Complete wastewater discoloration by a novel peroxidase source with promising bioxidative properties

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Research Article

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Abstract

The occurrence of micropollutants in aqueous matrices has become a global concern and a challenge for wastewater treatment plants. Monitoring their toxicity has shown that these compounds, even at low concentrations, pose risks to human and environmental health. Therefore, our study aimed to prospect immobilization strategies for non-commercial oxidoreductase enzymes and insert them in the context of pollutant remediation. The enzymatic extract was obtained by submerged fermentation of the fungus *Trichoderma koningiopsis* in an alternative substrate, consisting of fresh microalgal biomass from the phycoremediation process. The immobilization efficiency of peroxidase (POD) was evaluated by monitoring the residual activity (RA) and the discoloration potential (DP) of a synthetic dye solution. Concomitantly, the catalytic properties of free POD were explored, and the most promising storage strategy to maintain the enzymatic activity was studied. Guaiacol peroxidase from *T. koningiopsis* expressed specific activity of up to 7801.1 U mg^{-1} in the free form, showing stability when subjected to up 80°C in a pH range between 4.0–8.0. Furthermore, the bioproduct immobilized on magnetic nanoparticles expressed up to 688% RA and 100% DP. An increase in the RA of the enzyme, both in free and immobilized form, was also observe after storage for up to 8 months. The synthesized magnetic nanozymes showed good reusability, maintaining 13546.4 U mg^{-1} after ten cycles and removing 93% of color in a second batch. Toxicological evaluation with *Allium cepa* indicated that the enzymatic process of color removal with immobilized POD, despite maintaining unwanted cytotoxic effects, was essential to eliminate genotoxic effects. In this sense, the immobilization processes of *T. koningiopsis* peroxidase presented in our work are promising for the enzyme market and for the wastewater treatment sector.

Highlights

- High peroxidase production by submerged fermentation with non-commercial feedstock.
- Maintenance of peroxidase activity after storage at room temperature for months.
- Different immobilization approaches achieved an increase in specific activity.
- Low-cost and straightforward magnetic nanozymes synthesis showed good reusability.
- Immobilized peroxidase could successfully eliminate genotoxic effects.

1 Introduction

As part of the subclass of organic chemical compounds known as emerging pollutants, synthetic dyes have been in the environment for a long time. Still, the impacts on human and environmental health have been recognized recently. Their aromatic and highly complex structure makes them resistant to degradation processes and resistant to light and ultraviolet irradiation (Ali et al., 2013). Furthermore, it is estimated that the textile industry alone generates around 4500 million kiloliters of wastewater per year (Singh et al., 2013), with a significant amount (10-15%) of the total dyes used by this industry continuously discharged into the environment (Saroj et al., 2014).

Because conventional wastewater treatment systems show limited capacity to remediate these contaminants, alternative removal or remediation methods are required. In this context, enzymatic processes arise with catalytic and oxidative properties capable of biotransforming persistent pollutants. These properties put the global enzyme market in constant expansion, with projections of 3.4% annual growth between 2020-2027 (Research and Markets, 2021), starting to compete with chemical catalysts. Therefore, there is a growing interest in new enzyme sources and low-cost upstream processes, enabling its use as an environmentally and economically viable biocatalyst (Jun et al., 2019).

In the wastewater treatment sector, the oxidoreductase enzymes laccase (Lac) and peroxidase (POD), mainly obtained from fungi, have been explored for emerging pollutants degradation (Morsi et al., 2020; Viancelli et al., 2020), presenting competitive advantages as performance in mild environmental conditions, low aggressiveness to the environment, and high specificity (Abedi et al., 2011). However, it is essential to emphasize that there are some challenges considering enzymatic application in contaminants removal, mainly concerning the need to keep the reactions stable and favorable to obtain the maximum catalytic performance.

These challenges can be overcome with non-commercial enzymes, which are highly stable under extreme pH and temperature conditions and are also economically advantageous. In addition, enzymatic manipulation by immobilization is an exciting strategy to avoid enzymatic denaturation or inhibition and to enhance catalytic action, stability, and reuse by several cycles. Promising laccase and peroxidase immobilization results have been reported using organic supports, such as carbon, synthetic polymers, chitosan, sodium alginate, and inorganic supports, such as silica, clay, and metal oxides (Jun et al., 2019; Shakerian et al., 2020). Different strategies have also been promoting the contact between support and enzymes, such as traditional encapsulation and adsorption, and novel techniques by synthesizing magnetic nanozymes (Bilal et al., 2018).

The enzymatic immobilization opens interesting perspectives for novel technologies development, both for the enzyme market and the wastewater treatment sector, considering no universal protocols established for these processes (Anawar and Ahmed, 2019). Therefore, the present study aims to study immobilization strategies of non-commercial peroxidase, obtained through *Trichoderma* submerged fermentation supplemented exclusively by microalgal biomass, and evaluate its catalytic and toxicological performance in free and immobilized forms for synthetic dye discoloration.

2 Materials And Methods

2.1 Enzymatic extract obtention

The enzymatic extract was produced by a submerged fermentation process using *Trichoderma koningiopsis* MK860714, supplemented with microalgal biomass, both from noncommercial sources.

The fungus is cultivated by the Laboratory of Microbiology and Bioprocesses, in partnership with the Laboratory of Agroecology (Federal University of Fronteira Sul, Erechim, Brazil). It was isolated from the

weed *Digitaria ciliaris*, from soybean and corn cultivation areas in southern Brazil (Reichert Júnior et al., 2019).

The *Chlorella* spp. microalgal biomass was cultivated and kindly provided by Embrapa Swine and Poultry (Concórdia, Brazil). Its cultivation occurs in a biological reactor, aiming at the ammonia and phosphorus removal from swine wastewater digestate by phycoremediation. The biomass is composed of 56% protein, 35% carbohydrates, 2% lipids, and 7% minerals (Michelon et al., 2016).

The fermentation was carried out in Erlenmeyer with a practical volume of 100 mL. The medium was composed of 10 g fresh microalgal biomass (89% humidity), according to (Stefanski et al., 2020). After adding 90 mL of distilled water to the biomass, the Erlenmeyer was autoclaved at 120 °C and 1 atm for 20 min. Then, the medium was inoculated with 10 mL of a suspension containing 10^6 spores of *T. koningiopsis* per milliliter. The fermentation was conducted for 72 h in an orbital shaker (New Brunswick™, Germany) at 120 rpm and 28 °C.

After fermentation, the Erlenmeyer content was filtered to remove the fungal and microalgal biomass, and the liquid permeate was centrifuged (NT 815 - NovaTecnica, Brazil) at 2000 rpm and 4 °C for 10 min. The supernatant corresponds to the enzymatic extract, and the rest of the fermentation content was sterilized and discarded.

2.2 Enzymatic activity quantification

The quantified oxidoreductases enzymes were guaiacol peroxidase, according to Devaiah and Shetty (2009) method, and laccase, with methodology adapted from Hou et al. (2004).

For peroxidase, 1 mL of extract was added to the reaction medium composed of 0.5 mL of the substrate guaiacol 1% v v^{-1}, 1 mL of the cosubstrate hydrogen peroxide 0.08% v v^{-1}, 2 mL of distilled water, and 1.5 mL of sodium phosphate buffer at 5 mmol L^{-1} and pH 5.5.

