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Valorization of Yacon (*Smallanthus sonchifolius*) peel for peroxidase production and its immobilization on alginate beads for 2,4-dichlorophenol bioconversion

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Abstract

Yacon (*Smallanthus sonchifolius*) roots are emerging as an alternative source of peroxidase. This study aimed to extract, immobilize, and characterize the peroxidase from Yacon peel, herein designated as Yacon peel peroxidase (YPP), and apply it in the bioconversion of 2,4-dichlorophenol. The alginate beads, both with and without immobilized YPP, were characterized by Brunauer–Emmett–Teller (BET), thermogravimetric analysis, scanning electron microscopy (SEM), and Fourier transform infrared spectroscopy (FTIR) analyses. The immobilized peroxidase showed maximum specific activity at 50°C and pH 13, using pyrogallol as a substrate. The reaction mechanism was identified as following a Ping-Pong Bi-Bi model. In the 2,4-dichlorophenol bioconversion, the immobilized peroxidase showed maximum activity at 40°C, pH 6.8, and 0.5 mM H_2O_2 after 360 min of reaction, achieving more than 80% of bioconversion. The biocatalyst was reused for four cycles, and its application in continuous reactors should be explored. Moreover, to assess the influence of the 2,4-dichlorophenol molecules morphology and provide a better understanding of its bioconversion, density functional theory (DFT) was used to calculate chemical descriptors. The results suggest that immobilized Yacon peroxidase is a promising alternative for use in biocatalysis and the bioconversion of phenols.

KEYWORDS

characterization, extraction, molecular simulation, oxidation, peroxidase, Yacon

1 | INTRODUCTION

Peroxidases (EC 1.11.1.7) are heme proteins that reduce hydrogen peroxide while catalyzing the oxidation of organic and inorganic compounds.[1] These enzymes are identified as oxidoreductases and have been widely used to catalyze hydrogen peroxide-based redox reactions.[2] They are classified into two families, animal and plant[3] being found in diverse biological systems. Also, peroxidases can be distributed into three main classes,[4]

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including class III, which will be addressed in this study. Class III comprises plant-secreting peroxidases with a ferriprotoporphyrin IX prosthetic group.

It can be highlighted that, due to its multifunction and substrate specificity, it is found in several commercial applications,[4] as in the construction of biosensors and diagnostic kits,[5,6] in the biotransformation of organic compounds, in the treatment of industrial effluents containing dye,[7] and in the treatment of industrial effluents containing phenol,[8] among other processes. Phenolic compounds are priority pollutants due to their mutagenic effect and toxicity at low doses.[9] These compounds persist and accumulate in the environment and the food chain.[10] Phenol is reported to be a toxic and growth inhibitor for several microorganisms.[11] The group of chlorinated phenols, such as 2,4-dichlorophenol, or 2,4-DCP, is an example of an alarming compound found in herbicides used in agriculture.[12] Despite the use of 2,4-DCP being of great economic importance, the environmental impact generated, associated with problems in human health, causes great concern. Due to these facts, techniques have been applied for the environmental management of 2,4-DCP.[13] The enzymatic degradation of phenols into less toxic forms has been suggested as a treatment method. Thus, several enzymes that catalyze the oxidation of phenols, such as HRP, have been used for this purpose.[14,15] However, due to its high cost and commercial use in other processes, it becomes unfeasible for application in the treatment of effluents. Consequently, other sources of vegetable peroxidases have been studied for this purpose.

Regarding its origin, several sources of plant peroxidase have already been described in the literature, including cedar leaf peroxidase *Cedrela fissilis*,[7] date palm leaf *Phoenix dactylifera*,[16] and jackfruit bulbs *Artocarpus heterophyllus*.[17,18] However, the one that is commercialized is horseradish peroxidase (*Armoracia rusticana*), known as HRP,[4] a peroxidase of easy extraction and high availability, with use from laboratory to industrial level, including in the treatment of industrial effluents contaminated with phenol.[1]

The Yacon root (*Smallanthus sonchifolius*) becomes evident in the search for alternative enzymes. Native to the Andes and cultivated for its tubers, it is commonly used as food in South America.[19] Recently, this plant has been considered for several applications including its health benefits,[20,21] the presence of bioactive and high-value compounds.[22,23] Moreover, it was demonstrated that Yacon is a promising source of peroxidase with adequate characteristics for treating phenolic effluents after its extraction and purification.[8] Nevertheless, studies on Yacon peroxidase are scarce, particularly exploring its potential for environmental applications such as phenol removal. Consequently, there are no studies on strategies for Yacon peroxidase immobilization. It is well-known that immobilized enzymes form has been historically presenting a low impact on ecosystems and low cost,[24] in addition to presenting advantages over soluble enzymes, such as enzyme stabilization and the retention and repetition of their catalytic activities.[25]

Notably, there are no definitive studies on applying immobilized Yacon peroxidase to treat effluents containing phenolic compounds. Therefore, the present work aimed to characterize, biochemically and structurally, the immobilized form of the peroxidase obtained from Yacon root peels and apply it in the bioconversion of 2,4-DCP.

2 | MATERIALS AND METHODS

2.1 | Yacon roots

Yacon (*Smallanthus sonchifolius*) was purchased in Lages, SC, Brazil. The roots were placed in plastic bags suitable for food storage and preserved under refrigeration (5°C). The roots were immersed in distilled water for 15 min, dried with a paper towel, and manually peeled to extract the peels. The peels were immediately used for peroxidase extraction.

