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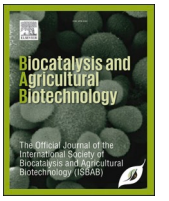

Yacon (*Smallanthus sonchifolius)* peel as a promising peroxidase source for the treatment of phenolic wastewater

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ABSTRACT

Peroxidases are versatile enzymes that catalyse the oxidation of many inorganic and organic substrates. There are numerous vegetable sources of peroxidases, including the yacon (*Smallanthus sonchifolius*), an easy-to-grow plant. This study aimed to extract, purify and characterise the peroxidases obtained from yacon roots. We partially purified the enzyme from yacon pell using an aqueous two-phase system (ATPS) and obtained 4.66 purification-fold and 33.87% activity recovery. The enzyme showed an optimal temperature of catalysis 60 °C and optimal pH 11 using pyrogallol as substrate. Considering the oxidation of 2,4-dichlorophenol, these conditions were 37.5 °C and pH 7. Furthermore, the enzyme presented substrate inhibition at high hydrogen peroxide concentrations. The presence of 5 mM Cu^{2+} inhibited, while and 5 mM Co^{2+} activated the enzyme. The enzyme remained stable at 50 °C for 60 min and showed improved thermostability compared to horseradish peroxidase. The kinetic mechanism of yacon peroxidase was investigated, and our studies suggested the enzyme follows a Ping-Pong Bi–Bi mechanism. Finally, 2,4-dichlorophenol was selected as a target pollutant, and we obtained as many as 86.26% of bioconversion using yacon peroxidase. The results presented in this article suggest that yacon is a promising source of peroxidase and can be an alternative for bioprocesses applications.

1. Introduction

Peroxidases (EC1.11.1.7) are predominantly heme proteins that use hydrogen peroxide or organic hydroperoxides as electron acceptors for the catalytic oxidation of diverse organic and inorganic substrates (Demarche et al., 2012; Elsayed et al., 2018; Pandey et al., 2017). These enzymes are classified into two families (plant and animal sources), and their molecular weight varies from 30 to 150 kDa (Elsayed et al., 2018). Several peroxidases obtained from plant sources have been characterised, and their potential application has been assessed. Their broad substrate specificity and the multifunctional properties of these enzymes justify their application in various analytical, industrial and biochemical processes (Vetal and Rathod, 2015). Some recently identified sources of peroxidases reported in the literature comprise *Cedrela fisilis* (Fritzke et al., 2020), *Commiphora gileadensis* (Almulaiky and Al-Harbi, 2019), *Ficus*

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carica (Elsayed et al., 2018), *Artocarpus heterophyllus* (Tao et al., 2018), *Azadirachta indica* (Pandey et al., 2017), *Panicum maximum* (Centeno et al., 2017), *Vigna radiata* (Basha and Prasada Rao, 2017) and *Caralluma umbellata* (Achar et al., 2017).

Peroxidases have been applied for the treatment of effluent-containing dyes (Fritzke et al., 2020; Jin et al., 2018; Zhang et al., 2018), phenolic compounds (Ely et al., 2017; Fernandes et al., 2020) and other contaminants (Morsi et al., 2020; Vo et al., 2019). Moreover, some studies evaluated its application in the development of biosensors (Díaz Nieto et al., 2018; Feizabadi et al., 2019; Kafi et al., 2018; Thenmozhi and Narayanan, 2017) and organic and polymer synthesis (Hamid and Khalil-ur-Rehman, 2009). Horseradish peroxidase (HRP) is the most well-known peroxidase. It is the choice of most high-value applications, including research, diagnostics, industrial and environmental use. Therefore, investigating alternative sources of peroxidases to attend to the global demand is crucial (Deva et al., 2014).

Yacon (*Smallanthus sonchifolius*) is an easy-to-grow plant from the Andes (Santana and Cardoso, 2008), which might be a promising alternative as a peroxidase vegetal source since it easily adapts to diverse climates. Furthermore, the tuberous yacon roots are valuable for nutrition and medicinal purposes (de Moura et al., 2012; Ojansivu et al., 2011; Perussello et al., 2015). Previous studies have already reported the presence of peroxidase enzymes in yacon roots that focused on the food industry (Neves and Da Silva, 2007; Padilha et al., 2009). Such studies investigated peroxidase inhibition to avoid polyphenols oxidation. Polyphenol oxidation affects taste, colour, aroma, and nutritional quality, which are undesirable events in food production (Campos et al., 2016; Fante et al., 2013). However, there are no studies on yacon peroxidases characterisation and its potential application in biochemical processes and environmental remediation to the best of our knowledge.

Therefore, we aimed to investigate the extraction, purification and characterisation of peroxidase obtained from yacon roots. Additionally, we demonstrate the application of yacon peel peroxidase (YPP) on 2,4-dichlorophenol bioconversion.

2. Material and methods

2.1. Material

Fresh yacon roots (*Smallanthus sonchifolius*) were purchased from local retailers in the municipality of Lages, Santa Catarina (Brazil) and stored under refrigeration (−10 °C) until use. The HRP enzyme was kindly provided by Toyobo (Brazil). All other chemicals used in this study were analytical grade.

2.2. Preparation of crude extract

Initially, the yacon roots were washed with distilled water, cut and peeled. For the protein extraction, 100 g of yacon peel were homogenised in 100 ml of potassium phosphate buffer pH 7.0 (100 mM) using mixer-type equipment for 5 min. Next, the extract was filtered using a vacuum pump, through filter paper (Whatman No. 1) and a layer of cotton gauze to remove suspended solids (Shahnawaz Khan et al., 2018). Then, 0.05% (w/v) of polyvinylpolypyrrolidone (PVPP) was added to the filtrate (Deva et al., 2014) under gentle stirring in an ice bath for 30 min. Finally, the solution was centrifuged at 4,000 rpm (2,150×*g*) for 20 min to obtain a clear liquid.

2.3. Partial peroxidase purification

2.3.1. Precipitation with ammonium sulphate

The crude extract remained under stirring, and ammonium sulphate was added to reach 80% saturation (0 °C) in the solution (Almulaiky and Al-Harbi, 2019). Next, the solution was centrifuged at 4,000 rpm (2,150×*g*) for 20 min. Finally, the supernatant was removed, the sedimented fraction was resuspended in potassium phosphate buffer pH 7.0 (100 mM) and subsequently dialysed.

2.3.2. Three-phase partitioning system (TPP) using yacon crude extract

This procedure was performed according to Vetal and Rathod (2015). The crude extract and *t*-butanol in a 1:1.5 (v/v) ratio and ammonium sulphate (50% w/v) were added to a 100 mL beaker. The solution remained under stirring for 80 min, in a magnetic stirrer, the temperature was controlled at 28 ± 2 °C. The mixture was centrifuged at 4,000 rpm (2,150×*g*) for 20 min to enhance the phase separation and then transferred to a separating funnel to rest for another hour. Three distinct phases were observed after separation in the funnel. The bottom phase was removed and dialysed as it is assumed to contain the more significant activity of peroxidase enzymes (Vetal and Rathod, 2015).

