compound to evaluate its potential effects on microbial growth, as this concentration is usually sufficient to promote the formation of ABS. These compounds were selected according to previous reports related to protein purification in ABS (Dos Santos et al., 2018; Ferreira et al., 2021; Silvério et al., 2013). Plates were incubated at 220 rpm for 3 days at 30 °C. Every 24 h, ca. 0.79 wt% (1.00% (v/v)) methanol was added, and the  $\text{OD}_{600}$  was monitored. A control was performed with a medium composed of 90.0 wt% BMMY and 10.0 wt% water. Each compound was tested in triplicate.

### 2.3.2. Effect of ABS phase-forming compounds on laccase activity

The effect of ABS phase-forming compounds, namely PEG 8000,  $\text{K}_2\text{HPO}_4/\text{KH}_2\text{PO}_4$  and dextran 40 was also investigated on the laccase enzymatic activity. These compounds were selected based on the results achieved in the yeast growth assays. To evaluate the effect of each constituent, solutions with a final weight of 0.5 g were prepared by mixing 10 or 15 wt% of each compound with 90 or 85 wt% of the fermentation broth containing laccase obtained from the standard fermentation conditions (as described in [Section 2.2](#)) over time. The following constituent concentrations were investigated: 15.0 wt% PEG 8000 and 15.0 wt%  $\text{K}_2\text{HPO}_4/\text{KH}_2\text{PO}_4$  (pH 6.0) for polymer-salt ABS, and 10.0 wt% PEG 8000 and 10.0 wt% dextran 40 for polymer-polymer ABS. These concentrations were chosen considering the ABS binodal curves available in the literature (Chen et al., 2023; de Souza et al., 2014). Enzymatic activity (further details in [section 2.5.3](#)) was then measured immediately ( $t = 0$  min) and after incubation at room temperature for 1, 5 and 72 h. Each compound was tested in triplicate. The relative laccase activity was determined according to Eq. (1):

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

where the enzymatic activity in the control corresponds to the enzymatic activity of laccase where the phase-forming compounds (wt% basis) was replaced by water and measured at  $t = 0$  min at room temperature, and enzymatic activity in sample corresponds to the enzymatic activity of laccase in the presence of the ABS phase-forming compounds.

## 2.4. Extractive fermentation for laccase production, recovery and concentration through ABS

For the production of laccase in ABS, PEG 8000,  $\text{K}_2\text{HPO}_4/\text{KH}_2\text{PO}_4$  and dextran 40 were added at different concentrations (wt%) to the BMMY fermentation medium. Extractive fermentations were performed in 250 mL Erlenmeyer containing 50 g of medium. The composition of extractive fermentation media is presented in [Table 1](#).

Control fermentations were performed by replacing the mass fraction of ABS phase-forming compounds with water. Fermentation conditions were carried out as described in [section 2.2](#). Samples were collected along time to monitor cell growth, pH, total protein concentration and enzymatic activity. At the end of the extractive fermentation, the phases separation was promoted by centrifugation at 1,500 rpm for 10 min at 25 °C. Then, samples were collected from each phase to evaluate the partition coefficient of *K. phaffii* cells ( $K_{\text{Cell}}$ ), partition coefficient of laccase ( $K_{\text{Lac}}$ ), partition coefficient of methanol ( $K_{\text{Meth}}$ ), volume ratio ( $V_R$ ), enzymatic activity, total protein quantification, methanol concentration (%), and pH. Each fermentation was carried out in triplicate. The laccase activity was quantified as described in [section 2.5.3](#), cell concentration was determined by  $\text{OD}_{600}$  measurements as described in [section 2.5.1](#), and  $K_{\text{Lac}}$ ,  $K_{\text{Meth}}$  and  $K_{\text{Cell}}$  were calculated according to Eq. (2):

$$K = \frac{C_T}{C_B} \quad (2)$$

**Table 1**  
Salt-polymer and polymer-polymer aqueous biphasic system composition.

| Salt-polymer ABS |                                                                |            | Polymer-polymer ABS |                  |            |
|------------------|----------------------------------------------------------------|------------|---------------------|------------------|------------|
| PEG 8000 (wt %)  | $\text{K}_2\text{HPO}_4/\text{KH}_2\text{PO}_4$ (pH 6.0) (wt%) | BMMY (wt%) | PEG 8000 (wt %)     | dextran 40 (wt%) | BMMY (wt%) |
| 10.0             | 15.0                                                           | 75.0       | 5.0                 | 10.0             | 85.0       |
| 15.0             | 10.0                                                           | 75.0       | 7.5                 | 7.5              | 85.0       |
| 15.0             | 7.0                                                            | 75.0       | 10.0                | 5.0              | 85.0       |

where  $C_T$  and  $C_B$  are laccase enzymatic activity (U/L) for  $K_{Lac}$ , methanol concentration (%) for ( $K_{Meth}$ ), and the  $OD_{600}$  value for  $K_{Cell}$  in the top and bottom phases, respectively. The bottom phase corresponds to the salt or dextran-rich phase, whereas the top phase corresponds to the PEG-rich phase.

The  $V_R$  was determined according to Eq. (3):

$$V_R = \frac{V_T}{V_B} \quad (3)$$

where  $V_R$  is the volume ratio between the top ( $V_T$ ) phase and bottom phase ( $V_B$ ).

## 2.5. Extractive fermentation monitoring

### 2.5.1. Microbial growth

To reduce possible interferences with ABS phase-forming compounds, fermentation broth was centrifuged (1,500 rpm, 10 min), and the pellet (*K. phaffii* cells) was resuspended in a volume of water equal to the initial sample. Then, cell growth was determined by measuring the samples'  $OD_{600}$  using a UV-Vis spectrophotometer (Shimadzu, UV-1280). Each measurement was performed in triplicate.

### 2.5.2. Total protein quantification

Total protein concentration was determined through UV-Vis spectrophotometer (Shimadzu, UV-1280) at a wavelength of 280 nm and using BSA as the standard. Each measurement was performed in triplicate.

### 2.5.3. Laccase activity assays

To quantify the laccase activity, 50  $\mu$ L of each sample was added to 950  $\mu$ L of 1.7 mM ABTS in citrate phosphate buffer (150 mM, pH 4.5). The increase in absorbance at 420 nm was measured using the kinetic mode of a UV-Vis spectrophotometer (Shimadzu, UV-1280). The enzymatic activity was determined using Eq. (4):

$$\text{Enzymatic activity} \left( \frac{U}{L} \right) = \frac{\text{abs/min} \times f_{dil} \times 10^6}{\epsilon} \quad (4)$$

where  $\epsilon$  is ABTS molar extinction coefficient (36000 L/(mol.cm) at 420 nm), abs/min is the increase in absorbance per min,  $f_{dil}$  is the sample dilution factor, and  $10^6$  is the conversion factor from M to  $\mu$ M. One unit (U) of laccase activity is defined as the amount of enzyme required to oxidise one  $\mu$ mol of substrate (ABTS) per min. Each measurement was performed in triplicate.

Total enzymatic units were calculated using Eq. (5):

$$\text{Total enzymatic units (U)} = \text{Enzymatic activity (U/L)} \times V_B (L) \quad (5)$$

where  $V_B$  (L) is the volume of the bottom phase.

The specific enzymatic activity was calculated using Eq. (6):

$$\text{Specific enzymatic activity (U/g)} = \frac{\text{Enzymatic activity (U/L)}}{\text{Protein concentration (g/L)}} \quad (6)$$

The purification levels of laccase were determined by the purification factor (fold) according to Eq. (7):

$$\text{Purification factor (fold)} = \frac{\text{ABS\_SEA (U/g)}}{\text{Control\_SEA (U/g)}} \quad (7)$$

where  $\text{ABS\_SEA}$  is the specific enzymatic activity in the ABS and  $\text{Control\_SEA}$  is the specific enzymatic activity in the control.

The extraction efficiency of laccase ( $EE_{Laccase\%}$ ) corresponds to the percentage ratio between the laccase activity in the bottom phase ( $\text{Laccase activity}_B$ ) to that in the top phase ( $\text{Laccase activity}_T$ ), and was determined according to Eq. (8):

$$EE_{Laccase\%} = \frac{\text{Laccase activity}_B \times W_B}{\text{Laccase activity}_B \times W_B + \text{Laccase activity}_T \times W_T} \times 100 \quad (8)$$

where  $W_B$  and  $W_T$  correspond to the weight of the bottom and top phases, respectively.

## 2.6. Methanol quantification

Methanol levels present in the different systems were assessed using a HPLC system coupled to a 1260 Infinity Refractive Index detector (RID) (Agilent, Santa Clara, CA, USA) and the Agilent Hi-Plex H analytical column (300  $\times$  7.7 mm i.d.; 8  $\mu$ m). The analysis was performed at 65 °C, using an isocratic elution of 5 mM sulfuric acid with a flow rate of 0.6 mL/min, as previously described (Pedro et al., 2015). All the samples were filtered prior the injection through a 0.22  $\mu$ m cellulose-acetate filter.

## 2.7. Polydopamine coating of PMMA

The polymerisation of dopamine to be used in the PMMA coating with PDA was performed using the produced laccase. Enzymatic polymerisation reactions were carried out by mixing 40 mg of dopamine in 24 mL of phosphate buffer (50 mM, pH 7.0) with 1 mL of laccase solution (recovered with ammonium sulphate – 90% saturation, room temperature, 2 h, under magnetic agitation – from the bottom phase of the selected ABS composed of 10.0 wt% PEG 8000 + 5.0 wt% dextran 40 + 85.0 wt% BMMY). Non-enzymatic polymerisation was performed as a control by replacing the laccase solution with 1 mL of water.

The dopamine polymerisation reactions for the PDA coating were carried out by immersing PMMA substrate in the mixture reactions at room temperature under continuous magnetic agitation. After 18 h, the PMMA was gently immersed in water to remove excess PDA precipitates and then dried at room temperature. Each test was performed in triplicate.

To evaluate the polymerisation efficiency, the coated samples were analysed by UV-Vis spectroscopy (Shimadzu, UV-1280). PMMA substrate was used as blank. The relative enzymatic polymerisation of dopamine in relation to the non-enzymatic reaction was determined according to Eq. (9):

$$\text{Relative Polymerisation (fold)} = \frac{\text{abs(enzymatic)}}{\text{abs(non - enzymatic)}} \quad (9)$$

where abs (enzymatic) and abs (non-enzymatic) correspond to the absorbance values at 380 nm of the PDA coated samples catalysed by laccase and by non-enzymatic polymerisation, respectively.