2.2 | Extraction and partial purification of Yacon peel peroxidase (YPP)

Peroxidase extraction was performed using a 1:1 ratio (m/v) of Yacon peels and 0.1 M potassium phosphate buffer solution (pH 6). The peels and buffer solution were homogenized with a mixer. To remove solid particles, the mixture was filtered (Whatman No. 1 filter paper and a layer of cotton gauze). The extract was centrifuged at 4000 rpm for 10 min to remove the insoluble material, and the supernatant was stored at 5°C[16]; after the extraction process, the loss was minimal, considering a yield of almost 100% of the extraction process. For partial purification of the enzymatic extract, 14% of polyethylene glycol 6000 (PEG) and 10% (m/v) of ammonium sulphate were added to the extract volume and kept at 100 rpm until the temperature remained at 5°C. Next, ammonium sulphate (10%) and PEG (14%) were added and kept under stirring for 2 h. The sample was stored at 5°C for 24 h. Then, the foam was removed, and the sample was centrifuged at 4000 rpm for 10 min. The supernatant, Yacon peel peroxidase (YPP), was stored at 5°C.[26]

2.3 | Immobilization of YPP

Calcium alginate capsules were obtained from a solution of 3.5% (m/v) of sodium alginate, homogenized at 50°C,

cooled, and dripped, using a hypodermic syringe with a needle (0.7 × 25 mm) in a calcium chloride solution 4% (m/v). They were stored in the calcium chloride solution itself at 5°C. After the preparation of the capsules, a covalent bond between the carboxyl group of the calcium alginate support and the amine group of the enzyme was applied.[27] The EDC (*N*-ethyl-N′-(3-(dimethylamino)propyl)carbodiimide)/NHS (*N*-hydroxysuccinimide) methodology was used in this case. Calcium alginate capsules, 7 mL of ultrapure water, 2.5 mL of 2-*N*-morpholino ethasulphonic acid (MES) buffer solution 200 mM (pH 6), and 0.25 mL of NHS solution (200 mM) were mixed. The suspension was maintained in a low-frequency ultrasound for 30 min. EDC (80 mM, 0.25 mL) was added, and the mixture remained under agitation (200 rpm) for 2 hours. Then, the capsules were washed by immersion with a 50 mM MES buffer solution (pH 6) at the end of the steps. Following this, for YPP immobilization, the capsules prepared in the previous step, ultrapure water, 200 mM MES buffer solution (pH 6), and the YPP extract were mixed in the proportion of 2:1:1. The mixture was kept under orbital agitation (200 rpm) at 20°C for 4 h. The YPP immobilized capsules were washed with ultrapure water and stored at 5°C.

2.4 | Capsule characterization

We used samples of the capsules without and with the immobilized YPP in the methodologies performed for the characterization. The diameter of each capsule was determined using a calliper rule. Three capsules ($N = 3$) were used for each test. To determine the dry mass of the capsules, porcelain crucibles (previously dried) were used, containing 10 capsules ($N = 10$), which were dried in an oven at 100°C for 24 h until constant mass. Scanning electron microscopy (SEM) was performed using the Jeol equipment/model JSM 6701F. The gold plating was performed on a BAL-TEC Sputter Coater (SCD 050) for 120 seconds. The magnification was 40, 100, and 1000 times. The Thermogravimetric analysis (TGA) was performed using the LECO brand TGA701 Thermogravimetric Analyzer equipment. The temperature increased from 25°C to 300°C with a heating rate of 10°C min^{-1}, under an atmosphere of 5.0 nitrogen (N_2). For the Fourier transform infrared spectroscopy (FTIR) analysis, samples were prepared from the dispersion in KBr pellets in the INVENIO-S equipment (brand: Bruker). The FTIR spectra (4000 to 400 cm^{-1}) were performed with a resolution of 4 cm^{-1} and 32 scans. The surface area, total volume, and pore diameter were determined by Brunauer–Emmett–Teller (BET), with nitrogen adsorption at low temperatures. The sample was prepared with activation at 45°C for 2 h and then cooled to 25°C for analysis. After preparation, the analysis was performed in Quantachrome brand equipment, Quadrasorb evo model, with QuadraWin processing software. The adsorbent gas used is 5.0 nitrogen.

2.5 | Enzymatic activity and specific activity of immobilized YPP

The YPP activity was determined according to Chance and Maehly[28] protocol by oxidizing the pyrogallol substrate into the purpurogallin product ($\varepsilon = 2.47\ mM^{-1}\ cm^{-1}$). The mixture was spectrophotometrically monitored at a wavelength of 420 nm. A unit of peroxidase activity (U) was defined as the amount of enzyme that catalyzes the formation of 1 mg of purpurogallin in 20 seconds, pH 6 and 20°C. The change in absorbance was used to calculate the specific activity (U g^{-1}), according to Equation (1), where V is the total volume of the assay (mL), m is the wet mass of the capsules with immobilized YPP (g), ΔA_{420} is the change in absorbance recorded at the wavelength of 420 nm, 12 is the extinction coefficient (based on mg mL^{-1}) of purpurogaline and C is the cuvette width (cm). For the blank, the capsules with the YPP immobilized were replaced by capsules without the enzyme.

$$\text{Specific activity}\left(\text{U g}^{-1}\right) = \frac{\left(\frac{\Delta A_{420}}{20\ \text{s}}\ \text{test} - \frac{\Delta A_{420}}{20\ \text{s}}\ \text{blank}\right) \times \text{V}}{12 \times m \times C} \quad (1)$$

2.6 | Influence of pH and temperature on the specific activity of immobilized YPP

To assess the influence of pH on the specific activity of immobilized YPP, McIlvaine buffer was used for pH between 2.2 and 8 and Sörensen buffer for pH between 10 and 13, in substitution for the buffer used in the enzyme activity test. To verify the influence of temperature on the specific activity of the immobilized YPP, incubation temperatures varied from 5°C to 90°C. The other procedures were like the one described in item 2.5.

2.7 | Influence of substrate concentration on the specific activity and kinetic study of immobilized YPP

The specific activity of immobilized YPP for different concentrations of pyrogallol and hydrogen peroxide was determined, as it is a catalyst whose activity depends on

two substrates. For pyrogallol, concentrations of 1.04, 2.08, 4.16, 8.32, 20.8, 41.6, and 83.20 mM were used, keeping the hydrogen peroxide concentrations at 2.08, 4.16, 20.8, 41.6, 104, 208, and 416 mM for each concentration of pyrogallol indicated above. In the kinetic study, the values of the constants of the Michaelis–Menten model (K_m and v_{max}) were calculated using SigmaPlot 12.0 software (Systat), which for both substrates were calculated from the Lineweaver–Burk plots. Based on the kinetics of two substrates, the oxidation mechanism was evaluated through the curves obtained for the different substrate concentrations.