2.3.3. Three-phase partitioning system (TPP) using yacon peel powder

This step was performed according to the methodology proposed by Panadare and Rathod (2017). At first, the yacon peel was dried at 40 °C for 24 h. The dry peels were gritted in a hand blender and sieved. The obtained average particle size was 0.225 mm. The partitioning was performed in a 100 mL beaker. Yacon peel powder (0.15 g/mL) was added to dimethyl carbonate (DMC) and water at a ratio of 0.75:1 (v/v), and 20% (w/v) sodium citrate. This suspension remained under stirring for 3 h in a magnetic stirrer at 28 ± 2 °C. Subsequently, the mixture was separated by centrifugation at 4,000 rpm (2,150×*g*) for 20 min. The bottom phase was separated and dialysed.

2.3.4. Aqueous two-phase system (ATPS)

As the previous methods were inefficient in removing undesirable compounds and concentrating the crude extract, we selected another alternative. Therefore, an aqueous two-phase system was used Al-Bagmi et al. (2019). According to this method, 14% (w/v) of solid polyethylene glycol (PEG 6,000) and 10% (w/v) of solid ammonium sulphate ($(NH_4)_2SO_4$) were added to the crude extract. The mixture remained under stirring at 4 °C until complete PEG dissolution and then was transferred to a separatory funnel for 24 h at 4 °C.

The bottom-phase, which is assumed to contain peroxidase (Shahnawaz Khan et al., 2018), was centrifuged at 4,000 rpm (2,150×*g*) for 20 min. Finally, the clarified liquid was removed and then dialysed.

2.3.5. Dialysis

The separated liquids were dialysed against potassium phosphate buffer pH 7.0 (100 mM) for 12 h at 4 °C using a cellulose membrane dialysis (MWCO 6000Da). The buffer was replaced three times (Pandey et al., 2017), and the final samples were analysed for protein concentration and enzyme activity.

2.3.6. Efficiency of the patrial purification methods

The evaluation of the partial purification methods in terms of specific activity, recovery and purification fold is defined according to Equations (1)–(3).

$$Specific\ activity\left(\frac{U}{mg}\right) = \frac{Total\ activity\ (U)}{Total\ protein\ (mg)} \tag{1}$$

$$Recovery_{(\%)} = \frac{Total\ activity\ in\ purified\ fraction\ (U)}{Total\ activity\ in\ crude\ extract\ (U)} * 100 \tag{2}$$

$$Purification\ fold = \frac{Specific\ activity\ in\ purified\ fraction\ \left(\frac{U}{mg}\right)}{Specific\ activity\ in\ crude\ extract\ \left(\frac{U}{mg}\right)} \tag{3}$$

2.4. Analytical methods

2.4.1. Protein concentration and enzyme activity

Total protein concentration was measured using Coomassie Brilliant Blue G-250 dye (Bradford, 1976). Bovine serum albumin (BSA) was used as standard. The absorbance was measured at 595 nm in a UV–vis spectrophotometer (Merck; Model Pharo 300). The concentration was expressed in mg/mL.

Peroxidase activity was determined according to Sigma-Aldrich, using pyrogallol as substrate. The reaction system (3 mL) consisted of 2.05 mL of ultrapure water, 0.32 mL of potassium phosphate buffer (0.1 M, pH 6), 0.16 mL of hydrogen peroxide solution (0.5%) and 0.32 mL of pyrogallol solution (5%). This mixture was incubated at 20 °C until the thermal equilibrium. Afterwards, 0.15 ml of the enzymatic extract was added and homogenised in a vortex-type device before absorbance read at 420 nm in a UV–vis spectrophotometer (Merck; Model: Pharo 300). According to this protocol, a peroxidase unit (U) is the amount of enzyme that catalyses the formation of 1.0 mg of purpurogallin from pyrogallol in 20 s, at pH 6.0 and temperature of 20 °C (Reaction 1).

$$H_2O_2 + Pyrogallol \xrightarrow{peroxidase} 2H_2O + Purpurogallin \tag{1}$$

A control test was performed for each assay. In this case, the enzyme solution was replaced by 0.15 mL of buffer. The absorbance variation recorded in this test was used to calculate the enzyme activity, as shown in Equation (4).

$$Activity\left(\frac{U}{mL}\right) = \frac{\left(\frac{\Delta A_{420}}{20\ s}\ assay - \frac{\Delta A_{420}}{20\ s}\ blank\right) V_{assay}}{12 * V_{enzyme}} \tag{4}$$

where, V_{assay} represents the total volume of the assay (3 mL) and V_{enzyme} refers to the volume of enzyme extract used (0.15 mL). ΔA_{420} is the variation in absorbance at 420 nm and, 12 is the extinction coefficient of 1 mg/mL of purpurogallin.

Besides, using 2,4-dichlorophenol (2,4-DCP) as substrate, peroxidase activity was determined according to Antonopoulos et al. (2001) with modifications. The reaction mixture consisted of 1 mL of phosphate buffer (100 mM, pH 7), 0.6 mL 2,4-DCP (25 mM), 0.6 mL 4-aminoantipyrine (16 mM), 0.2 mL of enzymatic solution and 0.6 mL of H_2O_2 (50 mM). The reaction was started by adding hydrogen peroxide. The absorbance variation was read in 510 nm during 1 min, using a UV–vis spectrophotometer (Merck; Model: Pharo 300). A peroxidase unity (U) was defined by the change in absorbance during the time interval analysed.

2.4.2. Electrophoresis

All the partially purified extract was submitted to electrophoresis on SDS-polyacrylamide gel (SDS-PAGE) with 12% (separation) and 4% (concentration) gel for 1.5 h (220 V, 70 mA). In addition, the proteins were stained with Coomassie Blue R-250 dye.

2.5. Effect of pH and temperature on enzyme activity

The effect of pH on the enzyme activity, using pyrogallol as substrate, was carried out using McIlvaine buffer (0.1 M citric acid/0.2 M sodium phosphate) pH from 2.6 to 8 and Sorensen glycine buffer (0.1 M glycine/0.1 M sodium chloride and 0.1 M sodium hydroxide) pH from 10 to 13. Enzyme activity was measured in triplicate. The effect of the temperature on enzyme activity was assessed according to the standard protocol (item 2.4.1) and incubating the assay mixture, excepting the enzyme solution at 0, 15, 30, 45, 60, 75 and 90 °C until reaching thermal equilibrium. Afterwards, the enzyme extract was added, and the absorbances were immediately

measured as described in item 2.4.1. The thermal stability of YPP was verified by incubating enzyme aliquots at 50, 60 and 70 °C for 60 min. Then, aliquots were sampled at 10 min intervals to measure the remaining enzyme activity.

A similar assay was conducted using 2,4-DCP. Based on previous experiments, we used McIlvaine buffer pH 3 to 8 and Sorensen glycine buffer pH between 10 and 11. The temperature effect was determined by incubating the assay mixture (5, 15, 30, 37.5, 45, 60 and 75 °C) until reaching thermal equilibrium. Afterwards, the enzyme extract was added, and the absorbances were immediately measured as described in item 2.4.1.