## 2.8. Surface characterization techniques

### 2.8.1. FTIR spectroscopy

To evaluate the chemical structure of the functionalized surfaces, Attenuated Total Reflectance Fourier Transform Infrared spectroscopy (ATR-FTIR) was performed. The analysis was conducted using a Bruker ALPHA II spectrometer equipped with an ATR accessory featuring a diamond crystal. The spectra for the pristine PMMA, non-enzymatic PDA, and laccase-assisted PDA coatings were acquired by performing 256 scans over the spectral range 350–4000  $\text{cm}^{-1}$  with a resolution of 4  $\text{cm}^{-1}$ . To accurately isolate and identify the functional groups of the deposited PDA layers, the baseline spectrum of the pristine PMMA substrate was mathematically subtracted from the spectra of the coated samples.

### 2.8.2. Scanning electron microscopy

To evaluate the surface morphology and structural uniformity of the coatings at the microscale, scanning electron microscopy (SEM) was performed. The imaging was conducted using a Hitachi SU-70 scanning

electron microscope operating at an accelerating voltage of 4 kV. Prior to analysis, the non-conductive pristine PMMA and PDA-coated samples were sputter-coated with a thin layer of carbon to prevent surface charging and minimize electron beam damage during image acquisition.

### 2.8.3. Atomic force microscopy

To quantitatively assess the nanoscale surface topography of the pristine PMMA and PDA-coated samples, atomic force microscopy (AFM) imaging was performed using a Park Systems FX40. The measurements were conducted in tapping mode using a silicon probe (AC160TS, NANOSensors, Neuchâtel, Switzerland). The cantilever possessed a nominal spring constant of 26 N/m, a resonant frequency of 300 kHz, and a length of 160  $\mu\text{m}$ . Images were acquired over a scan area of  $5 \times 5 \mu\text{m}$  at a scan rate of 0.20 Hz.

### 2.8.4. Confocal profilometry

To quantitatively evaluate the structural uniformity and macroscopic surface roughness of the pristine PMMA and PDA-coated samples, 3D optical profilometry was performed. The analysis was conducted using an S neox 3D Optical Profiler (Sensofar Metrology). Topographic measurements were acquired over a defined scan area of  $350.9 \mu\text{m} \times 264.2 \mu\text{m}$ . The acquired 3D surface data were processed using the instrument's integrated SensoSCAN software. Prior to calculating the surface roughness parameters, a standard spatial flattening process was applied to the acquired profiles to correct for sample tilt and remove underlying form.

### 2.8.5. Optical contact angle

To evaluate the surface wettability of the functionalized materials, optical contact angle (OCA) measurements were performed using the sessile drop method. The analysis was conducted on an OCA 15EC contact angle goniometer (DataPhysics Instruments, Germany). For each measurement, a 3.0  $\mu\text{L}$  droplet of Milli-Q water was dispensed onto the sample surface at room temperature. The droplet profile was captured optically, and the contact angle was calculated using the instrument's integrated image analysis software. To ensure statistical reliability and account for surface heterogeneity, measurements were performed in triplicate across different regions of each sample, and the results were expressed as the average contact angle  $\pm$  standard deviation.

## 3. Results and discussion

### 3.1. Effect of ABS phase-forming compounds on *K. phaffii* growth

In this work, the use of ABS for the extractive fermentation of laccase from *T. versicolor* using *K. phaffii* as a host was evaluated, with the aim of integrating the upstream process with the recovery and concentration

steps of the downstream stage. Designing ABS for extractive fermentation requires, as an initial step, the evaluation of microbial growth in the presence of different ABS compounds to determine whether these compounds negatively affect microbial growth. To evaluate the effect of ABS phase-forming compounds on *K. phaffii* growth, 10.0 wt% of different individual compounds were added into the BMMY fermentation medium. The tested compounds included an IL ([Ch]Cl), salts ( $\text{Na}_2\text{SO}_4$ ,  $(\text{NH}_4)_2\text{SO}_4$  and  $\text{K}_2\text{HPO}_4/\text{KH}_2\text{PO}_4$ ) and polymers (dextran, PPG and PEG). The ABS constituents were selected based on previous reported literature showing that they provide biocompatible phase behavior and are widely used for microbial cultivation, protein stability, and biomolecule recovery (Shibusawa et al., 2006). The *K. phaffii* growth was evaluated after 6 days of fermentation. The results, expressed as relative microbial growth (%) in relation to the control, are depicted in Fig. 1. The control, considered as 100%, consisted of the fermentation medium without ABS compounds, where these were replaced by the same amount of water (10 wt%).

Despite the biocompatibility characteristics of cholinium-based ILs, *K. phaffii* growth was inhibited in the presence of 10.0 wt% [Ch]Cl, making it an unsuitable choice for extractive fermentation. A similar trend has been reported for other ILs; for example, 1% (v/v) of 1-butyl-3-methylimidazolium tetrafluoroborate ([BMIM][BF<sub>4</sub>]) was shown to be toxic to *K. phaffii* cells, inhibiting their growth, whereas in the presence of 10% (v/v) of 1-butyl-3-methylimidazolium hexafluorophosphate ([BMIM][PF<sub>6</sub>]) the cell growth was unaffected (Ganske and Bornscheuer, 2006). With the exception of  $\text{K}_2\text{HPO}_4/\text{KH}_2\text{PO}_4$  at pH 6, the results indicate that the addition of 10.0 wt% of salts to the fermentation medium negatively impacted the growth of *K. phaffii*, with a strong inhibition in cell growth (Fig. 1A). Although  $(\text{NH}_4)_2\text{SO}_4$  is known to increase cell density in yeast cultures, as a nitrogen source, high concentrations can inhibit cell growth (Ardila-Leal et al., 2020; Vidotto et al., 1993). In the case of  $\text{Na}_2\text{SO}_4$ , a partial growth inhibition was observed, with the microbial growth reaching 50% of the control value. In contrast, the addition of potassium salts  $\text{K}_2\text{HPO}_4/\text{KH}_2\text{PO}_4$  (pH 6.0) presented promising results, achieving 91% of the cell growth compared to the control. Potassium phosphate buffer is commonly used in fermentation media (typically at concentrations between 0.15 and 1.5 wt%) as both a buffer and a nutrient source. It is well-established that salts can affect cell growth by increasing the osmotic pressure and ionic strength in the medium. Although 10.0 wt%  $\text{K}_2\text{HPO}_4/\text{KH}_2\text{PO}_4$  (pH 6.0) minimally affected the cell growth, increasing the concentration up to 20.0 wt% completely inhibited cell growth, achieving only 12% of the microbial growth compared to the control (see Supplementary Material). Therefore, even though  $\text{K}_2\text{HPO}_4/\text{KH}_2\text{PO}_4$  (pH 6.0) is a favourable option for use in extractive fermentation, the impact of high salt concentrations must be considered when choosing the mixture point

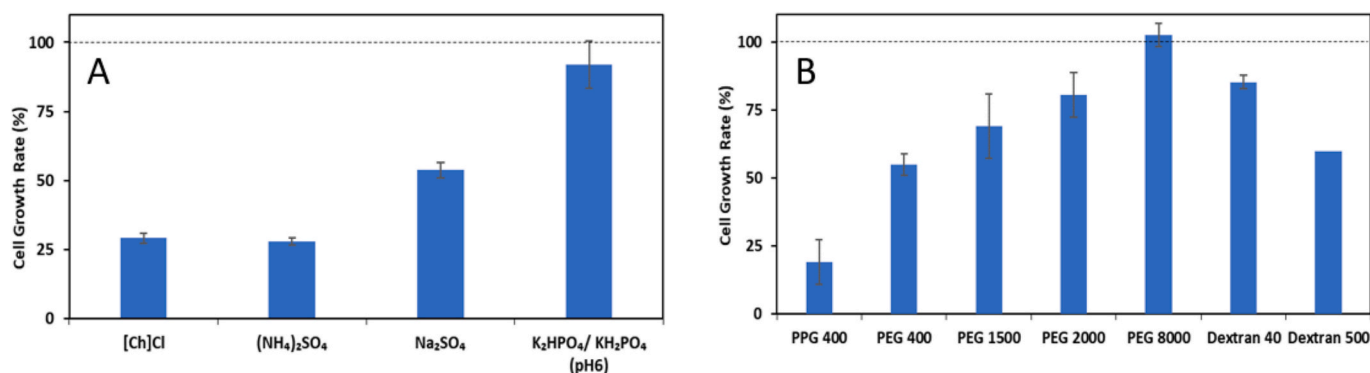


**Fig. 1.** (A) Effect of different salts/Ionic Liquid (10 wt%) on the cell growth rate of *K. phaffii* in relation to the control. Control was performed in 90 wt% of buffered complex methanol medium and 10 wt% water. Fermentation was performed in a shake-flask at 30 °C and 250 rpm for 6 days. Each value corresponds to the average of three independent samples. (B) Effect of different polymers (10 wt%) on the growth rate of *K. phaffii* in relation to control. Control was performed in 90 wt% of buffered complex methanol medium and 10 wt% water and was considered as 100%. Fermentation was performed in a shake-flask at 30 °C and 250 rpm for 6 days. Each value corresponds to the average of three independent samples.

for creating ABS.

Regarding the growth of *K. phaffii* in the presence of polymers (Fig. 1B), the addition of PPG 400 strongly inhibited its growth, achieving only 19% of the microbial growth. For PEG, increasing its molecular weight from 400 to 8000 leads to an improvement in the growth of *K. phaffii* from 55 to 102%, when compared to the control. These results are in agreement with previous studies using the bacteria *Burkholderia pseudomallei* (Ooi et al., 2011). On the other hand, the effect of the dextran molecular weight on cell growth was opposite to that observed with PEGs. This is expected given that the molecular weight of the heavier dextran is two orders of magnitude higher than those considered for the PEGs of higher molecular weight. Higher molecular weight polymers have a higher viscosity, creating thus more pronounced crowding effects in the medium, which can hinder nutrient and oxygen diffusion and physically restrict cell line movement and proliferation (Hyoungh Park et al., 2016). Although in the literature there is no evaluation of the growth of microorganisms with different molecular weights of dextran, dextran 500 has been used before in extractive fermentation with *Trichoderma reesei*, *Saccharomyces cerevisiae* and *B. pseudomallei* (Ooi et al., 2011; Sinha et al., 2000).

The results indicate that the presence of 10.0 wt%  $K_2HPO_4/KH_2PO_4$  (pH 6.0), PEG 8000 or dextran 40 is compatible with the growth of *K. phaffii*. Thus, these compounds were selected to study laccase activity and further ABS design to perform the extractive fermentation and bioprocess intensification.

### 3.2. Effect of ABS phase-forming compounds on laccase activity

After evaluating the impact of ABS phase-forming compounds on microbial growth, their effect on laccase activity was subsequently evaluated. In this assay, a comparative screening of the ABS constituents to determine whether they preserved, enhanced, or reduced enzyme activity under the tested conditions was carried out. According, the influence of  $K_2HPO_4/KH_2PO_4$  (pH 6.0), PEG 8000 and dextran 40 on the enzymatic activity was investigated using laccase produced under standard conditions in BMMY. For these assays, laccase was incubated in the presence of each compound and the relative enzymatic activity (%) over time was determined, whose results are shown in Fig. 2. The enzymatic activity at the initial time,  $t = 0$  min, under control conditions was considered as 100%. In the control, the compounds were substituted by water at the same concentrations (10 or 15 wt%). For the enzyme

activity values in U/L, see [Supplementary Material](#).