2.8 | Application of immobilized YPP in the bioconversion of 2,4-dichlorophenol (2,4-DCP)

2.8.1 | Determination of 2,4-DCP

To determine the 2,4-DCP, in a 10 mL glass vial, 3 mL of distilled water was added, 120 μL of the sample containing 2,4-DCP, 50 μL of ammonium buffer solution (pH 10), 50 μL of sodium persulphate ($Na_2S_2O_8$, 50 g L^{-1}) and 50 μL of 4-aminoantipyrine ($C_{11}H_{13}N_3O$, 20 g L^{-1}), in this sequence. After 10 minutes of reaction, the reading was performed in a UV–Vis spectrophotometer at 504 nm.[29]

2.8.2 | Bioconversion kinetics of 2,4-DCP by immobilized YPP

The evaluation of the bioconversion of 2,4-DCP over time, mediated by immobilized YPP, was performed by applying different amounts of capsules (0, 0.3, 0.65, 1, 1.3, 1.65, and 2 g) in an aqueous solution of 2,4-DCP 50 mg L^{-1} and pH 7. Samples were collected at 0, 60, 120, 240, and 360 min and then were conducted to determine the remaining concentration of 2,4-DCP.[29] The results were expressed through the relationship between the initial concentration of 2,4-DCP in the solution (C_0) and the concentration remaining at the end of the evaluated time intervals (C).

2.8.3 | Influence of pH and temperature on the bioconversion of 2,4-DCP by immobilized YPP

To evaluate the influence of pH on the bioconversion of 2,4-DCP, assays were performed at pH 2, 6.8, 10, and 13. The pH values were corrected in the 2,4-DCP solution using 0.1 M HCl or NaOH. To evaluate the influence of temperature on the bioconversion of 2,4-DCP, assays were performed at 20, 40, 60, and 80°C until they reached thermal equilibrium. It was mixed 50 mL of 2,4-DCP (50 mg L^{-1}), 1.65 g of capsules with immobilized YPP, and 0.63 mM of H_2O_2. After 6 h at 110 rpm, samples were collected and analyzed for the remaining 2,4-DCP concentration.

2.8.4 | Influence of hydrogen peroxide concentration on the bioconversion of 2,4-DCP by immobilized YPP

To evaluate the influence of hydrogen peroxide concentration on the bioconversion of 2,4-DCP, assays were performed with 0, 0.25, 0.50, 0.63, and 1 mM of hydrogen peroxide. We mixed 50 mL of 2,4-DCP (50 mg L^{-1}), 1.65 g of capsules with immobilized YPP and 0.63 mM of H_2O_2. After 6 h at 110 rpm, samples were collected and analyzed for the remaining 2,4-DCP concentration.

2.8.5 | Capsules reuse

To determine the number of cycles by which alginate capsules with immobilized YPP can be reused for bioconversion of 2,4-DCP (50 mg L^{-1}), the Erlenmeyer's flask with the samples were kept under agitation for 24 h, in the conditions of reaction (50 mL of 2,4-DCP 50 mg L^{-1}, 1.65 g of capsules with immobilized YPP, 0.63 mM of H_2O_2, pH 6.8 and 21°C). After the end of the contact period, the initial and final concentration of 2,4-DCP was analyzed. Then, the capsules used in the previous cycle were reinserted in a clean Erlenmeyer flask, and new solutions of 2,4-DCP were transferred. The same procedure was followed until there was no longer any difference in the concentration of 2,4-DCP before and after treatment.

2.9 | Molecular simulation

Molecular simulation was performed based on the density functional theory (DFT) to obtain the molecule geometric properties and chemical-quantum descriptors, including the highest energy occupied molecular orbital (HOMO, E_H), the lowest unoccupied molecular orbital energy (LUMO, E_L) and the energy gap (H). The 3D molecule of 2,4-DCP and its region of electrostatic potential was generated by Avogadro software. DFT calculations were conducted using the Gaussian 09 software. The simulations were conducted using the hybrid

Beck's three-parameter, the Lee–Yang–Parr functional correlation (B3LYP), which provides good results for intermediate-sized molecules.[30] The 6-31G(d) and 6-31G (d,p) basis sets were evaluated to alter the molecular geometry and lessen the system's energy to produce a more stable conformation. Based on these values, the reactivity descriptors were calculated as chemical potential (μ) Equation (2), global chemical hardness (η) Equation (3), and global electrophilicity index (ω) Equation (4):

$$\eta = \frac{(-EH + HL)}{2} \tag{2}$$

$$\mu = \frac{(-EH + EL)}{2} \tag{3}$$

$$\omega = \frac{\mu^2}{2\eta} \tag{4}$$

2.10 | Statistical analysis of experimental data

All experiments and analyses were performed in triplicate. First, the statistical analysis of the experimental results, using SigmaPlot 12.0 software (Systat), was performed using the Tukey test, where the average values were compared, applying the data regression, the isolated and interaction effects between the variables will be verified, indicating the 95% level of reliability.

3 | RESULTS AND DISCUSSION

3.1 | Capsules characterization by diameter, moisture, SEM, TGA, FTIR, and BET

Alginate capsules have more than 94% water in their composition and, consequently, a smaller dry diameter (Table 1). Comparing alginate capsules and those with immobilized YPP by EDC/NHS, it is observed that immobilization generates a slight increase in the moisture and diameter of the capsules.

The SEM (Figure 1S) shows the structure of the capsules before and after immobilization. After immobilization (Figure 1Sd, Figure 1Se, Figure 1Sf), the structure is less porous and more homogeneous than before immobilization (Figure 1Sa, Figure 1Sb, Figure 1Sc). This characteristic is due to the form of immobilization (by EDC/NHS), which can be associated with the crosslinking promoted by this type of immobilization, which connects the polymer chains, thus altering their entanglement.[31]

In the thermogravimetric analysis (Figure 1A), the total mass loss for the alginate capsule without the immobilized YPP was 31.95%. Up to 182.84°C, the sample lost 5.19% mass; between 182.84°C and 287.89°C, the first expressive loss of mass occurs (18.25%). Also, at 287.89°C, a new weight loss begins at 300°C (final test temperature). For the capsules with immobilized YPP, the total mass loss was 36.78%. At 148.65°C, the first mass loss event (27.44%) occurs, extending to 273.27°C. Between 273.27°C and 300°C, a new mass loss event occurs. The mass losses occurred basically in two regions, the first up to 200°C, which characterizes the mass losses related to dehydration and proteins, and the second region above 200°C, which is related to the decomposition of organic compounds, such as alginate.[32] The results for alginate capsules with immobilized YPP by EDC/NHS show an increase in mass loss in the first region, which indicates a more significant amount of moisture and the presence of the enzyme. The most significant loss of mass was for capsules with immobilized YPP. Similar results were found by Queiroz et al.[33] on the immobilized peroxidase obtained a higher loss of mass compared to the solid matrix (without the enzyme), which may be associated with better preservation of the enzyme's hydration layer and the maintenance of its catalytic activity.