The highest observed activity was considered 100% for both substrates, and the other results expressed relative activity (%).

2.6. Effect of substrate concentration on enzyme activity

We measured the enzyme activity according to the standard protocol (item 2.4.1) varying the concentrations of pyrogallol (1.04, 2.08, 4.16, 8.32, 20.8, 41.6 and 83, 20 mM) with H_2O_2 (0, 2.08, 4.16, 20.8, 41.6, 104, 208 and 416 mM) and 2,4-DCP (0.05, 0.5, 1, 3, 5, 7, 10 and 13 mM) with H_2O_2 (0.05, 0.1, 0.25, 0.5, 0.75, 1.25, 2.5, 5 and 10 mM).

The obtained data were applied to determine the Michaelis-Menten constant (K_m) and the maximum reaction speed ($V_{máx}$) in terms of pyrogallol and H_2O_2. We considered the following models for a single substrate: Michaelis-Menten (Equation (5)), uncompetitive inhibition by the substrate excess (Equation (6)) and mixed inhibition (Equation (7)). We also consider the Ping-Pong Bi–Bi (Equation (8)) mechanism as a two-substrate model. The Enzyme Kinetics Wizard tool from SigmaPlot 14.0 software (Systat) was applied for fitting the models to experimental data.

$$V_0\left(_{mM/min}\right) = \frac{V_{máx} * [A]}{K_m + [A]} \tag{5}$$

$$V_0\left(_{mM/min}\right) = \frac{V_{máx}}{K_m/_{[A]} + 1 + [A]/_{K_i}} \tag{6}$$

$$V_0\left(_{mM/min}\right) = \frac{V_{máx}}{\left(K_m/_{[A]}\right) * \left(1 + [I]/_{K_i}\right) + \left(1 + [I]/_{K_i'}\right)} \tag{7}$$

$$V_0\left(_{mM/min}\right) = \frac{V_{máx} * [A] * [B]}{K_b * [A] + K_a * [B] + [A] * [B]} \tag{8}$$

where, $[A]$ and $[B]$ are the substrate concentrations, K_a and K_b are the Michaelis-Menten constants for the two distinct substrates, $[I]$ represents the inhibitor concentration, K_i is the equilibrium constant of the inhibitor binding and K_i' is the equilibrium constant for binding the inhibitor to the enzyme-substrate complex.

2.7. Effect of selected metal ions and polyethylene glycol (PEG) on enzyme activity

The effect of selected metal ions on the enzyme activity was considered the following species: Ba^{+}, Ca^{2+}, Co^{2+}, Cu^{2+}, K^{+}, Na^{+}, Mg^{2+}, Mn^{2+}, Ni^{2+} and Zn^{2+}. We measured the enzyme activity, following the standard protocol (item 2.4.1), after incubating the enzyme solutions for 15 min in the presence of 1 and 5 mM of the selected ions (Tao et al., 2018). The effect of PEG on the enzyme activity was determined with the addition of PEG (4,000 and 6,000) in different dosages (0, 25, 50, 100, 250 and 500 mg/L) to the enzyme solution. A control test, i.e. without the addition of relevant metal ions in the solution or PEG, was considered 100%, and the other results were expressed in terms of relative activity (%).

2.8. Storage stability

For the storage stability study, a solution containing enzyme activity of 1 U/mL were kept at room temperature (21.31 ± 1.22 °C) and under refrigeration (−1.22 ± 0.74 °C) for two weeks. During this period, aliquots were taken for enzyme activity determinations. Similarly, for comparison purposes, the experiment was likewise performed using a solution containing 1 U/mL prepared with a commercial HRP enzyme (Toyobo do Brasil) instead. The activity measured on the first day was considered 100%, and the subsequent results were expressed as a relative activity.

2.9. Application of YPP in 2,4-dichlorophenol synthetic wastewater

2.9.1. Variation of enzyme concentration and reaction time

The 2,4-DCP bioconversion, mediated by YPP enzyme, was evaluated dosing enzymatic solution (0, 0.01, 0.02, 0.05 and 0.1 U/mL) in samples of synthetic wastewater (2,4-DCP 50 mg/L and pH 7), adjusted with potassium phosphate buffer (100 mM). Aliquots of the wastewater were kept under orbital agitation in shaker-type equipment at 30 °C. The time counting started with hydrogen peroxide (0.3 mM). Samples were collected at times of 0, 15, 30, 45, 60, 90, 120 and 180 min and subsequently conducted to determine the remaining concentration of 2,4-DCP by colourimetric analysis, according to the method 5530 D (APHA, 2005), using UV–vis spectrophotometer (Merck; Model Pharo 300). The results were expressed through the relationship between the initial concentration of 2,4-DCP (C_0) and the concentration remaining at the end of the evaluated time intervals (C).

Table 1
Summary of purification procedures for peroxidase from yacon peels. Enzyme activity assay: temperature 20 °C; pH 6; [H_2O_2] 7.73 mM; [Pyrogallol] 41.6 mM; contact time 20 s.

Sample	Total activity (U/mL)	Total protein (mg/mL)	Specific activity (U/mg)	Recovery (%)	Fold purification
Crude extract	31.00	85.00	0.36	100.00	1.00
$(NH_4)_2SO_4$ (80%)	23.10	34.10	0.68	74.52	1.88
TPP	17.75	16.00	1.11	57.26	3.08
TPP (powder)	0.11	0.30	0.37	–	–
ATPS	10.50	6.25	1.68	33.87	4.66

2.9.2. Hydrogen peroxide influence on the bioconversion of 2,4-DCP catalyzed by YPP in synthetic wastewater

Were observed the effect of hydrogen peroxide (0, 0.13, 0.25, 0.50, 0.63, 0.75 and 1 mM) in the wastewater treatment. In a synthetic wastewater sample (2,4-DCP 50 mg/L and pH 7) were added YPP in order to have an enzyme concentration in the reaction medium with an activity of 0.05 U/mL, and finally, the H_2O_2. The solution was kept under orbital agitation in shaker-type equipment (30 °C and 180 min). According to previously described, aliquots were collected and conducted to analyse the remaining concentration of 2,4-DCP.

2.9.3. Effect of controlled addition of enzyme and hydrogen peroxide

We also evaluated the effect of the controlled addition of hydrogen peroxide, which can avoid the enzyme's suicide mechanism. This effect occurs when excessive amounts of H_2O_2, compared to substrate availability, cause the enzyme to become catalytically inactive (Kennedy et al., 2002).

Thus, two distinct dosing procedures were established until a final H_2O_2 concentration of 0.63 mM and enzymatic activity of 0.05 U/mL were obtained in the reaction medium. The first (T1) consisted of five H_2O_2 applications of 25 μL and 40 μL of YPP solution at 0, 15, 30, 45 and 60 min. In the second (T2), dosages were performed at 0, 30, 60, 90 and 120 min. In addition, a control test was performed, in which all the enzyme and hydrogen peroxide were added right at the start. All assays were kept under agitation until the end of the 180 min period. Subsequently, samples were taken to determine the final 2,4-DCP concentration.