In the presence of 10.0 and 15.0 wt% PEG 8000, laccase activity decreased, losing 16% and 25% of its activity after 1 h, and 20% and 35% after 72 h, respectively. The effect of PEG on laccase activity and stability is therefore concentration dependent. Low concentrations of PEG 8000 (around 0.02% w/v) have been reported to enhance laccase activity and stability (Bou-Mitri and Kermasha, 2018). These low concentrations provide a protective hydration shell and reduce thermal inactivation rates (Bou-Mitri and Kermasha, 2018). However, higher concentrations can have the opposite effect, leading to decreased activity, possibly due to substrate diffusion limitations or unfavorable conformational changes in the enzyme structure (Bou-Mitri and Kermasha, 2018). For example, low concentrations of PEG 8000 increased laccase activity by up to 17%, higher concentrations (above 7.8% w/v) led to a 50% reduction in activity (Bou-Mitri and Kermasha, 2018), consistent with the losses observed in the present work.

In contrast, laccase maintained over 80% of its activity under control conditions for 72 h. A similar behaviour was observed in the presence of 15.0 wt%  $K_2HPO_4/KH_2PO_4$  pH 6.0. The best performance was achieved with the addition of 10.0 wt% dextran 40, which resulted in only 13% of activity loss after 72 h, compared to 20% of activity loss under control conditions, demonstrating that probably this result may be attributed to the protective effect of dextran over the laccase stability. Polysaccharides such as dextran 40 have been reported to stabilize enzymes, possibly by creating a favourable microenvironment and mitigating aggregation or denaturation (Bou-Mitri and Kermasha, 2018). These phenomena could explain the current results, as corroborated by the observed lower activity loss for laccase in the presence of dextran 40.

Overall, laccase maintains its activity in all tested compounds, with dextran 40 and  $K_2HPO_4/KH_2PO_4$  (pH 6.0) showing enhanced enzyme activity. Although the other compounds slightly affected the enzyme's activity, the activity of laccase remained high. While PEG is a common phase-forming compound due to its biocompatibility and ability to form ABS, its concentration must be carefully controlled to avoid compromising the enzyme activity. Dextran and phosphate buffers, on the other hand, can maintain and enhance laccase stability, and are therefore promising candidates for ABS aimed at maximizing enzyme recovery and activity. However, the salt concentration must be carefully balanced, since it can either preserve or impair laccase activity depending on the system composition. Therefore, all the studied compounds were considered suitable for the extractive fermentation process

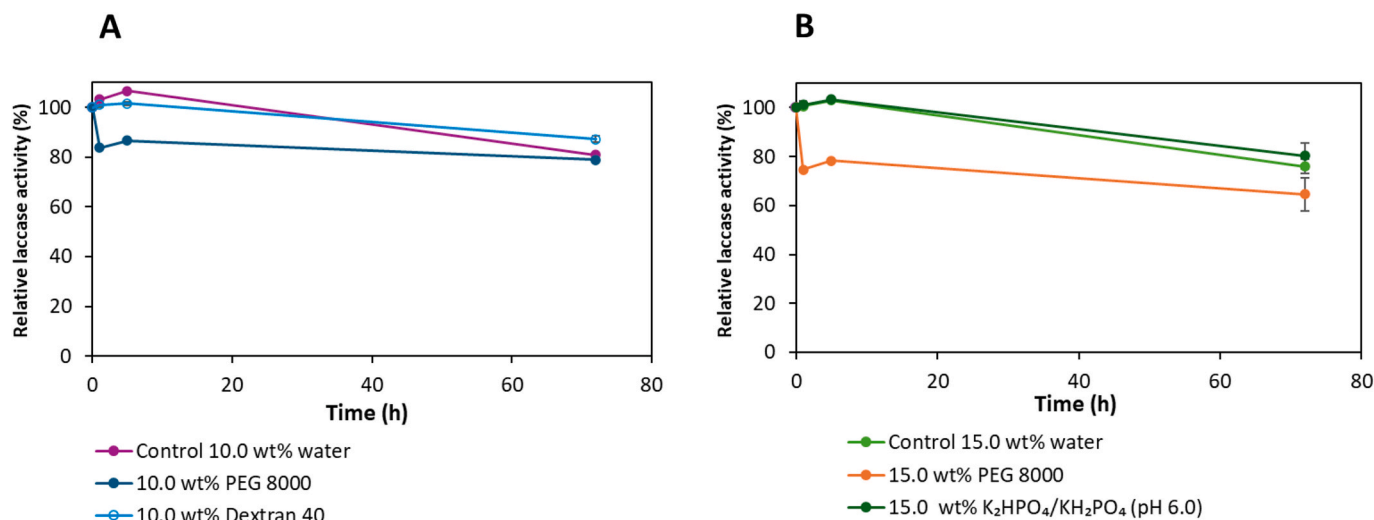


**Fig. 2.** Relative laccase activity (%) at room temperature with the addition of aqueous biphasic phase-forming compounds. The enzymatic activity at the initial time,  $t = 0$  min, under control conditions was considered as 100%. (A) 10.0 wt% polyethylene glycol 8000 and 10.0 wt% dextran 40; (B) 15.0 wt% polyethylene glycol 8000 and 15.0 wt%  $K_2HPO_4/KH_2PO_4$  (pH 6.0). For the control, the compounds were substituted by water at the same concentrations (10 or 15 wt%). Each value corresponds to the average of three independent experiments.

in the ABS design.

### 3.3. Designing the extractive fermentation process for laccase production

#### 3.3.1. Evaluation of salt-polymer and polymer-polymer ABS compositions

Based on the previous results regarding the effects of PEG 8000, dextran 40 and  $K_2HPO_4/KH_2PO_4$  on both yeast growth and laccase

activity, the extractive fermentation process for laccase production was evaluated using two types of ABS: i) a polymer-salt ABS composed of PEG 8000 and  $K_2HPO_4/KH_2PO_4$  (pH 6.0); and ii) a polymer-polymer ABS composed of PEG 8000 and dextran 40. For the polymer-salt ABS, three mixture points were chosen (Table 1) according to binodal curves reported in the literature (de Souza et al., 2014). In the polymer-salt system, the salt acts as a phase-forming component, and also act as a

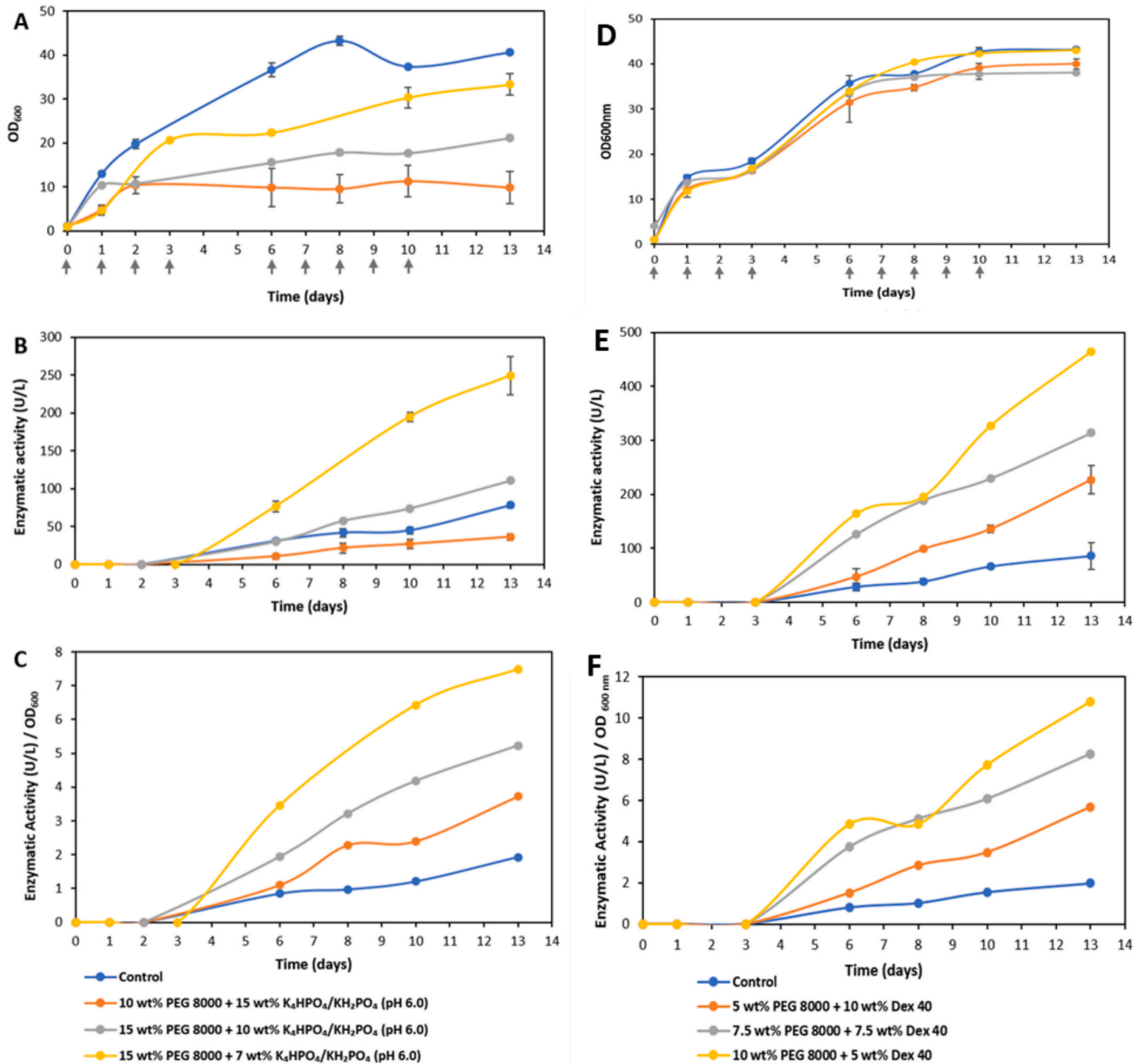


**Fig. 3.** (A) OD<sub>600</sub> values of recombinant *K. phaffii* during fermentation time (days). The arrows represent the days when methanol supplementation occurred. (B) Enzymatic activity (U/L) of laccase during fermentation time (days) in  $K_2HPO_4/KH_2PO_4$  (pH 6.0)-rich phase (bottom phase). Extractive fermentation in 3 mixture points of polymer-salt aqueous biphasic systems: 10.0 wt% polyethylene glycol 8000 + 15.0 wt%  $K_2HPO_4/KH_2PO_4$  (pH 6.0) + 75.0 wt% buffered complex methanol medium; 15.0 wt% polyethylene glycol 8000 + 10.0 wt%  $K_2HPO_4/KH_2PO_4$  (pH 6.0) + 75.0 wt% buffered complex methanol medium; 15.0 wt% polyethylene glycol 8000 + 7.0 wt%  $K_2HPO_4/KH_2PO_4$  (pH 6.0) + 78.0 wt% buffered complex methanol medium. (C) Enzymatic activity (U/L) per OD<sub>600</sub> values of recombinant *K. phaffii* cells. Control was considered fermentation with 25.0 wt% water + 75.0 wt% buffered complex methanol medium. Each value corresponds to the average of three independent experiments. (D) OD<sub>600</sub> values of recombinant *K. phaffii* during fermentation time (days). The arrow represents the days when methanol supplementation occurred. (E) Enzymatic activity (U/L) of laccase during fermentation time (days) in dextran 40-rich phase (bottom phase). Extractive fermentation in 3 mixture points of polymer-polymer ABS: 5.0 wt% polyethylene glycol 8000 + 10.0 wt% dextran 40 + 85.0 wt% buffered complex methanol medium; 7.5 wt% PEG 8000 + 7.5 wt% dextran 40 + 85.0 wt% buffered complex methanol medium; 10.0 wt% PEG 8000 + 5.0 wt% dextran 40 + 85.0 wt% buffered complex methanol medium. (F) Enzymatic activity (U/L) per OD<sub>600</sub> values of recombinant *K. phaffii* cells. Control was considered fermentation with 15.0 wt% water + 85.0 wt% buffered complex methanol medium. Each value corresponds to the average of three independent experiments.