The infrared spectra (FTIR) of alginate capsules without and with immobilized YPP (Figure 1B) observed that the absorption peaks' position and intensity were changed. The bands between 500 and 1500 cm^{-1} intensify, indicating a higher number of single bonds, which

Capsules	Wet diameter (mm)	Dry diameter (mm)	Moisture (%)
Calcium alginate capsules	3.67 ± 0.01	1.71 ± 0.01	94.60 ± 0.58
Calcium alginate capsules with immobilized YPP by EDC/NHS	3.95 ± 0.02	1.98 ± 0.01	94.01 ± 0.56

TABLE 1 Diameters and moisture of calcium alginate capsules, without and with immobilized Yacon peel peroxidase (YPP) by EDC/NHS. Three capsules ($N = 3$) an 10 capsules were used for diameter and moisture determination, respectively.

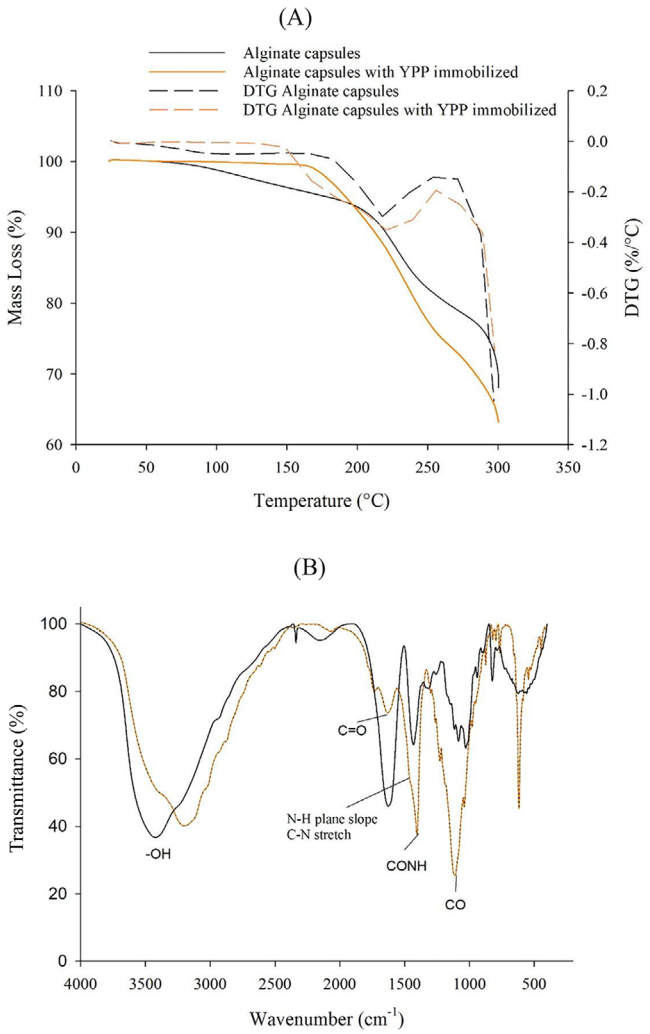


FIGURE 1 (A) Thermogravimetric analysis (TGA) of alginate capsules without and with immobilized Yacon peel peroxidase (YPP) by EDC/NHS; (B) Fourier transform infrared spectroscopy (FTIR) analysis of alginate capsules without and with immobilized YPP by EDC/NHS.

confirms that there was a change after peroxidase immobilization by EDC/NHS. Bands between 3300 and 3400 cm^{-1} are attributed to the elongation vibration of OH. The amide I band, which lies in the region of 1600 and 1700 cm^{-1}, is the most useful protein structure for FTIR, as it involves C═O stretch vibrations of peptide groups,[34] demonstrating the presence of the immobilized YPP. The amide II band (NH plane slope and C—N stretch mode) and the amide III band (CONH group plane mode) are observed at 1556 and between 1400 cm^{-1}, respectively, and the bands around 1076 cm^{-1} are attributed to asymmetric bridge oxygen and CO stretch. Şahin,[35] in a study with immobilized peroxidase also via EDC/NHS, found a similar behaviour for the immobilized peroxidase, mainly in the bands between 500 and 1500 cm^{-1} and the amide II band. This result confirmed the presence of the enzyme in the immobilization support.

The results of BET analysis for the alginate capsules and the capsules with immobilized YPP can be seen in Table 2. A larger superficial area provides many active sites and improves catalyst adsorption and catalytic activity.[36] Therefore, the result presented is advantageous for the values obtained with the capsules containing the immobilized YPP, as they were higher in the surface area, volume, and mean pore radius than the capsule containing only the alginate.