The U unit of peroxidase specific activity was defined as the enzyme amount capable of causing 0.001 increase in the absorbance unit per minute per milligram of total protein, when incubated at 25 °C for 10 min, and read in a spectrophotometer (UV-M51-Bel, Italy) at 470 nm. Total protein concentration was quantified by Bradford (1976) method, and specific activity was expressed in U mg^{-1}, dividing the enzymatic activity (U mL^{-1}) by the protein concentration (mg mL^{-1}) of the sample.

For laccase, 0.2 mL of extract were added to the reaction medium containing 0.4 mL of 2,2'-azino-di-(3-ethylbenzothialozin-6-sulfonic acid) (ABTS) 10 mmol L^{-1} and 3.4 mL of sodium acetate buffer at 50 mmol L^{-1} and pH 4.8. After incubation for 5 min at 40 °C, the substrate oxidation was monitored by absorbance read in a spectrophotometer at 420 nm (ε_{420}=36000 L mol^{-1} cm^{-1}). The U unit of laccase was defined as the enzyme amount capable of oxidizing 1 µmol of substrate per minute.

2.3 Discoloration studies

Preliminary discoloration studies were carried out with four commercial synthetic dyes (Guarany Ind. Ltda., Brazil) to verify the affinity between enzyme and dye and the relationship between enzymatic activity and discoloration potential (DP).

The dyes studied were red (455 nm), blue (595 nm), yellow (414 nm), and brown (530 nm). The tests were conducted according to previous studies (Klanovicz et al., 2020). The dye solution, prepared with distilled water at 100 mg L^{-1}, was treated with 5 or 10 mL of crude enzymatic extract and 40 mg L^{-1} of hydrogen peroxide 35% v v^{-1}.

Crude extracts with different peroxidase:laccase ratio, obtained in previous studies (Stefanski et al., 2020) varying operational parameters in fermentation, were applied in the dye removal study. The tests were carried out at a volume of 100 mL under constant agitation (160 rpm) and temperature (25 °C) for 5 h. The DP was quantified by the difference between initial (Abs_i) and final (Abs_f) absorbances of the treated samples (Equation 1).

$$DP\ (\%) = \frac{Abs_i - Abs_f}{ABS_i} * 100 \qquad (1)$$

2.4 Investigation of the peroxidase activity behavior

Initially, the reaction conditions and the substrate:enzyme ratio influence in enzymatic activity were evaluated. The effect of substrate (0.5-1.0 mL) and cosubstrate (0.5-1.0 mL) concentrations in the reaction medium, and the impact of pH (4.0-8.0) and incubation temperature (20-80 °C) were studied by the Plackett-Burman experimental design, conducted with 8 trials and 3 replicates under central point conditions.

Then, setting the conditions in the most favorable trial of the design, the behavior of specific activity, pH, and oxidation-reduction potential (ORP) were monitored over time to understand the catalytic cycle of the peroxidase oxidation reaction.

In addition, crude extract samples were stored, protected from direct sunlight, in three different conditions: at room temperature (ranging between 18-28 °C), in refrigerator (4 °C), and in freezer (−10 °C). The monitoring of residual activity (RA) was carried out in 15, 30, 60, 90, and 170 days and calculated by Equation 2, in which SA_i and SA_f correspond to the pre- and post-storage specific activity, respectively.

$$RA\ (\%) = \frac{SA_f}{SA_i} * 100 \qquad (2)$$

2.5 Prospection techniques for enzymatic immobilization

The *T. koningiopsis* enzymatic extract was submitted to three different immobilization strategies (Table 1), and their efficiency was measured by residual activity and discoloration potential.

Table 1 Strategies prospected to immobilize *T. koningiopsis* peroxidase.

Immobilization strategy	Support material	Acronym	Reference
Encapsulation	Ca-alginate beads	POD-beads	Rezvani et al. (2015)
		ModPOD-beads	
Adsorption	K10-montmorillonite	MK10-POD	Coghetto et al. (2012)
	Ornamental rock dust	OR-POD	
Magnetic nanozymes	Urea-NaOH	MN-POD	Sadaf et al. (2020)

The RA was calculated by Equation 2, but in this case, SA_i and SA_f correspond, respectively, to the specific activity of free and immobilized peroxidase. Both contents retained in the immobilization supports and the permeate were subjected to enzymatic quantification.

Preliminary tests were conducted to monitor immobilized enzyme activity after storage at room temperature. In addition, the recovery and reuse potential of MN-POD was first assessed in two ways: by activity after 10 catalytic cycles, renewing the peroxidase reaction medium described in Section 2.4; and by a new decolorization batch, according to the procedure detailed in section 2.3. The MN-POD recovery was made by the commercial magnet NdFeB (N45).

2.5.1 Encapsulation in Ca-alginate beads

The immobilization in beads was tested in two ways: in the presence and absence of the enzyme-substrate and cosubstrate. In both cases, the methodology was adapted from Rezvani et al. (2015), according to which initially a gel was prepared with sodium alginate and distilled water (1.75% w v^{-1}). In this step, the addition of 0.08% hydrogen peroxide and 1% guaiacol to the gel was tested, both in a proportion corresponding to the reaction medium of the enzymatic activity measurement.

The enzymatic extract was then added to the gel (6.7% v v^{-1}). After homogenization, it was dripped with a peristaltic pump (20 mL min^{-1}) to 100 mL of a calcium chloride solution (4.5% w v^{-1}), and kept in an ice bath under constant magnetic stirring.

The beads formed in the previous procedure were kept immersed in the calcium chloride solution for 20 min at 4 °C. Then, a sieve was used to retain the beads, which were then thoroughly washed with distilled water, thus obtaining the POD- and ModPOD-beads samples.

2.5.2 Adsorption on inorganic supports

The physical adsorption process was tested on two supports: K10-montmorillonite (Lot #STBH6207, Sigma-Aldrich, Merck, Brazil) and ornamental rock dust (donated from a local rock beneficiation process in Rio Grande do Sul, Brazil).

The ornamental rock is the acid volcanic rock rhyodacites, from the *Província Paraná Fácies Caxias* formation (K1αcx), composed with at least 50% of SiO_2 and several oxides. It arrived in the form of sludge (0.9% humidity) and went through an autoclave sterilization process (20 min, 1 atm, 120 °C), drying (2 h, 550 °C), and grinding before the immobilization process.

For both supports, the contact with enzymatic extract occurred under magnetic stirring in an ice bath for 10 min, following a methodology adapted from Coghetto et al. (2012). Initially, 2 g of support was homogenized with 60 mL of sodium phosphate buffer 5 mmol L^{-1} and pH 5.5. Then, the enzymatic extract was added at 3:10 v v^{-1} enzyme:buffer.

The suspension obtained from this procedure was vacuum-filtered to remove the liquid portion and kept in a desiccator over 48 h for complete drying. The MK10-POD and OR-POD samples were obtained after grinding the content retained in the filtration procedure.

2.5.3 Magnetic nanozymes synthesis

The methodology for magnetic nanozymes synthesis was adapted from Sadaf et al. (2020), in which initially a solution composed of 80 mL of distilled water, 12 g of urea, and 8 g of NaOH was prepared. Under constant stirring at room temperature, the enzymatic extract was added at 3% v v^{-1}, and the solution was kept under stirring for 15 min. The enzymatic solution was kept over 12 h in a refrigerator (4 °C). Then, 100 mL of $FeCl_3/FeSO_4$ (molar ratio 2:1) solution was added to the enzymatic solution under stirring and 60 °C.