determinant of ionic strength, enzyme stability, charge interactions, and partitioning behavior, making its concentration a critical parameter (Bekavac et al., 2024). These compositions were selected to explore the impact of varying polymer and salt concentrations on the partitioning and extraction efficiency of laccase, which is critical for optimizing its separation and recovery. The partition coefficients for cells ( $K_{cell}$ ), methanol ( $K_{Meth}$ ) and laccase ( $K_{Lac}$ ), were determined for each system (see [Supplementary Material](#)).

In all polymer-salt ABS tested, *K. phaffii* cells preferentially migrate to the bottom phase, i.e., the salt-rich phase, with a partition coefficient,  $K_{cell}$ , ranging from 0.05 to 0.12, indicating preferential partitioning of *K. phaffii* cells to the bottom salt-rich phase (see [Supplementary Material](#)). This behaviour may be related to the size and density effects of yeast cells. Cells are significantly large and dense, and especially in PEG-salt systems, the phase density differences favour the sedimentation of cells into the denser bottom phase (Walter and Krob, 1988). Moreover, partitioning of cells in ABS depends on the interactions of the surface properties of the cells with the physico-chemical properties of the systems (Cabral, 2007). The PEG-rich top phase is more hydrophobic for the hydrophilic cell surface, while the salt-rich bottom phase provides a more compatible, polar, and stabilising environment for the cells (Cabral, 2007). Importantly, the  $K_{Meth}$  was from 1.08 to 1.47 (see [Supplementary Material](#)), indicating a nearly equal distribution of methanol between the two phases. This suggests that methanol availability in the bottom phase is not limited by its phase partitioning and no methanol depletion have occurred during cell growth. This distribution of methanol between both phases can be explained by the fact that methanol is a small and water-miscible molecule, and therefore its partition is probably mainly governed by the relative composition and hydration of each aqueous phase rather than by strong phase-specific interactions (Kee et al., 2020). Laccase completely partitioned the bottom phase (salt-rich phase), in agreement with literature for similar systems, as reported by Rajagopalu et al. (2016). This behaviour is related to the increased hydrophobicity of the PEG-rich phase, which excludes laccase and favours its total migration to the opposite phase, the salt-rich phase.

When evaluating cell growth in the studied ABS, a reduction was observed compared to the control conditions, being more pronounced for ABS composed of higher salt concentrations, namely 10.0 and 15.0 wt% of  $K_2HPO_4/KH_2PO_4$  (pH 6.0) (Fig. 3A). The ABS composed of 15.0 wt% PEG 8000 + 7.0 wt%  $K_2HPO_4/KH_2PO_4$  (pH 6.0)) resulted in an 18% decrease in  $OD_{600}$ , while ABS with higher salt concentrations (15.0 wt% PEG 8000 + 10.0 wt%  $K_2HPO_4/KH_2PO_4$  and 10.0 wt% PEG 8000 + 15.0 wt%  $K_2HPO_4/KH_2PO_4$ ) led to reductions of 48 and 76%, respectively, by the end of the fermentation. This result is in agreement with the ones reported by Jawan et al. (2021)), confirming that high salt concentrations in ABS promote growth inhibition of microbial cells. To confirm that this behaviour was not related to pH variations, the pH was monitored throughout the experiments (see [Supplementary Material](#)), which showed to remain stable around 6.0, and thus confirming that the observed cell growth inhibition was primarily due to salt concentration.

Enzymatic activity and the enzymatic activity per  $OD_{600}$  values of recombinant *K. phaffii* cells were also evaluated in all systems. The results presented in Fig. 3 B and C confirm that the salt-rich phase of the ABS composed of 15.0 wt% PEG 8000 + 7.0 wt%  $K_2HPO_4/KH_2PO_4$  (pH 6.0) exhibits a 69% increase in laccase activity compared to the control fermentation. This higher recovered activity is attributed to the complete partitioning of laccase into the bottom phase and its concentration due to the phase volume ratio. Therefore, the observed increase reflects only the combined effects of enzyme separation and concentration within the ABS rather than direct evidence of enhanced laccase production. In contrast, the ABS with the highest salt concentration (10.0 wt% PEG 8000 + 15.0 wt%  $K_2HPO_4/KH_2PO_4$  (pH 6.0)) presented a 53% reduction in laccase activity, indicating that excessive salt can negatively impact both cell growth and enzyme activity. These results show the importance of optimising salt concentration in ABS, not only for

phase formation but also to balance biocompatibility with process performance, ensuring cellular viability and enzyme integrity.

For the polymer-polymer ABS, three mixture points were selected based on PEG 8000/dextran 40 binodal curves reported in the literature (Chen et al., 2023) (Table 1). The evaluated parameters  $K_{cell}$ ,  $K_{Meth}$ , and  $K_{Lac}$  are provided in the [Supplementary Material](#). *K. phaffii* cells and laccase preferentially migrate to the bottom phase (dextran-rich phase), with a  $K_{Lac}$  values between 0.02 and 0.05 and complete partition of cells. This partition of laccase is similar with previous studies on laccase partitioning in ABS, where the enzyme preferentially migrates to the more polar phases, driven by differences in phase polarity, salting-out effects, and protein-phase interactions (Ferreira et al., 2021). In polymer-polymer ABS, the PEG-rich top phase is expected to be relatively more hydrophobic and therefore less favourable for laccase, whereas the dextran-rich bottom phase provides a more compatible hydrophilic aqueous environment, which promotes laccase partitioning to that more polar phase. Although in the literature there is no evaluation of laccase partitioning in ABS composed of PEG 8000 + dextran 40, Silvério et al. (2013) reported a  $K_{Lac}$  of 0.939 in a polymer-polymer ABS composed of 6.06 wt% PEG 8000 and 12.41 wt% dextran 75. This result shows that lower molecular weight dextrans, as the ones considered in the current work, are beneficial for the laccase partitioning towards this phase.

In contrast to the polymer-salt ABS, the cell growth of *K. phaffii* in polymer-polymer ABS was not affected, presenting a similar  $OD_{600}$  values of 43 (Fig. 3 D). A minimal difference is observed for ABS composed of 5.0 wt% of PEG 8000 + 10.0 wt% Dextran 40 and 7.5 wt% of PEG 8000 + 7.5 wt% Dextran 40, with a decrease in  $OD_{600}$  values of 7 and 12%, respectively, in relation to the control. In this system, methanol preferentially partitions to the top phase (PEG-rich phase), with  $K_{Meth}$  3–4. This difference is consistent with the fact that even in water-rich biphasic systems, methanol remains a highly hydrophilic, fully miscible small molecule, so its distribution is governed by differences in phase composition and hydration rather than by complete phase exclusion. In PEG/dextran systems, the physicochemical contrast between phases is sufficient to the migration of methanol toward one phase, but not strong enough to remove it entirely from the other, which is why methanol can still be detected in both phases even when partitioning is high (Stenekes et al., 1999). Despite this preferential distribution, methanol remains present in both phases, ensuring its availability for the cells in the bottom phase. Therefore, no limitation was expected under these conditions, allowing sufficient methanol supply for *K. phaffii* metabolism. Similar to polymer-salt ABS, no significant differences in pH were observed between the control and ABS (see [Supplementary Material](#)). This result can be attributed to the generally biocompatible nature of polymer-polymer ABS, particularly those formed by PEG and dextran, which are known to provide a gentle and non-toxic environment for microbial cells. Moreover, dextran probably provides a more favourable environment for laccase activity. Dextran is a hydrophilic polysaccharide that is reported to promote a favourable hydration environment around proteins, reducing the denaturation, and providing an increasing macromolecular crowding/volume exclusion, and reducing protein aggregation (Li et al., 2024; Sun and Davison, 2001). Previous studies show that dextran-based formulations have been associated with improved enzyme stability by protecting enzymes against thermal or storage-induced inactivation (Bou-Mitri and Kermasha, 2018). Furthermore, unlike previous results with high concentrations of salts in the ABS, this polymer-polymer systems maintain a more favourable medium conditions and do not significantly alter the ionic strength of the medium, which helps maintain cell viability and metabolic activity during fermentation, as reported for *Lactococcus lactis* (Jawan et al., 2021). Furthermore, the absence of pH variation across the tested systems suggests that the observed cell growth is not influenced by acid-base imbalances but rather by the physicochemical properties of the phase-forming polymers.

The enzymatic activity and the enzymatic activity per  $OD_{600}$  values of recombinant *K. phaffii* cells were significantly higher in all

polymer–polymer ABS compared to the control fermentation (Fig. 3 E and F, respectively). As observed for the polymer-salt ABS, the higher recovered laccase activity in the polymer–polymer ABS is attributed to the selective partitioning of laccase into the dextran-rich bottom phase and its concentration due to the phase volume ratio. In addition, the more favourable environment of dextran 40 may have contributed to preserving laccase activity throughout the fermentation process. Therefore, the observed increase reflects only the combination effects of enzyme recovery, concentration, and stabilization.

The highest enzymatic activity was observed in the ABS composed of 10.0 wt% PEG 8000 + 5.0 wt% dextran 40, followed by 7.5 wt% PEG 8000 + 7.5 wt% dextran 40, and 5.0 wt% PEG 8000 + 10.0 wt% dextran 40, with an increase of 81, 73 and 62% of laccase activity, respectively, when compared to the control. This enhancement in enzymatic activity using polymer–polymer ABS is a result of the mild and non-denaturing environment provided by PEG and dextran, allowing to preserve the native conformation and catalytic function of laccase.