2.9.4. Comparative use of YPP and HRP on bioconversion of 2,4-DCP in synthetic wastewater

To demonstrate a potential application of YPP, we carried out a 2,4-dichlorophenol bioconversion experiment using a synthetic contaminated-water, containing 50 mg/L of 2,4-DCP. Based on preliminary tests, we define the following conditions for this reaction: H_2O_2 concentration of 0.63 mM, YPP activity of 0.05 U/mL, pH 7, the temperature of 30 °C, and contact time of 180 min. Analogously, we also performed a test using the enzyme HRP under the same experimental conditions. After the reaction, aliquots were sampled to determine the remaining concentration of 2,4-DCP via colourimetric analysis (method 5530 D) (APHA, 2005), using a UV–vis spectrophotometer (Merck; Model Pharo 300).

2.10. Statistical analysis

All experiments were carried out in triplicate. The data were analysed by ANOVA single factor and Tukey's test with 95% confidence, assisted by Statistica 12.0 software (StatSoft).

3. Results and discussion

3.1. Partial purification

The current study focused on different processes for the partial purification of yacon peel peroxidase and aimed at developing an effective method to obtain an enzymatic extract for use in lower purity applications, such as environmental remediation. The development of an easily handled purification protocol may lead to effective methodologies (Panadare and Rathod, 2018) such as TPP (three-phase partitioning system) and ATPS (aqueous two-phase system), which have been successfully applied to the extraction and partial purification of several enzymes (Nadar et al., 2017; Panadare and Rathod, 2018).

Our experiments observed that the crude extract showed an intense dark colour, possibly due to polyphenol oxidation. The oxidation was observed during the root peeling and suggested the presence of polyphenol oxidase producing o-quinones (Neves and Da Silva, 2007). Although polyphenols were precipitated by the PVPP method during the extraction, the solution remained dark-coloured, likely due to the presence of lignocellulosic materials in the yacon root peels. Moreover, yacon peel comprises approximately 54% of dietary fibre, which corresponds to a mixture of complex organic, including hemicelluloses, celluloses, lignins, pectins, gums, and non-digestible oligosaccharides (Pereira et al., 2013).

The ammonium sulphate-obtained extract, apart from proteins, concentrated many of the impurities from the crude extract. This hypothesis corroborates the dark colouration of the solution. In fact, it is well-known that precipitation by ammonium sulphate is not a selective technique (Panadare and Rathod, 2018). Nonetheless, we obtained 74.52% of enzymatic activity recovery and purification of 1.88-fold due to the salting-out effect (Table 1). Similar results were found by Tao et al. (2018), with a recovery of 78.90% and a purification of 2.3-fold and Pandey et al. (2017), with the recovery of 72.56% and purification of 2.04-fold. It is worth mentioning that both studies were on the extraction of peroxidase from different vegetable sources.

The TPP-based purification method similarly produced a partially purified dark-coloured extract. However, the recovery (57.26%) and purification yields (3.08-fold) suggest that the process can be optimised, being influenced by several factors such as the solvent and

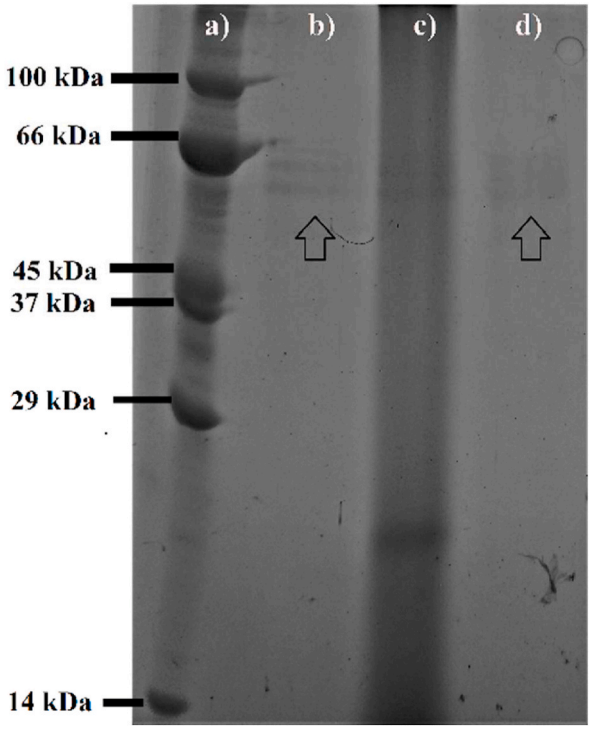


Fig. 1. SDS-PAGE of: **a)** protein marker, **b)** Three-phase partitioning (TPP) for the peel powder, **c)** Three-phase partitioning (TPP) for the crude extract, and **d)** Aqueous two-phase system (ATPS). (220 V, 70 mA, and 1.5 h).

salt concentrations, reaction time, pH, temperature and agitation (Narayan et al., 2008; Vetal and Rathod, 2015). Under optimised conditions, Vetal and Rathod (2015) obtained improved yields for TPP-based purification of orange peel peroxidase (*Citrus sinensis*), with a purification factor of 18.20-fold and 93.96% recovery of activity. Also, Narayan et al. (2008) reported 81% recovery and purification of 18-fold for *Ipomoea palmata* peroxidases.

The TPP process using yacon peel powder is not a partial purification of the crude extract but a direct extraction alternative. Therefore, the recovery and the purification factor for this methodology were not presented (Table 1). More importantly, the specific activity obtained via yacon peel powder and crude extract were similar. However, the peel powder extract was colourless, which indicates the absence of impurities observed from the crude extract. Despite this advantage, the total activity of 0.11 U/mL and protein concentration of 0.30 mg/mL was lower than the obtained results for the crude extract (17.75 U/mL and 16.00 mg/mL of total activity and protein concentration, respectively) (Table 1). This result may suggest that the protein migration from the powder to the solution was inefficient. Considering the variables that affect this process, the use of TPP may be interesting for the partial purification of YPP. Furthermore, it presents favourable results even at room temperature (Panadare and Rathod, 2017), making this application faster and cost-effective.

Alternatively, the ATPS process provided a partially purified extract with specific activity and purification factors superior to the other methods. Besides, the extract was likewise clear as the one obtained using TPP and peel powder. Accordingly, the ATPS technique was selected for partial YPP purification for the study. According to Nadar et al. (2017), this process has been used to purify a series of enzymes. The achieved purification factors are up to 123.89-fold, and the recoveries can reach 90%.