The nanozymes suspension was filtered, washed with distilled water, and then with 95% ethanol. The retained nanozymes film were dried at 70 °C for 2 h and kept in a desiccator over 24 h. The NM-POD sample was obtained after grinding the content retained in the filter.

2.6 Toxicological assessment of enzymatic treatment

The cytotoxicity and genotoxicity study of the dye solution before and after the enzymatic treatment, with free and immobilized peroxidase, was carried out with the organism *Allium cepa* according to the method described by Fiskesjö (1985).

Onion bulbs purchased in a local market were placed in water to germinate over 72 h, protected from direct sunlight. After root growth, they were exposed in duplicate to the treatments (pure or diluted in distilled water at a 1:8 ratio) for 48 h at room temperature, maintaining a negative control exposed to tap water. The exposed roots were hydrolyzed in HCl at 1 mol L^{-1} for 10 min at 60 °C, and then the slides with

the meristematic cells of *A. cepa* were prepared. This preparation is necessary for microscope analysis, so the cells were stained using the *Panótico Rápido*® kit (Laborclin).

Genotoxic effects were observed qualitatively using the Olympus CX21 Biological Microscope, in which changes such as micronuclei, binucleate cells, and nuclear buds were considered. The cytotoxic effects were quantified through mitotic changes, considering the different phases of cell division (metaphase, anaphase, and telophase). The mitotic index (MI) was calculated as the ratio between the number of cells observed in any of the division phases (NDC) and the total number of cells analyzed (NTC), according to Equation 3, with a count of up to 100 cells per slide.

$$MI\ (\%) = \frac{NDC}{NTC} * 100 \qquad (3)$$

2.7 Analytical procedures

Structural analysis of support and immobilized peroxidase were performed by the Nuclear and Energy Research Institute (IPEN/CNEN-SP, Brazil), namely infrared spectrometry (FTIR), using a Nicolet 6700 equipment (Thermo Scientific); and X-ray diffraction (XRD), using a Bruker D8 Advance 3 kW diffractometer, equipped with a Cu-K alpha radiation tube and scintillation detector, reading samples at 2θ angle ranging from 20 to 90 degrees with an increment of 0.05°. Crystalline phases in the XRD analysis were identified by comparing the sample diffractogram with the International Centre for Diffraction Data (ICDD) using X'Pert HighScore Plus software.

ORP and pH measurements were performed by pH/mV bench meter from HANNA Instruments HI2221. Using a silver chloride reference electrode, we quantified the oxidation potential of peroxidase according to Skoog et al. (2013). The obtained values were converted to mV *vs.* SHE, the standard unit of this electrochemical quantity (Brown et al., 2017).

2.8 Statistical analysis

The data obtained from the tests were analyzed by analysis of variance (ANOVA), using the software Statistica 8.0 and the online software Protimiza Experimental Design - http://experimental-design.protimiza.com.br/ (Rodrigues and Iemma, 2014).

3 Results And Discussion

3.1 Relationship between enzymatic activity and discoloration potential

Preliminary tests with four synthetic dyes were carried out in the present work, aiming to find the affinity between *T. koningiopsis* peroxidase and the structure of the dyes, expecting that enzymes use it as a substrate in the enzymatic bioxidative reaction. Table 2 shows the results achieved.

Table 2. Analysis of enzymatic treatment of synthetic dyes after five hours of reaction. Note: Means followed by equal letters indicate that the samples do not differ by Tukey test at 95% confidence level.

Enzymatic extract amount	Dye discoloration (%)			
	Red	Blue	Yellow	Brown
5 mL	2.8[a]	0.0[a]	3.2[a]	54.4[b]
10 mL	3.3[a]	22.4[c]	4.2[a]	45.5[d]

The most significant discoloration percentage occurred for the brown dye (54.4%), with statistical significance, comparing the enzymatic extract amount used (5 or 10 mL) and the four types of dyes. There was no statistically relevant difference for the red and yellow dyes, but for the blue dye, the enzymatic treatment with 10 mL of the crude extract showed a statistically adequate color removal (22.4%).

The different discoloration potential observed between dyes indicates the effect of structural diversity. Each dye has a specific molecular structure, which conveys the characteristics of color and dyeing. Considering the molecular aspect, the relationship between the structure and the enzymatic action depends on the type, number, and position (ortho or para) of the groups linked to the aromatic ring of the dyes. In this sense, the molecular aspect can accelerate, delay, or even cause complete inhibition of the enzymatic action (Dawkar et al., 2009).

Due to oxidative nature, there are several areas in which peroxidase could replace current techniques based on chemical catalysis (Hamid and Khalil-ur-Rehman, 2009). Although many peroxidases have already been extensively studied, most studies use commercial and high-cost enzymes, making environmental applications unfeasible. In this study, the crude enzymatic extract showed specificity for brown dye discoloration. This result is promising since studies that used peroxidases have reported affinity with other dyes. For example, peroxidase extracted from industrial soybean residues was efficient in degrading blue dye after 3.3 hours of reaction (70% degradation) (Miranda-Mandujano et al., 2018), and peroxidase from macrophytes performed well in the degradation of direct azo dyes such as amaranth and black starch (93% and 87% respectively, after 120 hours of reaction) (Haddaji et al., 2014). Also, for the blue dye, two peroxidase sources (*Ipomea palmata* and *Saccharum spontaneum*) showed removals of 15% and 70%, respectively, after 1 to 2 hours of treatment, indicating different specificities of plant peroxidase to the same dye (Shaffiqu et al., 2002). Enzymatic treatments conducted in other reactional systems also provided different color removal results, as shown by a previous study (Klanovicz et al., 2020), in which rice bran peroxidase removed 39% of the color after 3 hours of reaction in a microwave system, while complete removal was achieved after 24 h on an orbital shaker.

We verified the relationship between peroxidase and laccase activity with color removal in this study. For this purpose, crude extracts with different enzymatic activity profiles were applied in the brown dye discoloration process. In this experiment, the most remarkable discoloration (32.1%), statistically different

from the other assays, occurred with enzymatic activities of 350 U mL^{-1} and 0.03 U mL^{-1} for peroxidase and laccase, respectively. Even so, these activities did not represent the most considerable enzymatic amount among the 17 assays performed. The maximum and statistically relevant peroxidase activity was 826 U mL^{-1}, in which the color removal was 7.2%. For laccase, the maximum value was 0.07 U mL^{-1}, and the dye discoloration was only 7.9%.

Both for peroxidase and laccase, a statistically different enzymatic activity between the assays was observed, which can be explained by the wide range of operational conditions in the fermentation process for the crude extracts production, such as temperature, pH, and agitation. Thus, the fermentation manipulation can provide an enzymatic pool with different performances in application processes, although the maximum activity did not represent the maximum discoloration potential.