### 3.3.2. Purification of laccase in ABS

To comprehensively evaluate the potential of ABS as a tool for extractive fermentation, the recovered fraction containing the target enzyme was quantitatively evaluated by its catalytic performance, specific activity, and purification factor, since these parameters provide a more relevant assessment of process success. These parameters of laccase produced in ABS were assessed in the bottom phase at the end of the fermentation in ABS. The results are summarised in Tables 2 and 3 for the previously selected polymer-salt and polymer–polymer ABS, respectively.

In the polymer-salt ABS, the ABS composed of 15.0 wt% PEG 8000 + 7.0 wt%  $K_2HPO_4/KH_2PO_4$  (pH 6.0) presented a higher enzymatic activity and specific activity, which is only due to the complete partitioning of laccase into the smaller bottom phase ( $V_R = 1.63$ ), effectively integrating the steps of enzyme separation and concentration. In this ABS, laccase was concentrated 3.2-fold compared to the control fermentation, highlighting the advantage of ABS in concentrating enzymes, thereby reducing the working volumes required in the following purification steps. The specific enzymatic activity increased from 1.9 U/g (control) to 6.9 U/g, indicating a higher purity, compared to the control. The total enzymatic units produced, 3.6 U, were very similar to the control (3.3 U), demonstrating that the ABS components did not negatively affect the amount of laccase produced during the fermentation step. These findings highlight the advantage of extractive fermentation using ABS, which enables the integration of production, separation, concentration, and partial purification in a single step.

For polymer–polymer ABS, as shown in Table 3, there is an increase in the total enzymatic units, indicating that these ABS allowed the recovery of more total enzymatic units of enzyme at the end of fermentation, with values ranging from 4.3 to 5.0 U of laccase, corresponding to a 19–30% improvement over the fermentation control (3.5 U of laccase). This improvement could be related to the stabilising effect of dextran 40, probably helping laccase to maintain its activity for longer periods (as discussed in section 3.2). Including dextran 40 in the ABS design boosts the recovery of more active laccase at the end of fermentation. In

addition, similar to polymer-salt ABS, polymer–polymer ABS showed an increase in specific enzymatic activity at all mixture points, increasing the laccase purity. Notably, the ABS composed of 10.0 wt% PEG 8000 + 5.0 wt% dextran 40 allowed the highest laccase activity of 464.7 U/L and specific activity of 9.2 U/g, reflecting both effective enzyme stabilization and purification. These results indicate a potential protective effect of polymers and the ability of these systems to separate and concentrate the enzyme.

Based on the findings related to enzymatic activity and purification in both polymer-salt and polymer–polymer ABS, it is clear that the studied systems offer significant advantages in laccase separation, concentration and purification. In polymer-salt ABS, the optimal result was achieved with 15.0 wt% PEG 8000 + 7.0 wt%  $K_2HPO_4/KH_2PO_4$  (pH 6.0), enabling high enzyme concentration and purity in the ABS bottom phase. In the case of polymer–polymer ABS, the system composed of 10.0 wt% PEG 8000 + 5.0 wt% dextran 40 generated the best results, with the highest enzymatic activity (464.7 U/L), specific activity (9.2 U/g), and purification factor (4.5), achieved in the dextran-rich phase.

These findings highlight the ability of both ABS types to simultaneously separate, concentrate and partially purify laccase directly from the fermentation broth. The integration of enzyme recovery, and concentration into a single step, while improving the purity, is a major advantage, as it reduces the number of downstream processing steps and the overall working volume, which are often limiting factors in industrial bioprocessing. Notably, the polymer–polymer ABS provided an additional advantage by increasing the total enzymatic units recovered, indicating that these systems not only preserve but can even enhance enzyme stability. The observed increase in purification factor also suggests that these ABS can efficiently separate laccase from other proteins, facilitating subsequent purification steps if needed. Moreover, it should be noted that the main objective of the proposed extractive fermentation strategy was not to maximize laccase production, but to demonstrate an extractive fermentation strategy capable of integrating recovery, concentration, and partial purification in a single step. Although similar and higher laccase activities are reported in optimized conventional fermentations (Zhang et al., 2022), such processes generally require additional downstream processing steps. In contrast, the ABS developed in this work enabled the direct recovery of laccase in a concentrated and partially purified form, reducing processing complexity and potentially lowering downstream costs.

### 3.4. Enzymatic coating of PMMA with PDA using the produced and recovered laccase

Based on the successful separation and concentration, and purification of laccase achieved through polymer–polymer ABS, the practical applicability of laccase was further demonstrated by applying the enzyme in the laccase-assisted polymerization of dopamine for PDA coating of PMMA substrates. The laccase-assisted polymerisation of dopamine into PDA for coating PMMA surfaces was investigated with the laccase produced using the selected ABS composed of 10.0 wt% PEG 8000 + 5.0 wt% dextran 40 + 85.0 wt% BMMY, and compared to the conventional non-enzymatic (alkaline auto-oxidation) method to

**Table 2**

Enzymatic activity (U/L), total enzymatic units (U), specific enzymatic activity (U/g), Volume ratio ( $V_R$ ), and purification factor of laccase produced in aqueous biphasic systems composed of polyethylene glycol 8000 and  $K_2HPO_4/KH_2PO_4$  (pH 6.0) at the end of fermentation in aqueous biphasic systems. Control was considered fermentation with 15 wt% water + 75 wt% buffered complex methanol medium. Each value corresponds to the average of three independent experiments.

| PEG 8000 (wt%) | $K_2HPO_4/KH_2PO_4$ (pH 6.0) (wt%) | $V_R$       | Enzymatic activity <sup>a</sup> (U/L) | Total enzymatic units <sup>a</sup> (U) | Specific enzymatic activity <sup>a</sup> (U/g) | Purification factor |
|----------------|------------------------------------|-------------|---------------------------------------|----------------------------------------|------------------------------------------------|---------------------|
| Control        |                                    | n. a.       | 78.2 ± 0.0                            | 3.3 ± 0.0                              | 1.9 ± 0.0                                      | 1.0 ± 0.0           |
| 10.0           | 15.0                               | 0.40 ± 0.01 | 36.5 ± 3.5                            | 1.1 ± 0.1                              | 0.8 ± 0.1                                      | 0.4 ± 0.1           |
| 15.0           | 10.0                               | 0.88 ± 0.02 | 110.6 ± 4.1                           | 2.3 ± 0.1                              | 2.3 ± 0.1                                      | 1.2 ± 0.1           |
| 15.0           | 7.0                                | 1.63 ± 0.10 | 249.4 ± 25.2                          | 3.6 ± 0.4                              | 6.9 ± 0.7                                      | 3.6 ± 0.5           |

<sup>a</sup> Enzymatic activity, total enzymatic units and specific enzymatic activity in ABS refer only to the bottom phase.

**Table 3**

Enzymatic activity (U/L), total enzymatic units (U), specific enzymatic activity (U/g) and purification factor of laccase produced in aqueous biphasic systems composed of polyethylene glycol 8000 and dextran 40. Control was considered fermentation with 15.0 wt% water + 85.0 wt% buffered complex methanol medium. Each value corresponds to the average of three independent experiments.

| PEG 8000 (wt%) | Dextran 40 (wt%) | V <sub>R</sub> | Enzymatic activity <sup>a</sup> (U/L) | Total enzymatic units <sup>a</sup> (U) | Specific enzymatic activity <sup>a</sup> (U/g) | Purification factor |
|----------------|------------------|----------------|---------------------------------------|----------------------------------------|------------------------------------------------|---------------------|
| Control        |                  | n. a.          | 86.0 ± 0.2                            | 3.5 ± 0.0                              | 2.0 ± 0.0                                      | 1.0 ± 0.0           |
| 5.0            | 10.0             | 0.76 ± 0.03    | 227.2 ± 25.8                          | 5.0 ± 0.5                              | 3.1 ± 0.3                                      | 1.5 ± 0.3           |
| 7.5            | 7.5              | 1.67 ± 0.03    | 314.3 ± 26.5                          | 4.6 ± 0.4                              | 6.5 ± 0.5                                      | 3.2 ± 0.4           |
| 10.0           | 5.0              | 3.26 ± 0.10    | 464.7 ± 9.3                           | 4.3 ± 0.1                              | 9.2 ± 0.2                                      | 4.5 ± 0.3           |

<sup>a</sup> Enzymatic activity, total enzymatic units and specific enzymatic activity in ABS refer only to the bottom phase.

evaluate the advantages of the biocatalytic approach. This is the first time that laccase is used to produce PDA to coat PMMA surfaces.

To evaluate the textual characteristics of the coatings, the macroscopic and microscopic surface morphologies of the PMMA substrates were analyzed via optical imaging SEM and AFM (Fig. 4). The pristine PMMA substrate (Fig. 4 A1, B1) exhibits a visually clear macroscopic appearance, though SEM reveals some inherent micro-roughness and structural irregularities on the surface. Following the non-enzymatic dopamine polymerization, the optical image (Fig. 4 A2) displays dark macroscopic aggregates scattered across the surface. The corresponding SEM micrograph (Fig. 4 B2) confirms the presence of smaller PDA

agglomerates deposited across the underlying micro-irregularities of the substrate. This rough morphology is highly characteristic of conventional alkaline auto-oxidation, probably due to the rapid dopamine polymerization in the bulk solution leads to the disordered precipitation of insoluble clusters onto the substrate (Bogdan et al., 2022).

In contrast, the laccase-assisted PDA coating (Fig. 4 A3) presents a highly uniform dispersion without macroscopic defects. The SEM evaluation (Fig. 4 B3) reveals a smooth and continuous surface coating at the microscale. This controlled deposition appears to form a conformal layer that smooths over the inherent micro-irregularities of the underlying substrate.