Finally, it is noteworthy that the electrophoresis analysis was not considered for crude extract and the extract partially purified by salting out did not undergo due to impurities in the samples. Besides, impurities were still noticeable on the extract partially purified by TPP, despite being previously considered clearer (Fig. 1-c). Regarding the TPP (powder) (Fig. 1–b) and ATPS (Fig. 1–d) processes, they provided the highest purity extracts. In addition, for either method, bands near to 66 kDa are visible for YPP, which is analogous with peroxidases from other sources such as *Carica papaya* L. with 69.4 kDa (Chen et al., 2012), *Streptomyces* sp.com 60 kDa (Fodil et al., 2012) and *Brassica oleracea* with 73.2 kDa (Erdem et al., 2015).

3.2. *Effect of pH on enzyme activity*

The pH effect on YPP activity is shown in Fig. 2-a). We observed that the maximum enzyme activity using pyrogallol was at pH 11, and relative activity was higher than 60% at pH 10 and 12. These results contrast with many other peroxidases, whose optimal pH is close to 7.0. For instance, *Viscum angulatum* peroxidases (Das et al., 2011) and *Persea americana Mill* (Rojas-Reyes et al., 2014) reported maximum enzyme activity at pH 7 and peroxidases extracted from *Carica papaya* L (Bari et al., 2014) at pH 6. In these cases, pyrogallol was applied as the standard substrate. Besides, Ely et al. (2017), using pyrogallol as a substrate, studied a commercial Horseradish peroxidase and observed the highest enzymatic activity at pH 7. On the other hand, when applying the enzyme in the bioconversion of

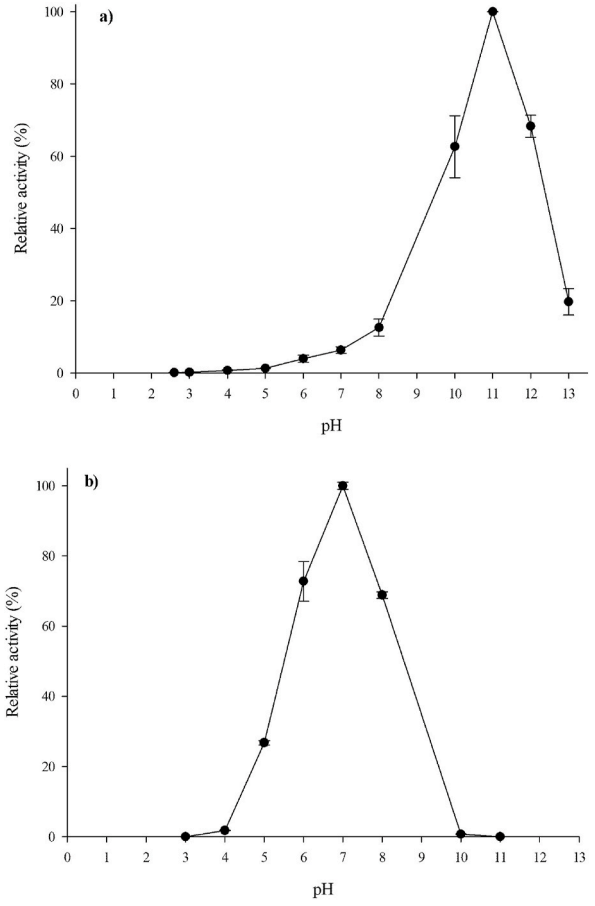


Fig. 2. Effect of pH on yacon peroxidase activity: **a)** substrate pyrogallol. Enzyme activity assay: temperature 20 °C; [H_2O_2] 7.73 mM; [Pyrogallol] 41.6 mM; contact time 20 s. **b)** substrate 2,4-DCP. Enzyme activity assay: temperature 20 °C; [H_2O_2] 10 mM; [2,4-DCP] 5 mM; contact time 60 s.

phenolic compounds in effluents, the optimum pH was 6.32. Considering the substrate 2,4-DCP, YPP showed maximum activity at pH 7, with reasonable performance between pH values from 6 to 8 (Fig. 2-b). Thus, a pH of 7 was selected for further testing.

Alternatively, some peroxidases showing optimal alkaline pH values have been reported. For example, Suzuki et al. (2006) studied peroxidases from *Fagopyrum esculentum* seeds and found optimum pH at 9 using guaiacol as a substrate. Al-Kassim et al. (1994) evaluated *Coprinus macrorhizus* peroxidases and obtained an optimum pH of 9 for the bioconversion of phenol, 2-chlorophenol and 2, 4-dichlorophenol and 9–10 for the bioconversion of 3-chlorophenol and 4-chlorophenol.

3.3. Effect of temperature on enzyme activity and thermal stability

YPP optimal activity was reached at 60 °C (Fig. 3-a) for pyrogallol, and similar results were previously reported for peroxidases extracted from *Jatropha curcas* and *Ricinus communis* (Cai et al., 2012; Kumar et al., 2008). Peroxidases extracted from *Persea americana Mill* and *Carica papaya* L (Bari et al., 2014), using pyrogallol as a substrate, expressed optimum temperatures at 35 and 38 °C, respectively. Also, Ely et al. (2017) demonstrated an optimal temperature of 50 °C of HRP when pyrogallol was used as substrate. Overall, the optimal temperatures for the peroxidase activity depend on the enzyme source, ranging from 20 °C for peroxidase from *Fagopyrum esculentum* (Suzuki et al., 2012) to 80 °C for peroxidases from *Streptomyces* sp. (Fodil et al., 2012). Such temperature-related variability might be attributed to the specific enzyme structure of each protein (Bouacem et al., 2018).

We also found that using 2,4-DCP as substrate, maximum enzyme activity was observed at 37.5 °C (Fig. 3-b). However, at 30 °C, it showed similar activity (98.37%), and at 45 °C, it still maintained, on average, 81.76% of the maximum observed activity. Thus, 30 °C was determined with the temperature to continue the treatability experiments, as it represents a more manageable condition to be achieved and can facilitate its application on an industrial scale. In terms of thermal stability, the enzyme was stable at 50 °C and maintained only 39.42% of the initial activity at 60 °C after 60 min. Nevertheless, the deactivation drastically increased at 70 °C as the enzyme presented only 8.02% of remaining activity after 10 min and was inactivated in 60 min (Fig. 4). This behaviour indicates that, although YPP showed optimal enzyme activity at 60–75 °C, its conformational structure is less favoured when exposed to higher temperatures. Indeed, the enzyme structure is thermosensitive and destabilises at high temperatures, which causes denaturation. Also, whereas the enzyme activity assay is a rapid test (20 s), in the thermal stability study, the enzyme is maintained at the same temperature for a more extended period before the enzyme activity measurement (Bisswanger, 2014). Consequently, although the