Similar findings were observed when manganese peroxidase (MnP) and manganese independent (MIP) activities were evaluated (Shrivastava et al., 2005). The authors found a difference between the enzymatic activity and the discoloration of synthetic sulfonephthalein dyes. The variations in the discoloration reflect the differences in the isoenzyme composition of the MnP and MIP, resulting in a difference in the kinetic constants (varying the maximum rate of reaction from 0.057 to 1 U mL^{-1}) and the substrate specificity (Shrivastava et al., 2005). Another study also concluded that the reaction conducted with laccase alone did not degrade certain types of dyes. When the redox mediator violuric acid was added, however, the degradation efficiency reached 90% using 2/5 of the enzymatic activity of the tests without mediator (Soares et al., 2001).

Thus, our results indicate that the change in operational parameters in fungal fermentation, in addition to providing enzymatic activity variations, must have influenced the composition of isoenzymes. In turn, it caused differences in specificity and interfered in decolorization activity. It is noteworthy that until this step of the work, no enzymatic manipulation method was employed. Later on, the reactions with immobilized peroxidase on different supports will be discussed.

3.2 Catalytic properties of free and crude peroxidase

To insert a novel enzyme source in biotechnological processes, it is essential to know and improve its catalytic properties and reactional conditions. For the first time, this work presents the preliminary characterization of guaiacol peroxidase produced by *T. koningiopsis* in submerged fermentation supplemented exclusively by fresh microalgal biomass.

The extracellular enzymes of this fungus are rarely mentioned in the literature due to the difficulty in offering favorable conditions for fermentation and supplementation for its expression, often involving costly processes for industrial applications (Jun et al., 2019). Mäkelä et al. (2020) report a wide variety of fungal peroxidases being applied as biocatalysts in reactions of environmental interest. In their study, the species *Trichoderma* is classified as Soft-Rot Fungi, about which little is known concerning peroxidase production. Azmi et al. (2019) reported a combination of lignin peroxidase production in more significant

proportion and manganese peroxidase in less proportion by *T. koningiopsis* when supplemented with oil palm fronds in solid-state fermentation.

Regardless the peroxidase source, enzymatic processes are influenced by reaction thermodynamics and need studies varying pH, temperature, and availability of substrate and cosubstrate - relevant factors in understanding the catalytic route (Nelson and Cox, 2012). In this sense, these variables were investigated in the present work for the *T. koningiopsis* POD following a Plackett-Burman design. The results indicated enzyme stability even when subjected to adverse reaction conditions.

When subjected to pH ranging from 4.0-8.0 and temperatures from 20-80 °C, the enzymatic activity was not significantly affected at 95% confidence level by the Tukey test. The enzyme:substrate and enzyme:cosubstrate ratios, on the other hand, negatively affected the enzymatic response with statistical relevance; that is, the POD activity was improved at the minimum levels studied. None of the reaction conditions led to enzyme denaturation, with the lowest activity obtained being 5733 U mg^{-1} in a test with pH 4.0, temperature 20 °C, and maximum levels of substrate and cosubstrate.

The peroxidase stability under varying conditions can be explained by the defense mechanisms previously developed by the fungus in its natural habitat (Baiyee et al., 2019). Our enzyme was isolated from weeds already adapted to its ecosystem. The supplementation of the fermentation medium with microalgae biomass induced higher peroxidase productivity compared to synthetic supplementation (Bordin et al., 2018), confirming that stress induction during the fungus growth improves peroxidase production.

In contrast to the production behavior of other enzymes, stressful environments induce higher productivity of oxidoreductase enzymes and, consequently, fermentation conditions with less nutrient availability can result in greater peroxidase expression. As well as the fungus fermentation process, the microalgae growth process occurred in a stressful environment, considering that it was cultivated with mainly ammonia and phosphorus availability (Michelon et al., 2016). Thus, according to a previous study (Stefanski et al., 2020), synergism between fungi and microalgae occurs, resulting in microalgae cells incorporated into the fungal hyphae mesh. This synergy may have led to the obtention of an enzymatic extract with high resilience capacity in peroxidase activity, which can be seen in Figure 1, where the interaction between the enzyme and reaction medium over time is shown under constant environmental conditions.

To ensure that the evaporation or natural oxidation of the enzymatic extract did not induce false positives, controls were performed and considered in the enzymatic activity quantification. During the follow-up of the reaction, in the first 192 h, the pH and ORP values were maintained without statistically significant differences. The pH remained between 7.3 ± 0.1 and 7.6 ± 0.1, and the ORP between 195.6 ± 3.3 and 214.2 ± 1.1 mV *vs.* SHE. On the other hand, the enzymatic activity varied from 7801.1 ± 179.1 to 11.3 ± 1.6 U mg^{-1}, significantly decreasing and reaching its minimum value in 96 h.

In the last experimental point studied (360 h), the pH significantly dropped to 6.5 ± 0.4, and the redox potential increased to 262.0 ± 20.4 mV *vs.* SHE. For this experimental point, the standard error became high and, therefore, the experiment was stopped. In this sense, it is understood that after 360 h, it is unfeasible to maintain the enzymatic reaction without providing maintenance.

The constant decrease in enzymatic activity in the first hours of reaction follows the same behavior as observed in a published study for guaiacol peroxidase extracted from rice bran (Klanovicz et al., 2021), in which in 24 h of reaction, the activity had already fallen more than half of the initial value. In the present study, intra and extracellular enzymatic system of the fungus can be critical in conducting the catalytic route through the quinone redox cycling mechanism. The presence of lignin and Fe^{2+} in class II heme peroxidases structure induces a reaction that produces hydroxyl radicals in the presence of hydrogen peroxide. The enzymatic behavior shown in Figure 1 can thus configure an advanced bioxidative process, in which the cosubstrate presence generates hydroxyl radicals, which in turn can regenerate cosubstrate. Thus, the enzymatic reaction is naturally maintained, without the need of external interventions (Vasiliadou et al., 2019).

The maintenance of positive ORP values during the reaction suggests that the reaction medium was receiving electrons, and the substrate was oxidized. At the end of the reaction, the increase in ORP may have been a reflection of pH decrease, these parameters being inversely proportional (Battistuzzi et al., 2010). The change in these parameters may have triggered the enzymatic reaction instability, which had already carried out multiple quinone redox cycles.

Considering the promising results of peroxidase activity and reaction maintenance even in adverse conditions, studies were conducted to monitor the enzymatic activity of the crude extract after storage. The results of this characterization are shown in Figure 2.

The enzymatic extract activity (initially 7801.1 U mg^{-1}) remained more stable at room temperature and in the refrigerator than in the freezer. For the storage condition between 18-28 °C, there was no significant difference in activity up to the 60^{th} day, reaching 105.4 ± 2.8% of residual activity. For storage at 4 °C, a considerable drop was observed between 30 and 90 days (91.4 ± 0.5%), but on the 170^{th} day, the RA returned to the initial value. In turn, the extract maintained at −10 °C, initiated a significant and current drop of enzymatic activity after 15 days, reaching 62.6 ± 1.2% of RA in 90 days.

From these tests, it was possible to determine that the enzymatic extract can be kept in the refrigerator for up to 170 days without sudden drops in peroxidase activity. In addition, keeping the extract at room temperature proved to be an interesting option for up to 60 days, but it still requires studies for longer periods, as on the 170^{th} day a significant drop was observed. Therefore, the activity behavior after storage will be discussed in the next section.