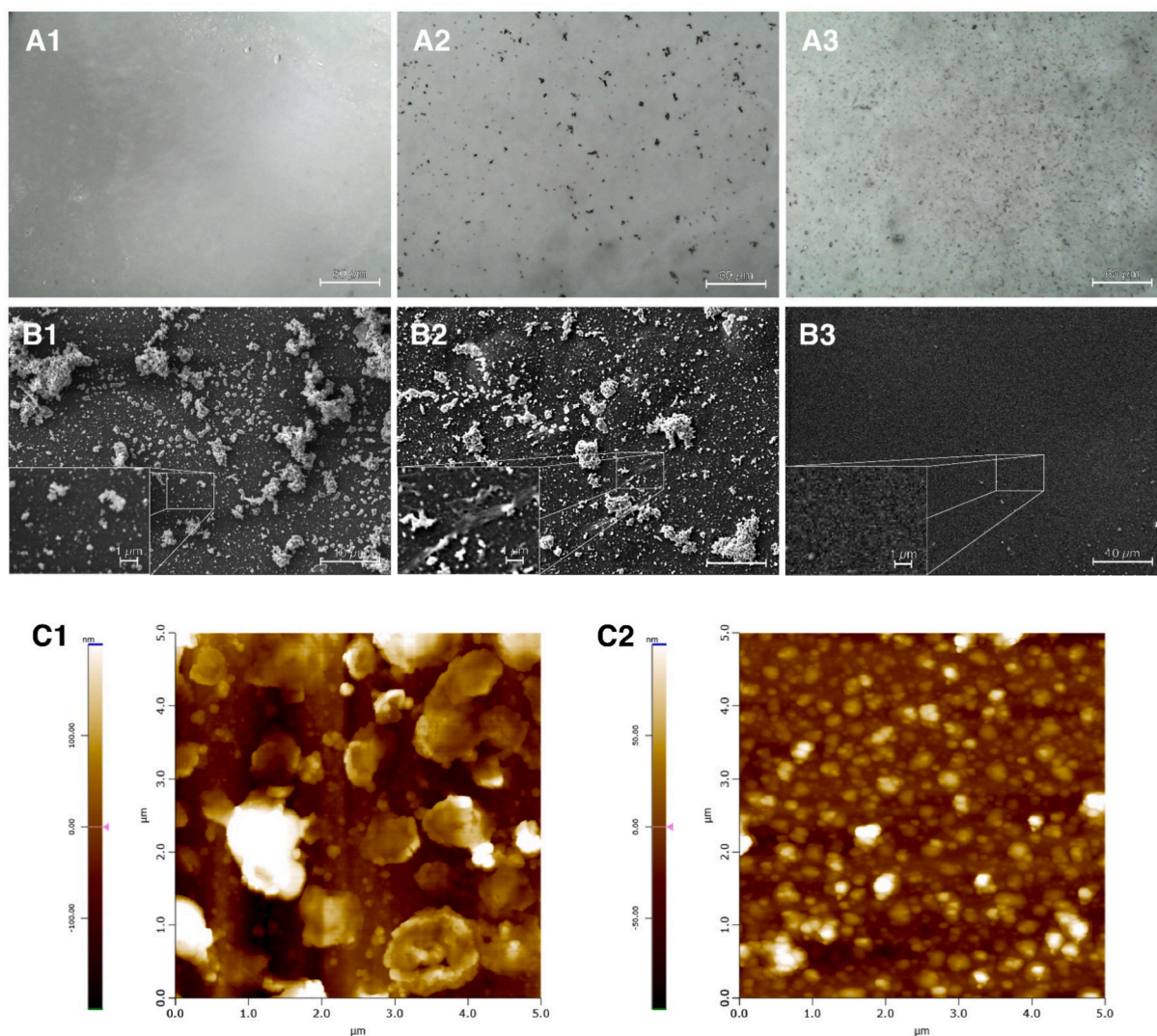


**Fig. 4.** Surface morphology and topography of polymethyl methacrylate before and after polydopamine functionalization. A1–A3) Optical images and B1–B3) scanning electron microscopy micrographs of pristine polymethyl methacrylate (A1, B1), non-enzymatic polydopamine (A2, B2), and enzymatic polydopamine (A3, B3). C1, C2) atomic force microscopy topography of the C1) non-enzymatic and C2) enzymatic polydopamine coatings.

AFM analysis further quantifies these morphological differences at the nanoscale and corroborates the SEM observations. The topography of the non-enzymatic PDA film (Fig. 4 C1) is dominated by the bulky, irregular agglomerates, resulting in a highly rough and chaotic surface profile. In contrast, the enzymatically catalysed PDA film (Fig. 4 C2) consists of densely packed, highly uniform nanoscale nodules. This fine, granular topography may be indicative of a more controlled dopamine polymerization process, resulting in reduced aggregate formation and a more homogeneous functional layer across the material surface.

Laccase-assisted PDA formation proceeds through enzymatic oxidation of dopamine to quinone intermediates using molecular oxygen, enabling polymerization under mild pH conditions and avoiding the harsh alkaline environment required for spontaneous dopamine autooxidation.

As the oxidation step is catalysed by laccase, the generation of reactive intermediates may occur in a more gradual and localized manner, which could contribute to reduced bulk precipitation and promote the formation of more uniform PDA coatings with fewer aggregates (Li et al., 2018). This interpretation is consistent with previous reports suggesting that laccase-catalysed dopamine polymerization can catalyse under milder and more controlled conditions (typically at neutral or slightly acidic pH), potentially leading to more uniform and stable PDA films while minimizing damage to sensitive substrates (Li et al., 2018).

UV-Vis spectroscopy (Fig. 5A) further confirmed the successful PDA deposition. The increased signal intensity across all investigated wavelengths, particularly at 380 nm (characteristic peak of PDA), confirms the successful modification of the material surface with PDA. Quantitatively, the enzymatic method resulted in a 1.2-fold higher polymerization efficiency compared to the non-enzymatic approach, as evidenced by the high absorbance intensity.

To evaluate the structural uniformity of the PDA coatings and corroborate the macroscopic observations, the surface roughness of the materials was analyzed via optical profilometry. The pristine PMMA substrate presented a relatively smooth baseline, with an arithmetic mean height ( $S_a$ ) of 69.8 nm and a root mean square height ( $S_q$ ) of 82.7 nm. Following the non-enzymatic dopamine polymerization, the surface roughness increased significantly ( $S_a = 108.3$  nm;  $S_q = 135.5$  nm). This increase indicates a chaotic deposition and the presence of bulk PDA aggregates on the surface, which is characteristic of the uncontrolled alkaline auto-oxidation process. In contrast, the enzymatic coating yielded a remarkably smoother surface ( $S_a = 78.1$  nm;  $S_q = 95.8$  nm), maintaining a topography that closely resembles the uncoated PMMA. These quantitative roughness parameters demonstrate that the laccase-assisted method successfully limits uncontrolled precipitation, facilitating the deposition of a highly uniform and controlled PDA functional

layer. Recent studies have demonstrated that laccase not only accelerates dopamine polymerization but also enables the formation of PDA films with improved adhesion, mechanical stability, and resistance to deformation (Li et al., 2018; Magalhães et al., 2025).

To evaluate the surface modifications induced by the PDA, the OCA of the uncoated and coated PMMA substrates was determined. As shown in Fig. 5B, the uncoated PMMA exhibited an initial OCA of  $79^\circ$ . Following the non-enzymatic dopamine polymerization, the OCA increased to  $83^\circ$ , a shift likely influenced by the microscopic roughness and irregular aggregates formed during the alkaline auto-oxidation process. In contrast, the enzymatic approach yielded a significantly higher OCA of  $95^\circ$ . Because AFM analysis confirmed that this enzymatic layer is highly smooth, uniform, and composed of densely packed nanoscale nodules, this shift towards increased contact angle reflects a more compact and homogeneous PDA surface architecture compared to the uncontrolled, chaotic precipitation of the non-enzymatic method. Thus, the distinct OCA corroborates the formation of a continuous, uniform functional layer.

To further confirm the surface modification and evaluate the chemical structure of the coatings, the samples were analyzed by FTIR spectroscopy. The pristine PMMA substrate exhibited its characteristic absorption bands (Fig. 6A), notably the strong acrylate carboxyl ( $\text{C=O}$ ) ester stretching vibration at  $1721\text{ cm}^{-1}$ , the C—H bending vibration at  $1434\text{ cm}^{-1}$ , and a highly intense, well-resolved C—O—C ester stretching region between  $1300$  and  $1100\text{ cm}^{-1}$  (Aziz et al., 2019).

To isolate the chemical signature of the deposited PDA layer, the PMMA baseline was subtracted from the spectra of the coated samples (Fig. 6B). Because spectral subtraction inevitably introduces residual artifacts and noise in regions where the underlying substrate absorbs heavily, particularly in the fingerprint region below  $1500\text{ cm}^{-1}$ , a reliable analytical window was established between  $1660\text{ cm}^{-1}$  and  $1500\text{ cm}^{-1}$ . This region is largely free from PMMA interference, allowing for the confident identification of newly formed functional groups.

Within this defined window, the subtracted spectra for both the enzymatic and non-enzymatic coatings reveal the characteristic features of polydopamine. Distinct peaks emerge at  $1598\text{ cm}^{-1}$  and  $1508\text{ cm}^{-1}$ . The peak at  $1598\text{ cm}^{-1}$  is attributed to N—H bending vibrations, while the peak at  $1508\text{ cm}^{-1}$  corresponds to the C=C stretching vibrations of the aromatic indole rings (Khosravi et al., 2022). The clear presence of these peaks confirms the successful polymerization of dopamine into PDA on the PMMA surface with and without laccase.

Furthermore, a comparison within this interference-free zone reveals some structural differences between the enzymatic and non-enzymatic PDA coatings. Notably, the laccase-assisted PDA spectrum exhibits additional bands at  $1646$ ,  $1557$ , and  $1522\text{ cm}^{-1}$  that are absent in the non-enzymatic control. The strong peak at  $1646\text{ cm}^{-1}$  is characteristic of

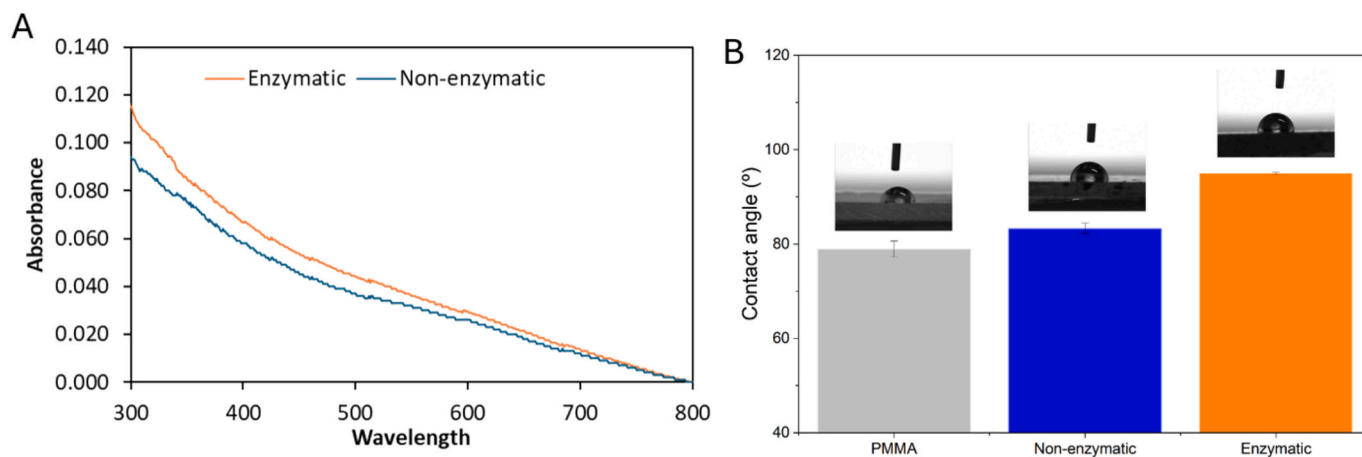


Fig. 5. (A) UV-Vis spectra of enzymatic and non-enzymatic samples obtained from the average of three replicates. (B) Water contact angle of pristine polymethyl methacrylate and polydopamine-coated samples prepared through non-enzymatic and enzymatic approaches.