3.2 | Influence of pH and temperature on immobilized YPP activity

For the influence of pH on immobilized YPP (Figure 2A), a profile of increased relative activity, from acidity to basicity, with maximum activity at pHs above 8, with pyrogallol as substrate, can be observed. Thus, effluents with alkaline characteristics could be treated using immobilized peroxidase, such as swine effluent in the removal of ammonia (pH <10),[37] and paper bleaching effluent (pH 11),[38] among others. Peroxidases from other sources also studied in immobilized form had varied optimal pHs. For example, the peroxidase of *Azadirachta indica*[26] and *Momordica charantia*[24] showed maximum activity at pH 5. On the other hand, the *Armoracia rusticana* peroxidase showed maximum activity at pH 8.[39] Moreover, *Zingiber officinale* peroxidase also showed maximum activity at pH 5, keeping it at more than 80% until pH 9.[40] It is noteworthy that in a study involving free-form YPP, the pH of maximum activity was also reported as alkaline (pH 11) and relative activity above 60% at pH 12.[8]

The maximum activity of the immobilized YPP was observed at 50°C (Figure 2B), a value that differs statistically from the others ($p < 0.05$). This result suggests that immobilization process affected the original structure of native enzyme which showed maximum activity at 60°C.[8] Moreover, at 40, 60, and 70°C, the YPP remains with a relative activity above 50%. For peroxidases of *Azadirachta indica*, *Momordica charantia*, and *Zingiber officinale* immobilized in chitosan capsules, concanavalin A and alginate and guar gum capsules mixed with agarose and alginate hydrogel, respectively, the maximum activity was obtained at 40°C (Ali and Husain, 2018[24,26]). For *Vigna radiata* peroxidase, the maximum activity was also at 40°C. However, it maintained a relative activity of over 50% at 50, 60, and 70°C.[41] Ely et al.,[42] studying HRP and using pyrogallol as a standard substrate, obtained the maximum activity at 50°C.

Capsules	Superficial area ($m^2\ g^{-1}$)	Total pore volume[a] ($cm^3\ g^{-1}$)	Average pore radius (Å)
Calcium alginate capsules	0.263	0.00030	22.887
Calcium alginate capsules with immobilized YPP by EDC/NHS	0.634	0.00191	37.584

TABLE 2 Brunauer–Emmett–Teller (BET) results with calcium alginate capsules without and with immobilized Yacon peel peroxidase (YPP) by EDC/NHS.

[a]For calcium alginate capsules, pores smaller than 883.5 Å radius at $P/P_0 = 0.98902$; for capsules with immobilized YPP, pores smaller than 1224.2 Å radius at $P/P_0 = 0.99211$.

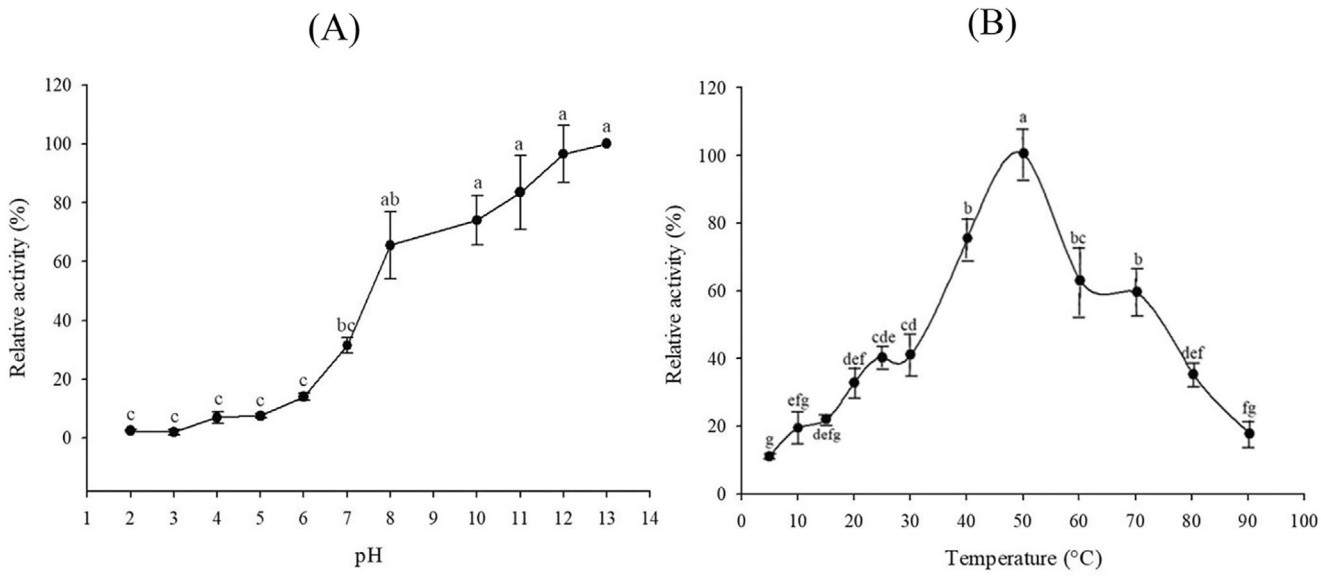


FIGURE 2 (A) Influence of pH on the relative activity of YPP immobilized in alginate capsules by EDC/NHS. Different letters indicate that, at the 5% significance level, there is significant difference between the averages: (B) influence of temperature on the relative activity of YPP immobilized in alginate capsules by EDC/NHS. Different letters indicate that, at the 5% significance level, there is significant difference between the averages.

Effluents with high temperatures, such as effluent from the textile industry (40°C to 50°C) and effluent from the bleached kraft pulp industry (55°C),[43] could be treated using immobilized YPP.

3.3 | Influence of substrate concentration on immobilized YPP activity and kinetic study

In enzymatic catalysis with two substrates, two general mechanisms are used to understand the interactions between enzyme and substrate: sequential or Ping-Pong.[44] In the region with no inhibition, the double-reciprocal plots of the initial pyrogallol oxidation rates by immobilized YPP (Figure 3A) provided approximately parallel lines, which proposes a mechanism of Ping-Pong Bi-Bi.[45] On the other hand, an excess concentration of H_2O_2 (Figure 3B) can cause the enzyme to pass into an irreversibly inactive state. That is, an inhibition by excess substrate occurs.[46] The immobilized YPP presented Km of 0.85 mM for the hydrogen peroxide and 1.4 mM for the pyrogallol, which indicates a higher affinity for the first substrate.