3.3 Strategies for *T. koningiopsis* peroxidase immobilization

Three different strategies were selected to conduct the immobilization process of crude peroxidase extract (Free-POD), obtained in the fermentation process of *Trichoderma* supplemented by *Chlorella* spp. Two parameters were chosen to determine the immobilization efficiency: the residual activity and the discoloration potential of the brown dye solution at 100 mg L^{-1}. This treatment process was selected because of its promising results in crude extract assays, the scarcity of studies on this dye, and the objective of inserting this bioproduct in the context of low-biodegradability contaminant remediation.

The immobilization strategies of this study were selected, taking into account the cost-benefit in the scenario of effluent treatment and circular economy. The strategies made it possible to obtain relevant results, with an increase in specific activity of up to 588.5% and color removal of up to 100%, as shown in Table 3.

Table 3 Immobilization of *T. koningiopsis* POD and results of residual activity (RA) and discoloration potential (DP) for brown dye.

Technique acronym	RA (%)	Treatment results	
		DP (%)	Final pH
Free-POD[1]	100.0	20.4	7.6
Control[2]	–	10.7	7.8
POD-beads	6.3	35.5	6.0
ModPOD-beads	3.1	36.4	5.9
MK10-POD	34.9	8.0	7.5
OR-POD	10.8	41.6	7.6
MN-POD	688.5	100.0	2.4

[1] Specific activity = 3066.2 U mg^{-1}; [2] Dye solution (100 mg L^{-1}) in the presence of 40 mg L^{-1} of hydrogen peroxide 35%.

RA values up to 100% indicate enzymatic retention in the support for the technique studied, and higher values demonstrate a positive interaction between support and enzyme. For the encapsulation method, it was possible to retain only 6.3% of enzyme in the POD-beads, and 22.0% was lost to the calcium chloride solution. When the technique was modified (ModPOD-beads), adding the POD substrate and cosubstrate to the beads to manipulate the affinity between enzyme and support, the retention was lower (3.1%), as well as the activity in the solution (20.7%). These results indicate that the encapsulation technique is not suitable for *Trichoderma* peroxidase because of the low enzymatic retention in the beads. There was also a loss of enzymatic activity in the process, since the RA balance considering beads and solution does not reach the reference value (100%).

Two different supports were studied in the adsorption method: the commercial clay MK10, widely used in enzymatic immobilization studies, and noncommercial ornamental rock dust (OR). The choice of this alternative support was motivated by the environmental problem related to the disposal of this waste material, which is constantly generated by rock processing industries. For both supports, enzymatic activity was lost to the buffer solution, in which the contact process with peroxidase was conducted (18.5% for MK10 and 15.0% for OR). However, the commercial support had a greater affinity with the enzyme, with a 34.9% activity retention, when compared to rock dust (10.8%). When making the RA balance, a loss of activity was observed in this case. However, compared to the encapsulation strategy, adsorption proved to be more promising and retained higher enzymatic activities in the supports.

The most advantageous process from the perspective of maintenance and even increasing enzymatic activity after immobilization was through magnetic nanozymes synthesis, conducted with low-cost reagents. In this strategy, although part of the activity was lost to the solution in which the nanoparticles precipitated, the bond between POD and support favored the expression of catalytic activity. This process made it possible to obtain 21111.1 U mg^{-1} of specific activity in MN-POD, representing a RA of 688.5%, and relevant potential for application in biotechnological processes because of their magnetic properties.

As in the present study, commercial horseradish peroxidase was immobilized by magnetic nanocomposites by Chang et al. (2015), exhibiting biochemical properties superior to those of the free enzyme, maintaining the RA in 100% and improving resistance to temperature and pH variations. In turn, Monteiro et al. (2019) investigated the immobilization of commercial *Candida antarctica* lipase on magnetic nanoparticles and achieved RA values of up to 120% for a pH range between 8 and 10. This same RA value was achieved for POD magnetic nanozymes in Sadaf et al. (2020) study, however for a pH range between 3 and 4. Although for each nanoparticle synthesis study different methods are used, and will give them particular biochemical characteristics, Zdarta et al. (2018) highlight that the presence of many functional groups in the materials used for the synthesis gives to the nanozymes a high surface area and porosity. These characteristics may justify the fixation of a large amount of enzyme in the nanoparticles and the increase in catalytic activity compared to the free enzyme.

Among the techniques studied in the present work, MN-POD showed the most significant potential for discoloration, removing 100% of the color of the dye solution in 5 hours. However, in this enzymatic treatment, the acidic pH of the post-treatment solution would require adjustment before its final disposal. For treatments with POD immobilized by encapsulation and adsorption techniques, the pH remained close to neutrality; however, color removals of only up to 41.6% were obtained.

From the data presented in Table 3, it is not possible to determine a direct relationship between RA and DP values. It is worth mentioning that control tests with dye solution and support, in the absence of enzyme, were conducted. However, they did not bring conclusive contributions, since the control results were mostly influenced by pore size and contact surface, and the results of the enzymatic treatment depend on the cross-interaction between support, enzyme and dye, giving complexity to the process to be investigated in future works. Accordingly, the immobilization process becomes relevant to provide, in

addition to enzymatic retention, a combined action between support and substrate that enhances the catalytic reaction. In addition to the enzymatic activity retention, the immobilization process must provide the possibility of reusing and storing the enzyme, maintaining catalytic efficiency. In this sense, MN-POD was applied in the second batch brown dye treatment, in which 93.8% of discoloration was obtained in 5 hours, maintaining the same conditions as the first batch.

In the storage test, MK10-POD, OR-POD, and MN-POD samples were stored at room temperature protected from direct sunlight, and their activity was measured after 2.5 months of the immobilization process. Comparing to free-POD, the MK10-POD, OR-POD, and MN-POD remained with residual activities of 192.5%, 3.0%, and 10637.2%, respectively, after the storage period. Additionally, MN-POD activity was verified after about 8 months, and it has 9237.9% of RA.

The data in Table 3 reveal that OR-POD lost enzymatic activity after storage, while MK10-POD and MN-POD increased. For all samples, the specific activity of the immobilized enzyme remained higher than in free form. The same behavior of activity increase after storage was observed for the crude extract. This phenomenon can be attributed to changes in the biochemical conformation of the protein or to an increase in specificity with the substrate, which is already reported in the literature for enzymes submitted to upstream processes, such as concentration and enzymatic purification (Abedi et al., 2011).

The MN-POD sample stored for about 8 months, with 283256.5 U mg^{-1}, was subjected to 10 cycles of recovery and reinsertion in a reaction medium to monitor the residual activity behavior. In cycles 2 and 3, a statically significant decay in RA was observed, arriving at the end of cycle 3 with 37.2%. From cycles 4 to 10, there was no considerable decay in RA. At the end of the experiment, the MN-POD had 13546.4 U mg^{-1} of specific activity, corresponding to 4.8% of RA. Both activity and degradation efficiency decay are expected in reusability assays, considering that the enzyme active site and the support pore voids are filled over cycles (Sastre et al., 2020; Shakerian et al., 2020).

In this study, the enzymatic activity improvement was possible using simple immobilization techniques and interesting cost-benefit, factors of great relevance in industrial applications. In addition, the bioproduct storage under mild conditions, dispensing chemical supplement addition, or freeze-drying processes, are advantages to its insertion in the enzyme market for industrial applications, which is in constant expansion according to the latest Global Industry Analysts report (Research and Markets, 2021).