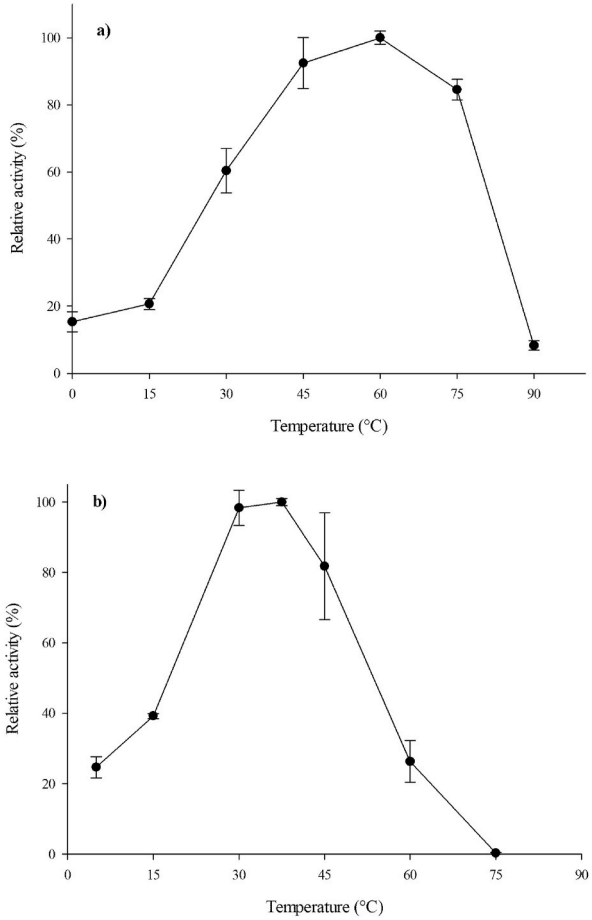


Fig. 3. Effect of temperature on yacon peroxidase activity: **a)** substrate pyrogallol. Enzyme activity assay: pH 6; [H_2O_2] 7.73 mM; [Pyrogallol] 41.6 mM; contact time 20 s. **b)** substrate 2,4-DCP. Enzyme activity assay: pH 7; [H_2O_2] 10 mM; [2,4-DCP] 5 mM; contact time 60 s.

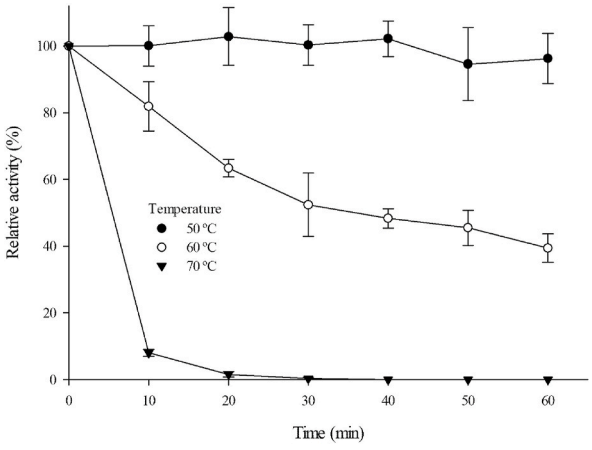


Fig. 4. Thermal stability of yacon peroxidase at 50, 60 e 70 °C. Enzyme activity assay: pH 6; enzyme concentration 0.42 ± 0.03 U/mL; [H_2O_2] 7.73 mM; [Pyrogallol] 41.6 mM; contact time 20 s.

maximum enzyme activity was recorded at 60 °C, the application of the enzyme is limited to processes that do not exceed 50 °C.

Similar behaviour was reported by Mogharrab et al. (2007) for the HRP, which showed a reduction of 96.4% in the enzyme activity at 70 °C after 10 min. Similarly, Lavery et al. (2010) observed maximum activity at 30 °C for HRP and the enzyme remarkably denatured at temperatures above 42 °C. Liu et al. (2006) reported that HRP retained only 30% of activity at 50 °C after 3 h of incubation.

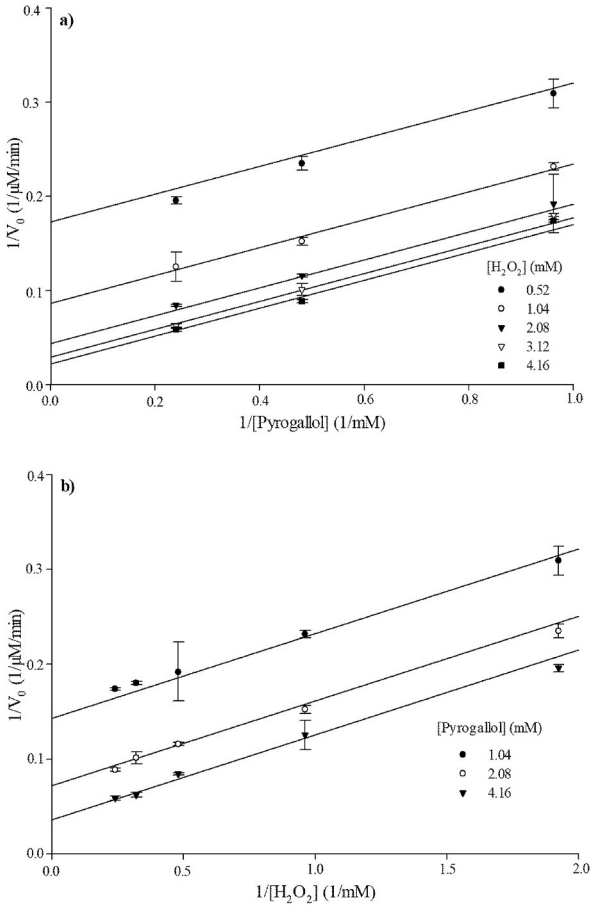


Fig. 5. Lineweaver-Burk plots for: **a)** pyrogallol concentration for different H_2O_2 concentrations and **b)** H_2O_2 concentration for different pyrogallol concentrations. Enzyme activity assay: temperature 20 °C; pH 6; enzyme concentration 0.42 ± 0.03 U/mL; contact time 20 s.

Finally, Al-Bagmi et al. (2019) comparatively analysed the peroxidase extracted from *Phoenix dactylifera* and a commercial HRP and highlighted that the extracted peroxidase remained stable at 75 °C for 60 min, while the HRP considerable decreased its activity (>90%) at the same conditions. These results suggest that YPP has thermostability equivalent or superior to HRP and may be a viable alternative for bioprocess. Furthermore, YPP and recently characterised peroxidases have shown promising thermal stability results. In particular, peroxidase of *Artocarpus heterophyllus* maintained enzyme activity without significant reduction during 60 min of incubation at 65 °C (Tao et al., 2018). Similarly, Pandey et al. (2017) showed that *Azadirachta indica* peroxidase was stable at 65 °C for 60 min and Elsayed et al. (2018) reported thermostable peroxidases extracted from *Ficus carica* thermostable at 50 °C.

3.4. Effect of substrate concentration and reaction mechanism

Two possible general mechanisms describe the interactions between enzyme and substrate in a two-substrate enzyme-catalyzed reaction: sequential or Ping-Pong Bi–Bi mechanisms (Galende et al., 2015). According to Fig. 5, a set of parallel lines fit the double-reciprocal data of the YPP activity as a function of non-inhibitory substrate concentrations, suggesting that YPP follows a Ping-Pong Bi–Bi reaction mechanism (Nelson and Cox, 2001; Voet and Voet, 1995).