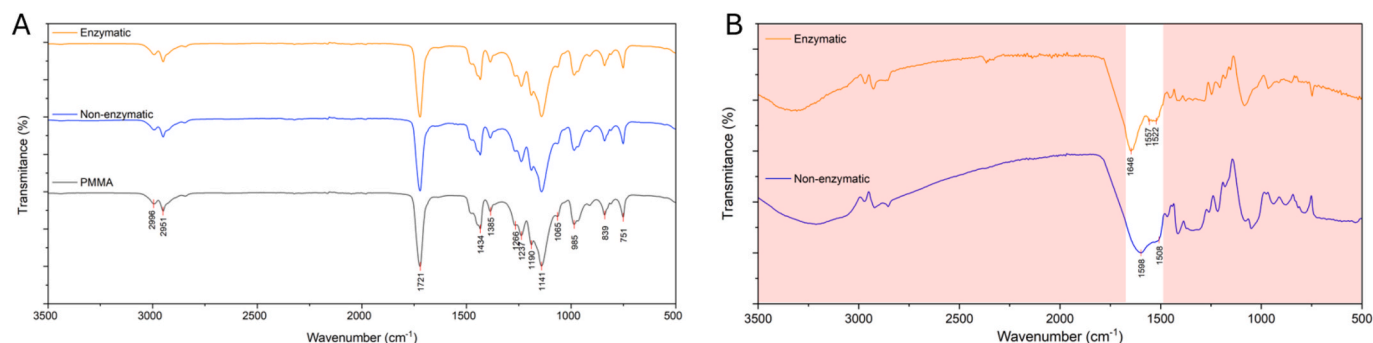


**Fig. 6.** Fourier transform infrared spectroscopy showing (A) polymethyl methacrylate substrates with and without polydopamine coating, and (B) spectra of polydopamine-coated samples with the polymethyl methacrylate signal removed to reveal characteristic peaks.

the Amide I band, arising primarily from the C=O stretching vibrations of peptide linkages. The peaks at 1557 and 1522  $\text{cm}^{-1}$  correspond to the Amide II band, associated with in-plane N–H bending and C–N stretching vibrations of the protein backbone (Lakshmanan and Ramasamy, 2022). These protein markers confirm that the laccase enzyme did not merely act as a transient aqueous biocatalyst, but was successfully co-deposited and immobilized within the PDA matrix during the continuous polymerization process on the PMMA surface.

The superior performance of the laccase-assisted method is significant since the enzymatic polymerization occurs under milder and more environmentally friendly conditions compared to chemical oxidants, probably reducing the risk of damaging sensitive substrates. These results demonstrate that the laccase produced in this work has a high potential to be used in polymer synthesis and materials covering, promoting process intensification, making the approach more economically and environmentally attractive.

#### 4. Conclusions

This work demonstrated a sustainable and integrated strategy for the production, recovery and concentration of *T. versicolor* laccase using extractive fermentation in aqueous biphasic systems. By selecting biocompatible phase-forming compounds, polymer-salt and polymer-polymer systems were applied to enable the integration of upstream and downstream in a single step. Compared to the control fermentation, the polymer-polymer system showed the most promising results, improving laccase activity by 19%, increasing its specific activity with a 4.6-fold purification. The recovered enzyme retained its catalytic performance and was applied in the polymerisation of dopamine into polydopamine functional coating on polymethyl methacrylate. The biocatalytic-assisted coating showed superior uniformity and efficiency compared to the non-enzymatic methodology.

Although the proposed extractive fermentation strategy showed promising results at laboratory scale, future industrial implementation will require addressing challenges associated with aqueous biphasic systems scale-up, polymer recycling, phase separation efficiency, and process economics.

#### CRedit authorship contribution statement

**Flávia F. Magalhães:** Writing – original draft, Validation, Methodology, Investigation, Formal analysis, Data curation. **Ana M. Gonçalves:** Writing – review & editing, Validation, Investigation, Data curation. **Bernardo L. Tavares:** Writing – review & editing, Visualization, Validation, Methodology, Data curation. **Luís A. Passarinha:** Writing – review & editing, Validation, Supervision, Resources, Funding acquisition. **Augusto Q. Pedro:** Writing – review & editing, Validation, Supervision, Resources, Investigation, Conceptualization. **Mara G. Freire:** Writing – review & editing, Validation, Supervision, Resources, Project administration, Funding acquisition, Conceptualization. **Ana**

**Paula M. Tavares:** Writing – review & editing, Validation, Supervision, Resources, Project administration, Investigation, Funding acquisition, Conceptualization.

#### 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.

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#### Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.biortech.2026.135829>.

#### Data availability

Data will be made available on request.

#### References

- Aghaei, M., Salehipour, M., Rezaei, S., Mogharabi-Manzari, M., 2024. Bioremediation of organic pollutants by laccase-metal-organic framework composites: a review of current knowledge and future perspective. *Bioresour. Technol.* 406, 131072. <https://doi.org/10.1016/J.BIORTECH.2024.131072>.