3.4 | Bioconversion kinetics of 2,4-DCP by immobilized YPP

The mass of the alginate capsules containing YPP immobilized by EDC/NHS that led to the best bioconversion results were 1.65 and 2 g (Figure 4A), with 2,4-DCP bioconversion rates of 70.42% and 70.61%, respectively, at the end of 360 minutes of reaction (the curves are superimposed). This performance was inferior to that obtained with free enzyme which resulted in around 86% of 2,4-DCP bioconversion.[8] This difference might be associated with the alteration in the enzyme microenvironment and partition

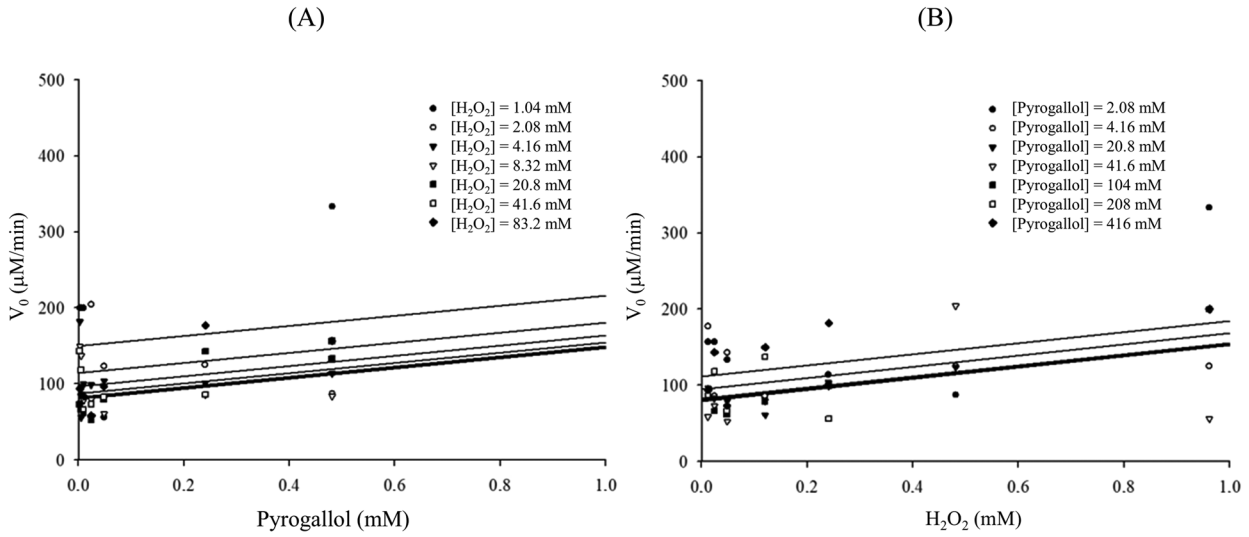


FIGURE 3 Linearization by Lineweaver-Burk of the specific activity data of the immobilized YPP for (A) variation of the concentration of pyrogallol at different fixed concentrations of H_2O_2 and (B) variation of the concentration of H_2O_2 at different fixed concentrations of pyrogallol.

effects due to the covalent immobilization process.[47] Additionally, diffusional limitations in alginate beads might, to a certain extent, reduce the enzyme activity.[48] These possible changes in the immobilized enzyme were demonstrated by the shift in optimal temperature (Figure 2) and will also be observed during the investigation on hydrogen peroxide concentration effect. Although immobilized enzymes presented lower substrate conversion, the reuse of the biocatalyst in many cycles of reaction might be the key feature which justify the immobilization process. For the other stages of the study, 1.65 g of capsules containing the immobilized YPP were used, because the statistics show no significant difference ($p > 0.05$) between the conversions when using 1.65 and 2 g. Still, the smaller amount of enzyme can affect the treatment cost, making it cheaper, but it should be noted that the cost of treatment is not the object of this study. The reaction time was fixed at 360 min.

3.5 | Influence of pH and temperature on the bioconversion of 2,4-DCP by immobilized YPP

For the influence of pH on the bioconversion of 2,4-DCP (50 mg L^{-1}) (Figure 4B), the highest bioconversion was observed at pH 6.8, with a value of 74% and statistically different ($p < 0.05$) from the others. Bioconversion at pH 2 had the lowest value, below 40%. It is verified that, although the pH range of maximum activity of the immobilized YPP is from 8 to 13 (previously determined), for the conversion of 2,4-DCP, pH 6.8 presented the best result. This result is justified due to the difference in the substrates. Different substrates promote changes in the biochemical affinity of the enzyme.

For the influence of temperature on the bioconversion of 2,4-DCP (50 mg L^{-1}) (Figure 4C), the greatest bioconversion, approximately 90%, occurred between 20°C and 40°C, values being statistically equal ($p > 0.05$). At 60°C, the immobilized YPP presented a bioconversion of 70%. This reduction in the bioconversion, when the reaction medium is subjected to 60°C, can be explained by the loss of stability of the enzyme at this temperature (evaluated previously). The results of the influence of temperature, in a wide range and combined with pH 6.8, provide opportunities for its application in treatments of contaminated water with 2,4-DCP in situ and ex-situ. In a previous study carried out with free YPP, similar pH and temperature results were obtained for the bioconversion of 2,4-DCP, pH 7 and 37.5°C.[8] For soy peroxidase (*Glycine max*), the optimal pH condition was reported at 6.5 and 50°C for removing 2,4-DCP.[13]

3.6 | Influence of H_2O_2 concentration on the bioconversion of 2,4-DCP by immobilized YPP

For the influence of the H_2O_2 concentration on the bioconversion of 2,4-DCP (50 mg L^{-1}), whose profiles are shown in Figure 5, it is verified that 0.25 and 0.50 mM of H_2O_2 provided the highest bioconversions, with results statistically equal ($p > 0.05$) and above 80%.[8] Free YPP