This mechanism is described briefly: the first substrate combines with the enzyme creating an intermediate enzymatic state, releasing the first product. The second substrate interacts with the enzyme to form the second product, allowing the enzyme regeneration to the native state (Galende et al., 2015). In fact, the Ping-Pong Bi–Bi mechanism has already been described for other peroxidases, such as HRP (Šekuljica et al., 2015). The catalytic cycle of HRP involves the native enzyme oxidation to compound I by hydrogen peroxidase. Then, compound I is reduced by a reducing substrate to compound II, which is also reduced by another reducing substrate leading the enzyme to the native state, completing the cycle. However, an excess of hydrogen peroxide oxidises compound II to compound III, associated with the enzyme inhibition (Nicell and Wright, 1997; Veitch, 2004). Likewise, we verified this behaviour for the YPP (Fig. 6-a). In this case, concentrations of 20 mM H_2O_2 showed a strong inhibitory effect on the enzyme activity. The experimental data were described by an uncompetitive inhibition model ($R^2 = 0.98146$) due to excess substrate. In that case, the presence of a distinct site alternative to the active one is suggested, where the substrate interactions result in conformational changes in the enzyme structure, affecting the activity (Nelson and Cox, 2001; Voet and Voet, 1995). However, regardless of the inhibition effects,

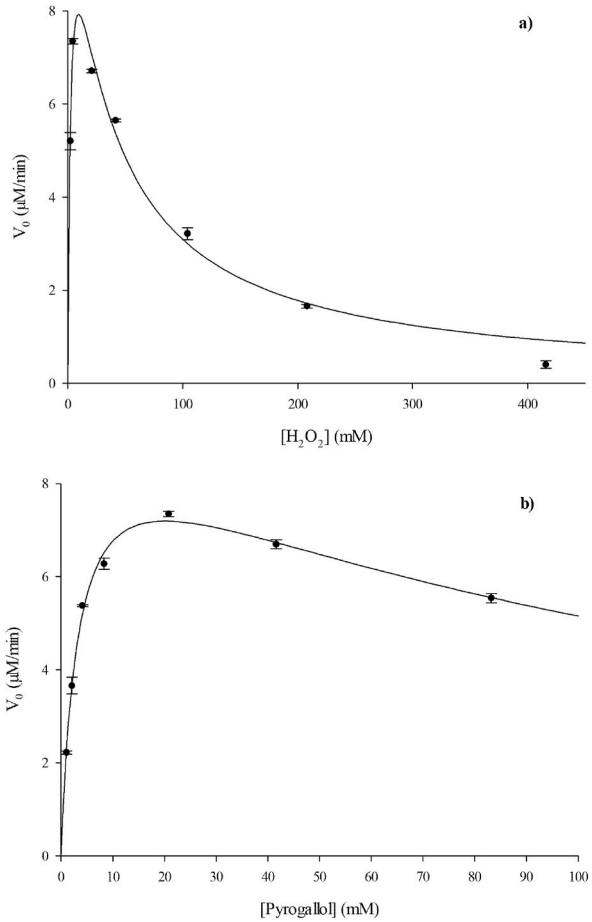


Fig. 6. a) Effect of H_2O_2 concentration increase on enzymatic activity with a saturating non-inhibitory pyrogallol concentration (20.80 mM). Enzyme activity assay: temperature 20 °C; pH 6; contact time 20 s. **b)** Effect of pyrogallol concentration increase on enzymatic activity with a saturating non-inhibitory H_2O_2 concentration (4.16 mM). Enzyme activity assay: temperature 20 °C; pH 6; contact time 20 s.

the low demand of H_2O_2 (<10 mM) is useful as the YPP reaches the maximum catalytic activity at a low electron acceptor concentration. It may imply lower costs associated with the enzyme application. The uncompetitive inhibition mechanism was similarly associated with the excess of the substrate ($R^2 = 0.9868$), in which concentrations above 41.60 mM caused reduced enzyme activity (Fig. 6-b).

As a result, considering the uncompetitive inhibition by either substrate and, as the behaviour of the enzyme in a ping-pong mechanism is quite similar (Galende et al., 2015), we would assume the possibility of competitive inhibition (indicated by Fig. 8). Furthermore, according to the Lineweaver-Burk graph, the determination coefficient ($R^2 = 0.9869$) and the lines pattern suggest the possibility of mixed inhibition. In other words, a possible competition between the substrates for the active site is assumed, including a binding to the enzyme-substrate complex (in an allosteric site).

Substrate competitive inhibition was also reported by Galende et al. (2015) for *Cytisus multiflorus* peroxidase. However, mixed inhibition was not identified. Besides, the authors also report variations in K_m relative to H_2O_2 it is used, with values from 0.71 to 4.10 mM. In our study, YPP presented K_m of 2.5 mM for peroxide and 3.4 mM for pyrogallol, which indicates a greater enzyme affinity for the first substrate. Other studies involving pyrogallol as a substrate reported K_m of 42.06, 46.42 and 40 mM for peroxidases from *Ipomoea carnea*, *Viscum angulatum* and *Persea americana Mill*, respectively (Das et al., 2011; Patel et al., 2008; Rojas-Reyes et al., 2014). These results suggest a higher affinity of yacon peel peroxidase in pyrogallol than the other cited enzymes.

Similarly to the behaviour observed using pyrogallol as a standard substrate, when using 2,4-DCP, the enzymatic activity showed high sensitivity to variations in the concentration of hydrogen peroxide (Fig. 7-a). Although, in this case, H_2O_2 concentrations higher than 0.5 mM showed inhibitory effects. The uncompetitive inhibition model also showed an excellent fit to the data ($R^2 = 0.94320$) and, according to the predicted values, the hydrogen peroxide concentration for the maximum enzymatic activity in the oxidation catalysis of 2,4-DCP was 0.3 mM. For 2,4-DCP, high substrate concentrations also suggest a tendency to inhibit enzymatic activity (Fig. 7-b). However, the customarily found concentrations of the contaminant in water and effluent are well below the maximum concentration evaluated in this study. Thus, concerning the treatment of effluents, the correct peroxide dosage is the factor that demands greater attention.