3.4 Support and immobilized peroxidase characterization

OR-POD, MN-POD, and MK10-POD samples were submitted to characterization to understand the interactions between enzyme and support. In addition, support samples without enzyme were also characterized.

The FTIR and XRD graphs are presented in Figure 3, and in a first look it is possible to observe the similarities when comparing the spectrum of the support and support with enzyme; the transmittance

peaks are in the same wavenumber range in FTIR analysis, as well as the intensity peaks in the same 2q values in XRD patterns.

These findings indicate that the enzyme-support interaction did not modify the characteristic chemical bonds and the structural arrangement of the support material atoms during the immobilization processes. This aspect is positively relevant considering that immobilization aims to maintain both enzymatic and material properties. In the current work, this objective was achieved, and the immobilization maintained peroxidase catalytic activity without affecting the physical-chemical characteristics of the materials.

Another relevant observation is the similarities, for the two analyzes, of the peak behavior for the adsorption methods (samples OR-POD, OR, MK10-POD, and MK10), indicating that ornamental rock dust is very similar to the commercial and widely used clay MK10. The FTIR spectra of these samples show weak peaks between 820 and 947 cm^{-1} (Figure 3a), corresponding to Al–Al–OH, Al–Fe–OH and Al–Mg–OH deformation, and Si–O stretching mode. The absence of peaks around 1000 cm^{-1} indicates no water content in the samples (Ahmed et al., 2018).

For MN and MN-POD samples, small intensity peaks were observed at 935, 1059, and 1144 cm^{-1}, suggesting the presence of a significant amount of specifically adsorbed sulfate groups. These can occupy external and internal surfaces (sulfated goethite), and the amount of these groups is related to the acidic pH. The peak at 1591 cm^{-1} indicates H_2O bending vibrations (Gotić and Musić, 2007).

In XRD analysis of magnetic nanoparticles, the peaks indicated in Figure 3b, between 27 and 50°, confirm goethite presence as a single phase, characteristic of superparamagnetic particles (Gotić et al., 2008). Also, characteristic peaks corresponding to planes of Fe_3O_4 and MgO crystals were observed, whose low intensity is due to the small size of the synthesized particles (Beyki et al., 2016).

The amorphous characteristic of the MN and MN-POD samples is another positive relevant finding from XRD analysis. It is beneficial for many applications due to the superior catalytic activity, superparamagnetic behavior, and large surface area compared with crystalline structures (Machala et al., 2007). Experimental conditions are relevant in amorphous Fe_3O_4 synthesis, especially regarding particle size and structure homogeneity (Jolivet et al., 1994). In this sense, the method we used can be considered adequate for obtaining excellent magnetic nanoparticles.

From the XRD patterns in Figure 3b, it is possible to verify very similar crystalline structures of OR-POD, OR, MK10-POD, and MK10. The 2q values variation between samples do not exceed 0.5 degrees, but there are some differences in intensity. The main highlight is the occurrence of an intense peak at 60.2° for OR, confirming that this material has SiO_2, which was expected since it is the primary mineral of this material. Small intense peaks associated with SiO_2 are also clearly observed at 26.7 and 27.5°, both for OR and MK10. The remaining low intense peaks in Figure 3b are natural mineral signatures and were expected for these materials (Ahmed et al., 2018).

3.5 Toxicological assessment of enzymatic treatments

The test organism selected was *A. cepa*, in which disorders in cell division can be counted and compared using the mitotic index. With a negative control as a standard (cells exposed to tap water), higher or lower mitotic index (MI) values indicate disorders in the mitosis process, as shown in Table 4.

According to Fiskesjö (1985), one of the precursors of this test, the meristematic onion cells study, is indicated for environmental monitoring, presenting advantages such as low cost, easy execution, and possibilities of microscopic research, being possible to evaluate the chromosomal damage caused by an aqueous matrix and disorders in cell division.

Table 4 Disorders in cell division of *A. cepa* after being exposed for 48 h to enzymatically treated dye solutions.

Treatment assay	Solution parameters		MI (%)
	DP (%)	pH	
Negative control[1]	–	~ 7.0	17.9
Crude dye solution	0.0	8.3	19.0
Free-POD	20.4	7.6	7.7 e 6.9*
ModPOD-beads	36.4	5.9	65.4 e 22.4*
MK10-POD	8.0	7.5	28.9 e 9.9*
OR-POD	41.6	7.6	6.9*
MN-POD	100.0	2.4	88.9 e 3.0*

[1] Tap water; * Diluted at 1:8.

By qualitative observations on slides, it was possible to detect chromosomal aberrations caused by enzymatic treatments. In Figure 4 these damages are indicated to emphasize them in some of the treatments. The presence of micronuclei in free-POD treatment is one of the main genotoxic highlights, indicating that the crude enzymatic extract can induce uncontrolled cell division and possibly tumor formation (Fernandes et al., 2007). In contrast, chromosomal aberrations were not observed for the other treatments, indicating that enzymatic immobilization is relevant to eliminate genotoxic effects. However, for all assays, disorders in the mitosis process were quantified, as shown in Table 4.

In the MN-POD assay, for which complete discoloration was achieved, the acceleration phenomenon of cell division was quantified, increasing by 5.0 times compared with the negative control. When the sample was diluted at 1:8, a delay in cell division occurred (0.2 times), being possible to verify this by MI values (Table 4) and visual comparison between images (a), (c), and (d) of Figure 4. As in negative

control, for the enzymatic-treated samples the cells were predominantly in the prophase phase, i.e, the first stage of cell division. On the other hand, in the dye solution before treatment, all the division phases were visualized, although the MI value was only 1.1 times greater than the negative control.

The findings regarding the cell division phases are relevant since cells in metaphase and anaphase are more susceptible to chromosomal changes and DNA abnormalities (Monarca et al., 2000). In this sense, cytotoxic and genotoxic analyses provide complementary results since mitotic indexes close to the negative control value do not guarantee the absence of chromosomal aberrations. According to Fiskesjö (1985), toxicological assessment can provide a diagnose of possible behaviors of the test organism when exposed to treatments, but it is a result of multiple factors combined. For example, the author found a relationship between the pH of the solution and toxicological effects; however, it was not possible to find this direct relationship between disorder in cell division and pH in our work.

Miranda-Mandujano et al. (2018) and Feng et al. (2021) attributed the increase in toxic effects after enzymatic reaction to the formation of transformation products, during the catalytic route. It is believed that in our study, these products with distinct toxic effects may have been formed after enzymatic treatment because of the disorders caused by cell division. These disorders are possibly related to the different enzymatic conformations obtained by the immobilization strategies, considering that other cytotoxic behaviors occurred between tests, even with dye concentration and operating conditions being equal.

The samples dilution before exposure to *A. cepa* cells resulted in a change in cell division behavior, as indicated by the results in Table 4. It is understood that the search for a dilution factor in the context of the treatment proposed in this work can be one of the strategies for reaching MI values closer to the negative control. It is worth mentioning that the degradation of different compounds by the enzymatic route presents different responses from the toxicological point of view. Therefore, it is relevant that treatments inserted in other contexts are deeply analyzed in multiple aspects.