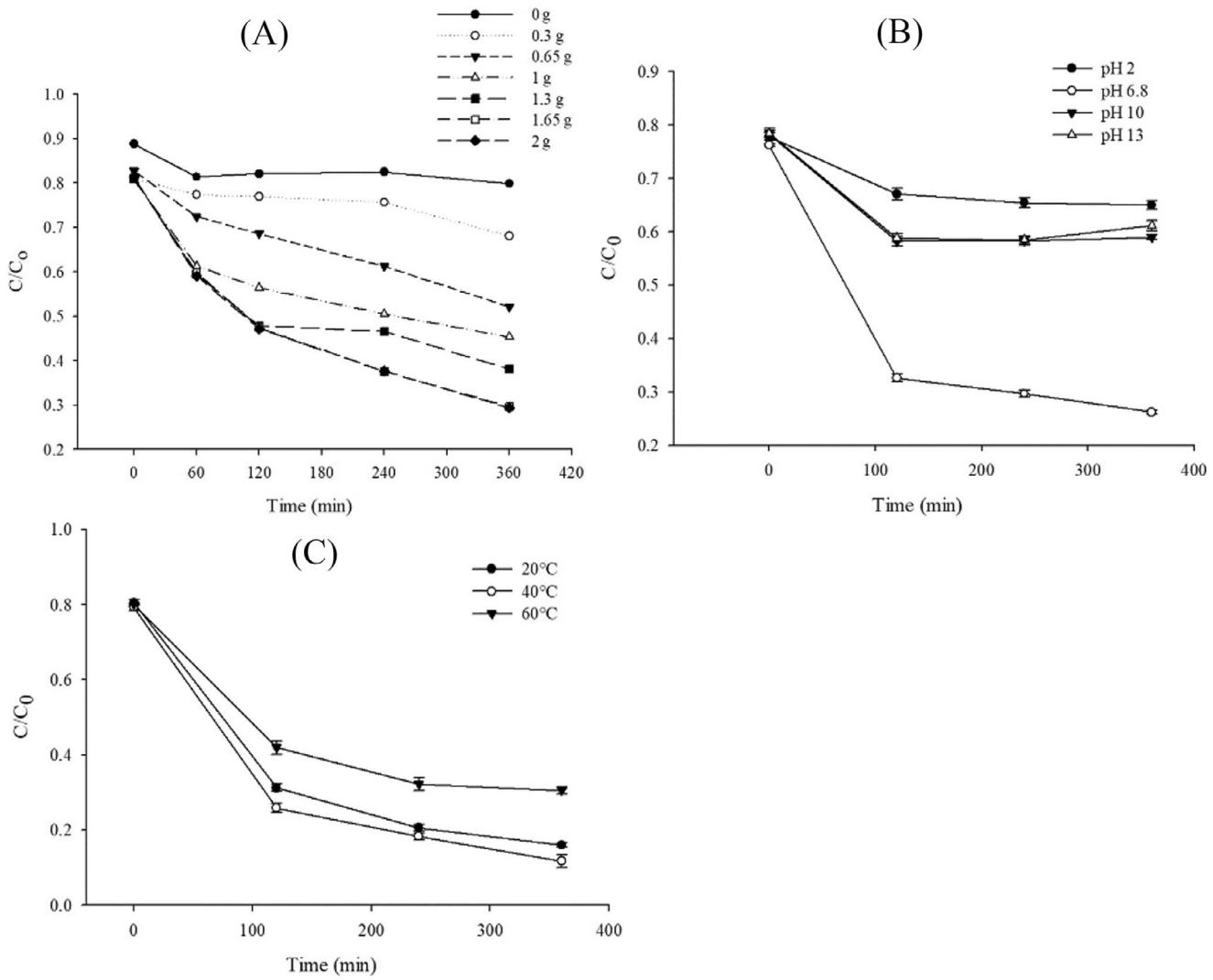


FIGURE 4 Influence of enzyme concentration (A), pH (B), and temperature (C) on the bioconversion of 2,4-DCP by immobilized peroxidase in alginate capsules by EDC/NHS.

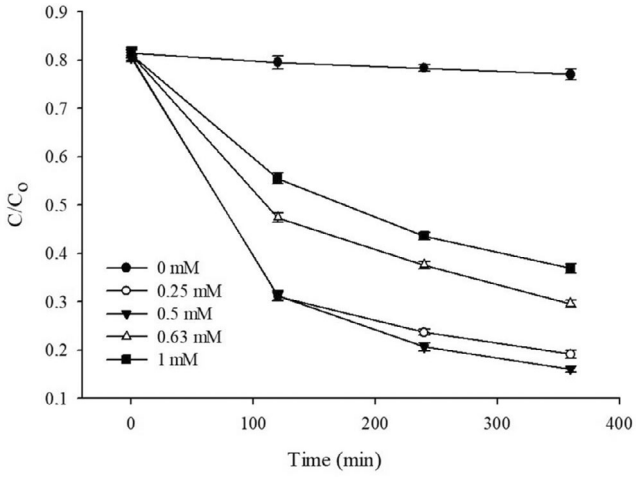


FIGURE 5 Influence of hydrogen peroxide concentration on the bioconversion of 2,4-DCP by immobilized peroxidase in alginate capsules by EDC/NHS.

obtained the highest bioconversion of 2,4-DCP with 0.63 mM of H_2O_2. For the other concentrations tested (except the experiment without H_2O_2), it appears that there was bioconversion; however, there was less. It is also verified that the highest bioconversions were obtained for the lowest concentrations of H_2O_2, demonstrating that the excess of this substrate can inhibit the enzyme and harm the process, making it more costly.

3.7 | Capsules reuse on bioconversion of 2,4-DCP by immobilized YPP

The conditions for reusing alginate capsules with YPP immobilized via EDC/NHS in the bioconversion of 2,4-DCP were: 50 mg L^{-1} of 2,4-DCP, 1.65 g capsules, 50 mM H_2O_2, pH 6.8, and 40°C. In the first cycle, there

was a bioconversion of 75.86 ± 2.01% of 2,4-DCP. In the other cycles, the bioconversion of 2,4-DCP was 39.80 ± 0.26% % (2nd cycle), 19.68 ± 0.39% % (3rd cycle), and 5.52 ± 0.01% % (4th cycle). The results demonstrate the possibility of reusing the capsules with immobilized peroxidase. However, the bioconversion percentage decreases over the cycles. This loss can be associated with by-products that interact with the immobilized enzyme and reduce their activity.[49] In our case, we observed precipitated compounds, which could affect the enzyme activity. Additionally, since the reaction occurs via free radicals, these compounds might be another factor responsible for the loss of activity.[50] Therefore, we suggest the investigation of continuous flow as a strategy to constantly remove the by-products from the reactor. This removal could enhance the enzyme reusability, which is the most desirable feature associated with immobilization.