- Ardila-Leal, L.D., Alvarado-Ramírez, M.F., Gutiérrez-Rojas, I.S., Poutou-Piñales, R.A., Quevedo-Hidalgo, B., Pérez-Flórez, A., Pedroza-Rodríguez, A.M., 2020. Low-cost media statistical design for laccase rPOXA 1B production in *P. pastoris*. *Heliyon* 6, e03852. <https://doi.org/10.1016/j.heliyon.2020.e03852>.
- Aziz, S.B., Abdullah, O.G., Brza, M.A., Azawy, A.K., Tahir, D.A., 2019. Effect of carbon nano-dots (CNDs) on structural and optical properties of PMMA polymer composite. *Results Phys.* 15, 102776. <https://doi.org/10.1016/j.rinp.2019.102776>.
- Badhwar, P., Kumar, P., Dubey, K.K., 2019. Extractive fermentation for process integration and amplified pullulan production by *A. pullulans* in aqueous two phase system. *Sci. Rep.* 9, 1–8. <https://doi.org/10.1038/s41598-018-37314-y>.
- Bekavac, N., Benković, M., Jurina, T., Valinger, D., Gajdoš Kljusurić, J., Jurinjak Tušek, A., Šalić, A., 2024. Advancements in aqueous two-phase systems for enzyme extraction, purification, and biotransformation. *Molecules* 29, 3776. <https://doi.org/10.3390/MOLECULES29163776>.
- Bogdan, D., Grosu, I.-G., Filip, C., 2022. How thick, uniform and smooth are the polydopamine coating layers obtained under different oxidation conditions? An in-depth AFM study. *Appl. Surf. Sci.* 597, 153680. <https://doi.org/10.1016/j.apsusc.2022.153680>.
- Bou-Mitri, C., Kermasha, S., 2018. Lyoprotection and stabilization of laccase extract from *Coriolus hirsutus*, using selected additives. *AMB Express* 8, 152. <https://doi.org/10.1186/S13568-018-0683-3>.
- Cabral, J.M.S., 2007. Cell partitioning in aqueous two-phase polymer systems. *Adv. Biochem. Eng. Biotechnol.* 106, 151–171. <https://doi.org/10.1007/10.2006.045>.
- Chen, F., Li, X., Yu, Y., Li, Q., Lin, H., Xu, L., Shum, H.C., 2023. Phase-separation facilitated one-step fabrication of multiscale heterogeneous two-aqueous-phase gel. *Nat. Commun.* 14, 2793.
- Combes, J., Imatoukene, N., Couvreur, J., Godon, B., Brunissen, F., Fojcik, C., Allais, F., Lopez, M., 2021. Intensification of p-coumaric acid heterologous production using extractive biphasic fermentation. *Bioresour. Technol.* 337, 125436. <https://doi.org/10.1016/j.BIORTECH.2021.125436>.
- de Souza, R.L., Campos, V.C., Ventura, S.P.M., Soares, C.M.F., Coutinho, J.A.P., Lima, Á. S., 2014. Effect of ionic liquids as adjuvants on PEG-based ABS formation and the extraction of two probe dyes. *Fluid Phase Equilib.* 375. <https://doi.org/10.1016/j.fluid.2014.04.011>.
- Dos Santos, N.V., Martins, M., Santos-Ebinuma, V.C., Ventura, S.P.M., Coutinho, J.A.P., Valentini, S.R., Pereira, J.F.B., 2018. Aqueous biphasic systems composed of choline chloride and polymers as effective platforms for the purification of recombinant green fluorescent protein. *ACS Sustain. Chem. Eng.* 6, 9383–9393. <https://doi.org/10.1021/ACSSUSCHEMENG.8B01730/ASSET/IMAGES/LARGE/SC-2018-01730R.0004.JPEG>.
- Ferreira-faria, D., Aires-barros, M.R., Azevedo, A.M., 2020. Continuous aqueous two-phase extraction: from micro fluidics to integrated biomanufacturing. *Fluid Phase Equilib.* 508, 112438. <https://doi.org/10.1016/j.fluid.2019.112438>.
- Ferreira, A.M., Valente, A.I., Castro, L.S., Coutinho, J.A.P., Freire, M.G., Tavares, A.P.M., 2021. Sustainable liquid supports for laccase immobilization and reuse: Degradation of dyes in aqueous biphasic systems 400, 0–1. <https://doi.org/10.1002/bit.27764>.
- Ganske, F., Bornscheuer, U.T., 2006. Growth of *Escherichia coli*, *Pichia pastoris* and *Bacillus cereus* in the presence of the ionic liquids [BMIM][BF<sub>4</sub>] and [BMIM][PF<sub>6</sub>] and organic solvents. *Biotechnol. Lett.* 28, 465–469. <https://doi.org/10.1007/s10529-006-0006-7>.
- Gutiérrez-Rangel, P.C., Mayolo-Deloya, K., Torres-Acosta, M.A., 2024. Decisional tool development and application for techno-economic analysis of fungal laccase production. *Bioresour. Technol.* 402, 130781. <https://doi.org/10.1016/j.BIORTECH.2024.130781>.
- Hyoung Park, J., Sin Lim, M., Rang Woo, J., Won Kim, J., Min Lee, G., 2016. The molecular weight and concentration of dextran sulfate affect cell growth and antibody production in CHO cell cultures. *Biotechnol. Prog.* 32, 1113–1122. <https://doi.org/10.1002/BTPR.2287>.
- Isozaki, K., Matsuda, H., Agata, R., Jeon, J., Wu, B., Pincella, F., Ikenaga, M., Tachibana, Y., Ohta, Y., Nakamura, M., 2024. Synthetic urushiol from bioresource: chemical conversion of enzymatic degradation products of wood lignin to an ancient yet future coating material. *RSC Sustain.* 2, 1358–1362. <https://doi.org/10.1039/D3SU00446E>.
- Jawan, R., Abbasiliasi, S., Tan, J.S., Halim, M., Mustafa, S., Lee, B.H., Kwa, J.S., Ariff, A. B., 2021. Extractive fermentation for recovery of bacteriocin-like inhibitory substances derived from *Lactococcus lactis* Gh1 using PEG2000/Dextran T500 aqueous two-phase system. *Fermentation* 7, 257. <https://doi.org/10.3390/FERMENTATION7040257>.
- Kee, P.E., Ng, T.C., Lan, J.C.W., Ng, H.S., 2020. Recent development of unconventional aqueous biphasic system: characteristics, mechanisms and applications. 40, 555–569. <https://doi.org/10.1080/07388551.2020.1747388>.
- Kee, P.E., Yim, H.S., Kondo, A., Lan, J.C.W., Ng, H.S., 2022. Extractive fermentation of *Kytococcus sedentarius* TWHK01 using the aqueous biphasic system for direct recovery of keratinase. *J. Taiwan Inst. Chem. Eng.* 137, 104232. <https://doi.org/10.1016/J.JTICE.2022.104232>.
- Khosravi, H., Naderi, R., Ramezanzadeh, B., 2022. Synthesis and application of molybdate-doped mussel-inspired polydopamine (MI-PDA) biopolymer as an effective sustainable anti-corrosion substance for mild steel in NaCl solution. *Biomass Convers. Biorefinery* 14, 27557–27573. <https://doi.org/10.1007/S13399-022-03514-W>.
- Lakshmanan, R., Ramasamy, U., 2022. Idiosyncratic investigation of *Trametes versicolor* yellow laccase using organic fruit exocarp in solid-state fermentation. *Biomass Convers. Biorefinery* 13, 14803–14819. <https://doi.org/10.1007/S13399-022-03192-8>.
- Leong, Y.K., Show, P.-L., Lan, J.-C.-W., Krishnamoorthy, R., Chu, D.-T., Nagarajan, D., Yen, H.-W., Chang, J.-S., 2019. Application of thermo-separating aqueous two-phase system in extractive bioconversion of polyhydroxyalkanoates by *Cupriavidus necator* H16. *Bioresour. Technol.* 287, 121474. <https://doi.org/10.1016/j.biortech.2019.121474>.
- Li, F., Yu, Y., Wang, Q., Yuan, J., Wang, P., Fan, X., 2018. Polymerization of dopamine catalyzed by laccase: comparison of enzymatic and conventional methods. *Enzyme Microb. Technol.* 119, 58–64. <https://doi.org/10.1016/j.enzmictec.2018.09.003>.
- Li, J., Wang, H., Wang, L., Yu, D., Zhang, X., 2024. Stabilization effects of saccharides in protein formulations: a review of sucrose, trehalose, cyclodextrins and dextrans. *Eur. J. Pharm. Sci.* 192, 106625. <https://doi.org/10.1016/J.EJPS.2023.106625>.
- Li, X., Wang, L., Zhen, Y., Li, S., Xu, Y., 2015. Chicken egg yolk antibodies (IgY) as non-antibiotic production enhancers for use in swine production: a review. *J. Anim. Sci. Biotechnol.* 6. <https://doi.org/10.1186/S40104-015-0038-8>.
- Magalhães, F.F., Alfieri, M.L., Romanyuk, K., Kholkin, A., Freire, M.G., Napolitano, A., Tavares, A.P.M., Panzella, L., 2025. Laccase-mediated dopamine polymerization: a bioinspired and sustainable coating technology. *Prog. Org. Coatings* 207, 109399. <https://doi.org/10.1016/J.PORGCOAT.2025.109399>.
- Magalhães, F.F., Neves, M.C., Pedro, A.Q., Freire, M.G., Santos-Ebinuma, V.C., Tavares, A.P.M., 2024. Recombinant laccase biosynthesis for efficient polydopamine coating. *Biochem. Eng. J.* 212, 109483. <https://doi.org/10.1016/J.BEJ.2024.109483>.
- Ooi, C.W., Hii, S.L., Kamal, S.M.M., Ariff, A., Ling, T.C., 2011. Extractive fermentation using aqueous two-phase systems for integrated production and purification of extracellular lipase derived from *Burkholderia pseudomallei*. *Process Biochem.* 46, 68–73. <https://doi.org/10.1016/j.procbio.2010.07.014>.
- Pan, Y., Yang, J., Wu, J., Yang, L., Fang, H., 2022. Current advances of *Pichia pastoris* as cell factories for production of recombinant proteins. *Front. Microbiol.* 13, 1059777. <https://doi.org/10.3389/FMICB.2022.1059777/XML>.
- Pedro, A.Q., Oppolzer, D., Bonifácio, M.J., Maia, C.J., Queiroz, J.A., Passarinha, L.A., 2015. Evaluation of MutS and Mut+ *Pichia pastoris* strains for membrane-bound Catechol-O-Methyltransferase biosynthesis. *Appl. Biochem. Biotechnol.* 175, 3840–3855. <https://doi.org/10.1007/S12010-015-1551-0>.
- Pereira, J.F.B., Freire, M.G., Coutinho, J.A.P., 2020. Aqueous two-phase systems: towards novel and more disruptive applications. *Fluid Phase Equilibria J.* 505. <https://doi.org/10.1016/j.fluid.2019.112341>.
- Popkov, A., Su, Z., Sigurdardóttir, S.B., Luo, J., Malankowska, M., Pinelo, M., 2023. Engineering polyelectrolyte multilayer coatings as a strategy to optimize enzyme immobilization on a membrane support. *Biochem. Eng. J.* 193, 108838. <https://doi.org/10.1016/j.bej.2023.108838>.
- Preethi, P.S., Gomathi, A., Srinivasan, R., Pavan Kumar, J.G.S., Murugesan, K., Kodiveri Muthukailannan, G., 2020. Laccase: Recombinant Expression, Engineering and Its Promising Applications. pp. 63–85. [https://doi.org/10.1007/978-981-15-1710-5\\_3](https://doi.org/10.1007/978-981-15-1710-5_3).
- Rajagopal, D., Show, P.L., Tan, Y.S., Muniandy, S., Sabaratnam, V., Ling, T.C., 2016. Recovery of laccase from processed *Hericium erinaceus* (Bull.:Fr) Pers. fruiting bodies in aqueous two-phase system. *J. Biosci. Bioeng.* 122, 301–306. <https://doi.org/10.1016/j.jbiosc.2016.01.016>.
- Ryu, J.H., Messersmith, P.B., Lee, H., 2018. Polydopamine surface chemistry: a decade of discovery. *ACS Appl. Mater. Interfaces* 10, 7523–7540. <https://doi.org/10.1021/acsami.7b19865>.
- Santos, A.G., Buarque, F.S., Ribeiro, B.D., Coelho, M.A.Z., 2022. Extractive fermentation for the production and partitioning of lipase and citric acid by *Yarrowia lipolytica*. *Process Biochem.* 122, 374–385. <https://doi.org/10.1016/j.procbio.2022.09.011>.
- Sarkari, S., Khajehmohammadi, M., Davari, N., Li, D., Yu, B., 2022. The effects of process parameters on polydopamine coatings employed in tissue engineering applications. *Front. Bioeng. Biotechnol.* 10, 1005413. <https://doi.org/10.3389/fbioe.2022.1005413>.
- Shibusawa, Y., Takeuchi, N., Sugawara, K., Yanagida, A., Shindo, H., Ito, Y., 2006. Aqueous-aqueous two-phase systems composed of low molecular weight of polyethylene glycols and dextrans for counter-current chromatographic purification of proteins. *J. Chromatogr. B* 844, 217–222. <https://doi.org/10.1016/J.JCHROMB.2006.07.028>.
- Silvério, S.C.C., Rodríguez, O., Tavares, A.P.M.P.M., Teixeira, J.A.A., MacEdo, E.A.A., 2013. Laccase recovery with aqueous two-phase systems: enzyme partitioning and stability. *J. Mol. Catal. B Enzym.* 87, 37–43.
- Sinha, J., Dey, P., Panda, T., 2000. Extractive fermentation for improved production of endoglucanase by an intergeneric fusant of *Trichoderma reesei*/*Saccharomyces cerevisiae* using aqueous two-phase system. *Biochem. Eng. J.* 6, 163–175. [https://doi.org/10.1016/S1369-703X\(00\)00082-6](https://doi.org/10.1016/S1369-703X(00)00082-6).
- Sondhi, S., Chopra, N.K., Kumar, A., Gupta, N., 2023. Laccase: a green solution for environmental problems. *Adv. Environ. Eng. Res.* 04, 1–32. <https://doi.org/10.21926/aer.2302030>.
- Stenekes, R.J.H., Franssen, O., Van Bommel, E.M.G., Crommelin, D.J.A., Hennink, W.E., 1999. The use of aqueous PEG/dextran phase separation for the preparation of dextran microspheres. *Int. J. Pharm.* 183, 29–32. [https://doi.org/10.1016/S0378-5173\(99\)00038-1](https://doi.org/10.1016/S0378-5173(99)00038-1).
- Straathof, A.J.J., 2011. The Proportion of Downstream Costs in Fermentative Production Processes, in: *Comprehensive Biotechnology*. Elsevier, pp. 811–814. <https://doi.org/10.1016/B978-0-08-088504-9.00492-X>.
- Sun, W., Davison, P., 2001. Effect of dextran molecular weight on protein stabilization during freeze-drying and storage - PubMed. *Cryo Lett.* 22, 285–292.
- Vidotto, V., Benesh, S., Maina, A.M., Bruatto, M., 1993. Influence of different levels of ammonium concentrations on cell growth, RNA and protein production by *Candida albicans*. *Mycopathologia* 123, 19–25. <https://doi.org/10.1007/BF01103484>.

- Walter, H., Krob, E.J., 1988. Effect of rapidity of phase separation on the efficiency of cell fractionation by partitioning in aqueous two-phase systems. *Biochim. Biophys. Acta - Gen. Subj.* 966, 65–72. [https://doi.org/10.1016/0304-4165\(88\)90129-8](https://doi.org/10.1016/0304-4165(88)90129-8).
- Yang, J., Li, W., Ng, T.B., Deng, X., Lin, J., Ye, X., 2017. Laccases: production, expression regulation, and applications in pharmaceutical biodegradation. *Front. Microbiol.* 8. <https://doi.org/10.3389/fmicb.2017.00832>.
- Zhang, Z., Shah, A.M., Mohamed, H., Zhang, Y., Sadaqat, B., Tsiklauri, N., Sadunishvili, T., Song, Y., 2022. Improved laccase production in *Pleurotus djamor* RP by atmospheric and room temperature plasma (ARTP) mutagenesis. *Electron. J. Biotechnol.* 58, 1–9. <https://doi.org/10.1016/J.EJBT.2022.03.005>.