4 Conclusions

The search for solutions to problems caused by human activities has become increasingly necessary. This work discusses proposals for the production and immobilization of peroxidase using low-cost processes and reagents and providing a bioproduct with oxidative potential for pollutant remediation in aqueous matrices.

The enzymatic extract was produced by submerged fermentation of the fungus *T. koningiopsis*, supplemented exclusively by microalgae cultivated in swine wastewater digestate, placing it in the context of circular economy. The extract showed potential for insertion in advanced bioxidative reactions due to the presence of guaiacol peroxidase with good stability when subjected to extreme reaction conditions. The bioproduct presented an adequate behavior, expressing specific activity of up to 7801.1 U

mg^{-1} in the free form and 21111.1 U mg^{-1} when immobilized. It is worth highlighting the promising results in storage tests at room temperature for up to 8 months, mantaining the catalytic efficiency over time.

The *T. koningiopsis* peroxidase and the immobilization processes studied in the present work were also promising, considering its potential for synthetic dye discoloration, removing 100% of the color in 5 hours, and 93% in the enzyme reuse test, even with drop of the specific activity. The technique of magnetic nanozyme synthesis proved to be the most promising both from the point of view of discoloration potential and the maintenance of residual activity. Nanozymes also have the advantage of direct recovery with the aid of a magnetic field, enabling reuse for multiples catalytic cycles. Howevert, free-POD showed cytotoxic and genotoxic effects in assays with the test organism *A. cepa*, while immobilized POD did not show genotoxicity.

Declarations

Declaration of competing interest

The authors declare that they have no conflict of interest

List of abbreviations

Not applicable

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

All authors agreed with this publication

Availability of data and materials

The datasets generated for this study are available on request to the corresponding author

Competing interests

There are no competing interests

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Authors' contributions

N Klanovicz: Conceptualization, experimental investigation, data curation, formal analysis, writing – original draft preparation. FS Stefanski: Conceptualization, experimental investigation, data curation, formal analysis, writing – original draft preparation. AF Camargo: Conceptualization, experimental investigation, data curation, formal analysis. W Michelon: Conceptualization, experimental investigation, data curation, formal analysis. ACSC Teixeira: Supervision, project administration, funding acquisition, writing – original draft preparation. H Treichel: Supervision, project administration, funding acquisition, writing – original draft preparation.

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Figures

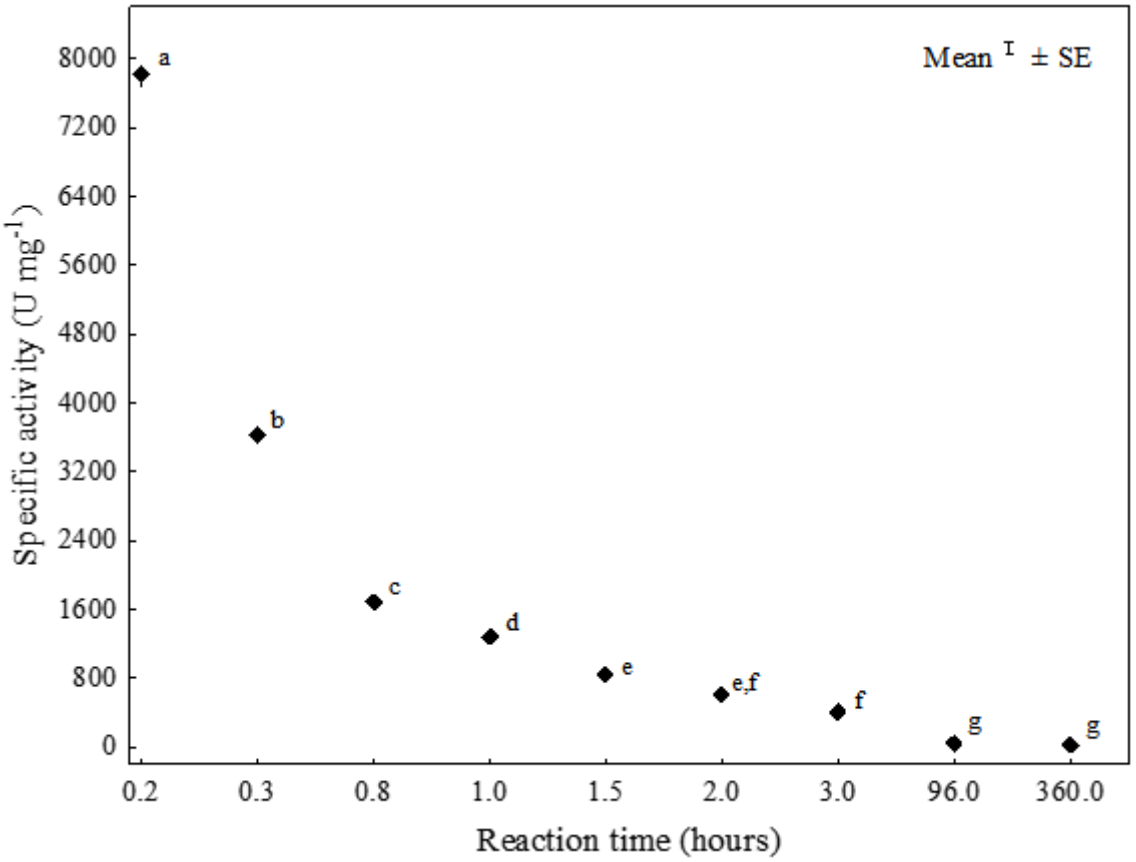

Figure 1

Enzymatic activity of T. koningiopsis over reaction time at pH 5.5 and 25 °C. Note: Equal letters indicate that the samples do not differ by the Tukey test at 95% confidence level. SE: standard error.