When comparing YPP to HRP studies reported in the literature for the conversion of phenolic compounds, Liu et al.[51] reported that HRP functionalized on biochar achieved 80% removal with 20 mg/mL of the enzyme after 60 min, maintaining 60% of its activity after seven reuse cycles. For commercial HRP immobilized in calcium alginate-starch, Weber et al.[50] observed 56% phenol removal in 90 min, with 66.56% of the initial biodegradation capacity retained after four reuse cycles. Lastly, in the study by Zeyadi and Almulaiky,[52] immobilized NH2-MOF-Zr@HRP achieved 49% removal after a 360-min reaction, maintaining 93% of its stability even after three cycles. Overall, YPP demonstrates promising potential, achieving more than 70% 2,4-DCP bioconversion. Although further studies are needed to optimize its stability and reusability, these results indicate that YPP is an effective alternative peroxidase for phenolic compounds degradation.

3.8 | Molecular simulation

In order to improve the understanding of the 2,4 dichlorophenol bioconversion, the molecular and electronic properties of the contaminant were carried out. The bases 6-31G(d) and 6-31G(d,p) were used to acquire molecules with optimized energy. The results showed that as the obtained molecular energy was lower in the 6-31G(d) base, this one provides a more stable configuration. Figure 6 displays the optimized 2,4-DCP molecule obtained through 6-31G(d), the region of electrostatic potential, and 3D plots of HOMO and LUMO orbitals.

From Figure 6A, it is possible to observe the optimized structure and the size of the 2,4-DCP molecule. The knowledge of the molecule size is of supreme importance because it affects the efficiency of the process,

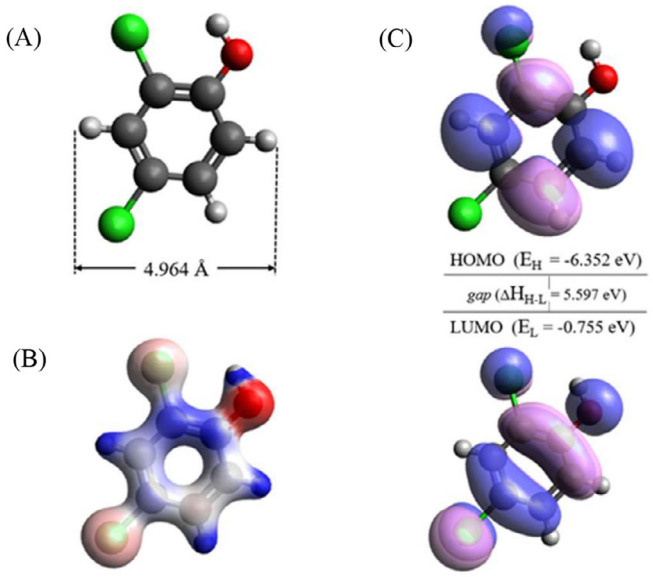


FIGURE 6 (A) Optimized 2,4 dichlorophenol molecule obtained through the 6-31G(d) base set; (B) electrostatic potential region, where blue corresponds to low electron density and red to strong electron density; and (C) 3D plots of the HOMO and LUMO orbitals where blue refers to the positive phase and pink to the negative phase.

generating steric hindrance when the contaminant molecule is more significant than that of the adsorbent.[53] Figure 6B shows the electrostatic potential of the molecule, wherein the red region is. The electron density is stronger; the more it goes blue, the lower the electron density.[54] It can be observed that for 2,4-DCP, the highest electron density of the molecule is found in the oxygen atom due to its higher electronegativity, while the lowest electron densities are found close to the hydrogen atoms. In Figure 6C, the HOMO and LUMO orbitals were represented, which provides information about the molecule's chemical reactivity and charge transfers. Higher values of HOMO (E_H) energy indicate a greater ability to donate electrons, whereas low values of LUMO (E_L) energy are related to the ease of receiving electrons.[55] The gap value is associated with molecule reactivity, as the smaller the gap, the less reactive and more stable the molecule.[56] 2,4-DCP has a higher E_L (−0.755 eV), indicating the molecule's tendency to receive electrons.

The chemical descriptors were also calculated from the values of E_H and E_L. They are the chemical hardness ($\eta = 5.974$ eV), which determines the resistance and stability of the molecule to deformation; the chemical potential ($\mu = 3.553$ eV), which shows the tendency of electrons to escape equilibrium; and the electrophilicity index ($\omega = 1.776$ eV) that indicates the ability of the molecule to receive electrons.[57] It is important to emphasize that calculating the quantum

chemical descriptors and the molecular simulation of 2,4-DCP enhances understanding of how this contaminant behaves in bioconversion.

4 | CONCLUSION

The YPP immobilized in alginate capsules by EDC/NHS proved effective in the bioconversion of 2,4-dichlorophenol, showing potential for application in effluent treatment processes. The best performance was observed under conditions close to pH 6.8, temperatures between 20°C and 40°C, hydrogen peroxide concentrations around 0.25–0.50 mM, and with an enzyme mass of 1.65 g per 50 mL, which supports its use in real effluent treatment and bioremediation. Although the results obtained are noteworthy, further advances are still required to enhance capsule stability and resistance, thereby enabling their reuse over multiple cycles. Therefore, additional studies focusing on immobilization and capsule production are essential. Overall, Yacon root peel peroxidase in its immobilized form represents a promising and sustainable alternative for biocatalysis and for the bioconversion of phenolic compounds in water and effluents, as well as in other oxidation-based applications.

AUTHOR CONTRIBUTIONS

Mariana Gomes Oliveira: Conceptualization; formal analysis; investigation; methodology; validation; writing – original draft preparation. **Marcela Pires Spaolonzi:** Writing – original draft; methodology; formal analysis; data curation; investigation. **Carla Ivane Ganz Vogel:** Conceptualization; methodology; validation. **Aniela Pinto Kempka:** Supervision; writing – review and editing; validation; investigation; formal analysis; methodology. **Everton Skoronski:** Validation; writing – review and editing; supervision; resources; project administration; methodology; funding acquisition; investigation; formal analysis.

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CONFLICT OF INTEREST STATEMENT

The authors have no relevant financial or non-financial interests to disclose.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author, Everton Skoronski, upon reasonable request.

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