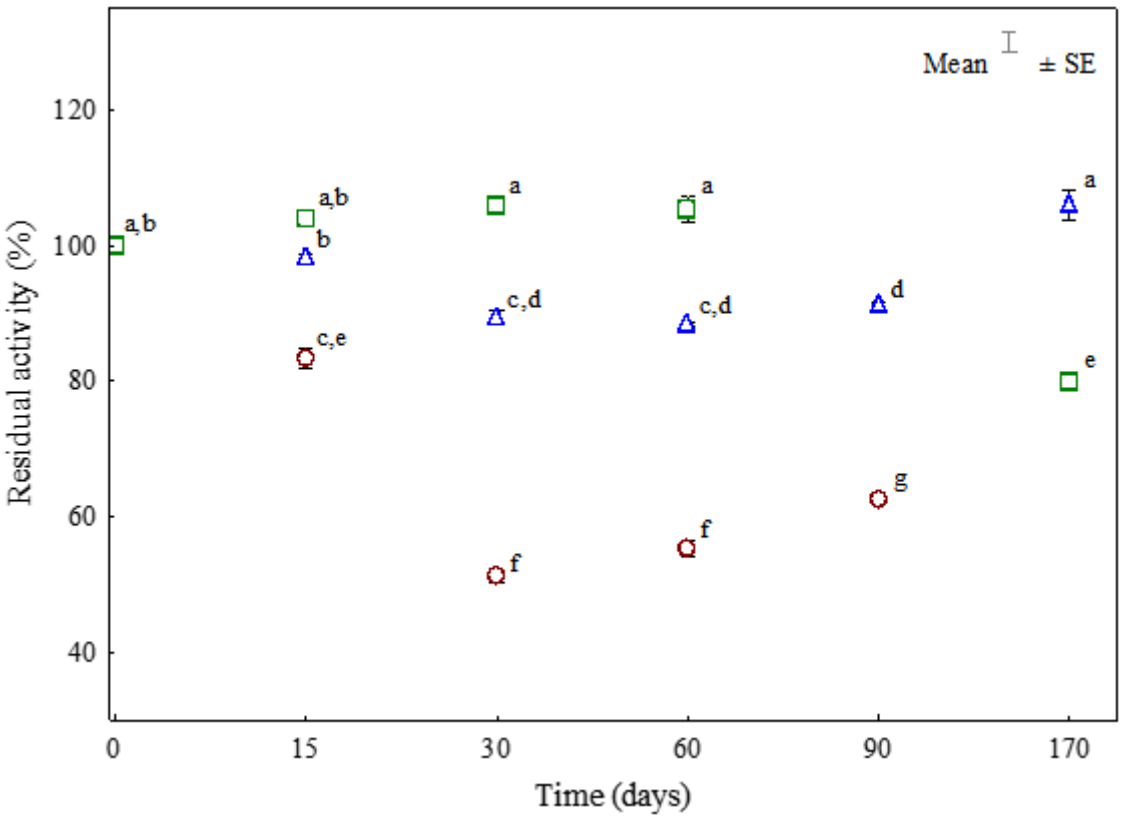

Figure 2

Monitoring free peroxidase activity stored in room temperature in the range 18-28 °C (□), refrigerator at 4 °C (△), and freezer at −10 °C (●). Note: Equal letters indicate that the samples do not differ by the Tukey test at 95% confidence level. SE: standard error.

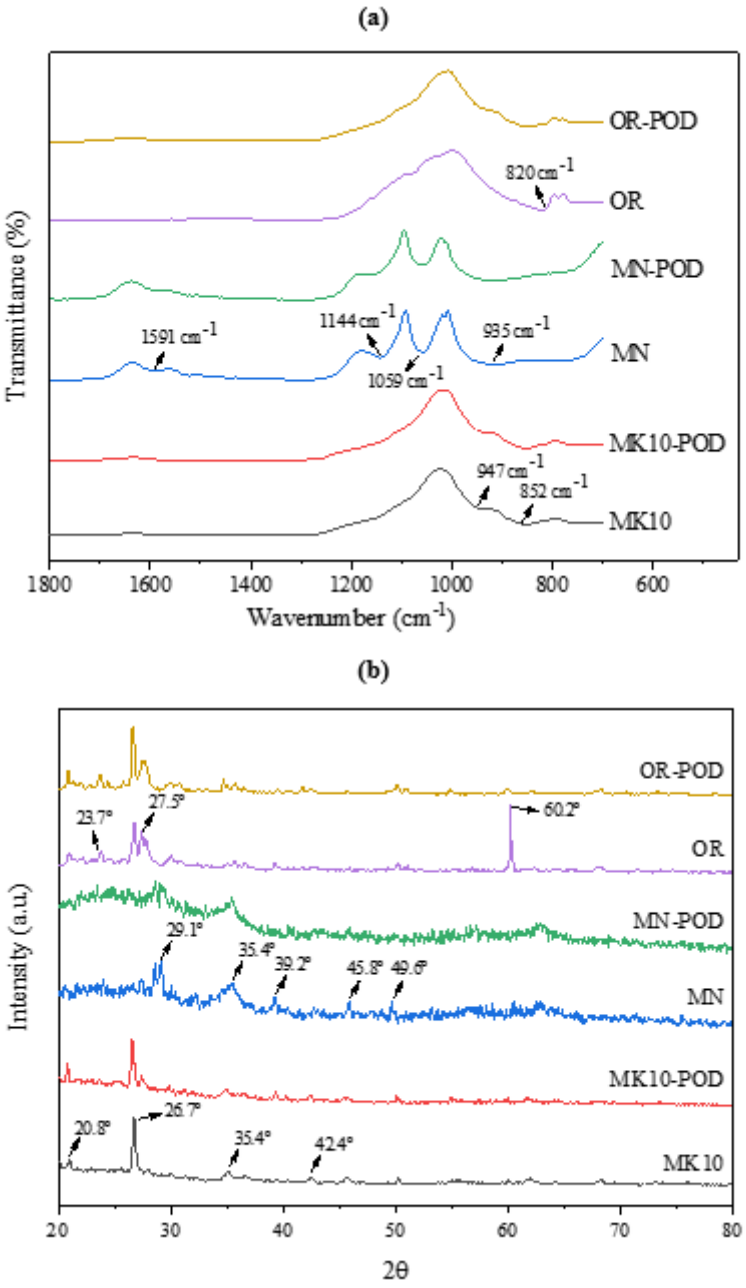

Figure 3

FTIR (a) and XRD (b) analysis of support and immobilized peroxidase.

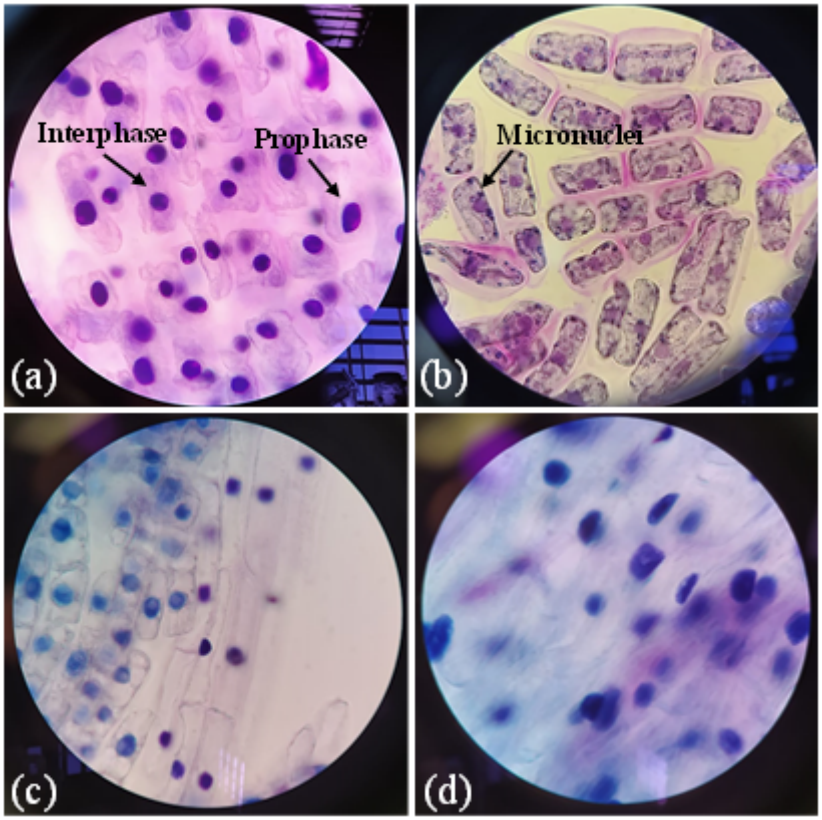

Figure 4

Microscopic observations in A. cepa cells, with 100x magnification lens, for (a) negative control, (b) free-POD at 1:8 dilution, (c) MN-POD, and (d) MN-POD at 1:8 dilution.

Supplementary Files

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