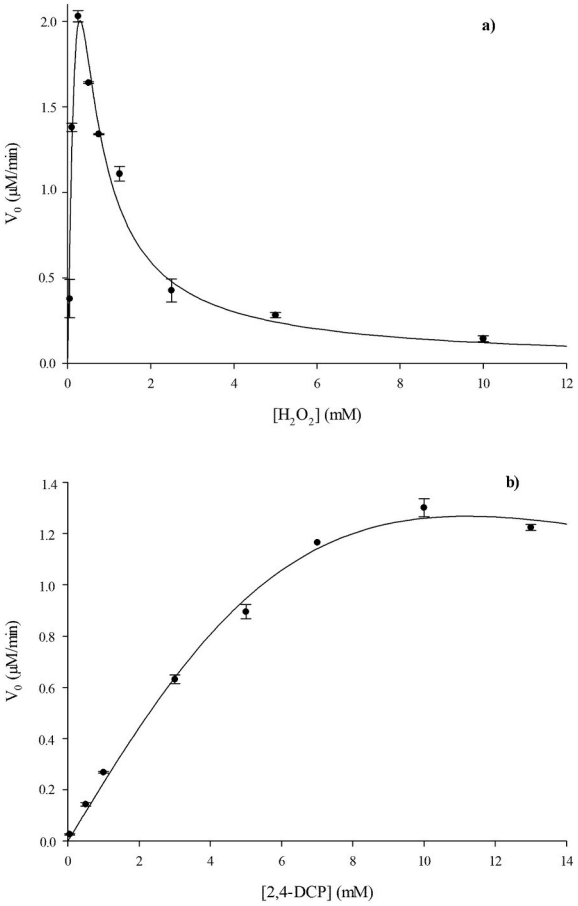


Fig. 7. a) Effect of H_2O_2 concentration increase on enzymatic activity with a saturating non-inhibitory 2,4-DCP concentration (5 mM). Enzyme activity assay: temperature 60 °C; pH 7; contact time 60 s. **b)** Effect of 2,4-DCP concentration increase on enzymatic activity with a saturating non-inhibitory H_2O_2 concentration (0.25 mM). Enzyme activity assay: temperature 20 °C; pH 7; contact time 60 s.

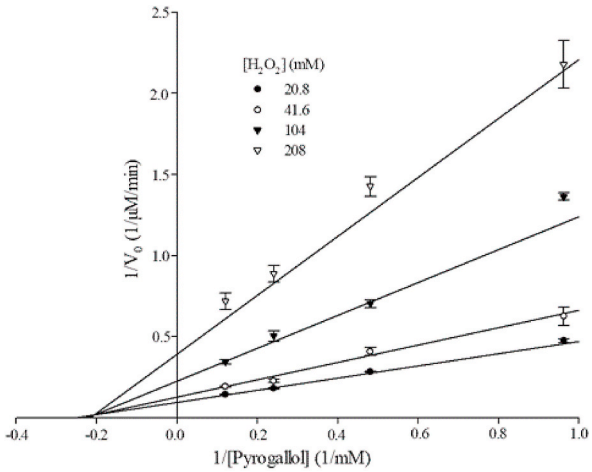


Fig. 8. Lineweaver-Burk plot with adjustment of a mixed inhibition model. Enzyme activity assay: temperature 20 °C; pH 6; enzyme concentration 0.42 ± 0.03 U/mL; contact time 20 s.

Table 2
Effect of metal ions on yacon peroxidase activity. Enzyme activity assay: temperature 20 °C; pH 6; enzyme concentration 0.42 ± 0.03 U/mL; [H_2O_2] 7.73 mM; [Pyrogallol] 41.6 mM; contact time 20 s.

Metal ions	Relative activity at different metal ions concentrations (%)	
	1 mM	5 mM
Control	100.00	100.00
Ba^{+}	94.78 ± 9.01	88.90 ± 9.07
Ca^{2+}	99.06 ± 7.99	85.11 ± 8.47
Co^{2+}	97.01 ± 5.14	129.37 ± 10.35 *
Cu^{2+}	92.01 ± 10.19	40.88 ± 9.32 *
K^{+}	114.38 ± 8.30	88.47 ± 8.29
Mg^{2+}	93.27 ± 6.12	94.83 ± 7.84
Mn^{2+}	100.85 ± 6.62	92.89 ± 5.83
Na^{+}	104.47 ± 8.87	106.33 ± 9.64
Ni^{2+}	87.76 ± 5.60	80.34 ± 6.27
Zn^{2+}	97.49 ± 12.70	97.59 ± 3.13

Values represent the mean ± standard deviation. Asterisks (*) indicate a significant difference between control and treatment ($p < 0,05$).

Table 3
Effect of polyethylene glycol (PEG) on yacon peroxidase activity. Enzyme activity assay: temperature 20 °C; pH 6; enzyme concentration 0.42 ± 0.03 U/mL; [H_2O_2] 7.73 mM; [Pyrogallol] 41.6 mM; contact time 20 s.

PEG	Relative activity at different concentrations (%)	
	4.000	6.000
0 mg/L (control)	100.00	100.00
25 mg/L	98.84 ± 12.47	112.64 ± 8.07
50 mg/L	109.70 ± 10.02	107.19 ± 13.38
100 mg/L	115.45 ± 13.87	107.54 ± 13.83
250 mg/L	118.39 ± 17.40	115.66 ± 14.34
500 mg/L	122.54 ± 19.22	104.25 ± 6.46

Values represent the mean ± standard deviation.

3.5. Effect of metal ions and PEG on enzyme activity

The effects of different metal ions on the activity of partially purified YPP are shown in Table 2. No significant change in enzyme activity was observed in the metal ions Ba^{+}, Ca^{2+}, K^{+}, Na^{+}, Mg^{2+}, Mn^{2+}, Ni^{2+} and Zn^{2+} at 1 and 5 mM. However, at a concentration of 5 mM, activation by Co^{2+} and inhibition by Cu^{2+} were observed.

Pandey et al. (2017) also reported activation of *Azadirachta indica* peroxidase by Co^{2+} in higher than 15 mM concentrations. In addition, they showed that the enzyme was stable in the presence of Na^{+}, Mg^{2+}, Mn^{2+}, Ca^{2+} and Ni^{2+}, remaining more than 90% of the control test. Nevertheless, Mg^{2+}, Mn^{2+}, Ca^{2+} was inhibited at higher concentrations as 500 mM. Similarly, Fritzke et al. (2020) observed that the presence of Na^{+}, Mg^{2+}, K^{+}, Ca^{2+} and Ni^{2+} (10 mM) did not affect the activity of *Cedrela fisilis* peroxidase. However, the author did not observe inhibition in the presence of Cu^{2+}. Likewise, Tao et al. (2018) reported that *Artocarpus heterophyllus* peroxidase was significantly inhibited, in the presence of Cu^{2+} and Ca^{2+} (5 mM), with a slight inhibitory effect in the presence of K^{+} (5 mM). In contrast, Zn^{2+} and Ba^{2+} (5 mM) provided an increase of 45.10% and 22.50%, respectively, in enzymatic activity. On the other hand, Elsayed et al. (2018) found different effects studying the effect of metal ions (1, 5 and 10 mM) on three *Ficus carica* peroxidase isoenzymes. For instance, one was inhibited by a high concentration of Cu^{2+}, while the others were activated. Moreover, within the three isoenzymes, Ni^{2+}activated one of them while Co^{2+} (5 and 10 mM) inhibited all the proteins.

Table 3 shows the results obtained for the enzyme activity in the presence of PEG. Despite increasing activity as the concentration of PEG 4,000 increases, there was no statistically significant difference between the tests and the control (i.e. no PEG). In contrast, Deva et al. (2014) reported that PEG provided significant protection to *Brassica oleracea* peroxidase, enhancing phenol removal from water by the enzyme. Moreover, this effect increased with the molecular mass of PEG (4,000, 6,000 and 10,000). However, concentrations above 150 mg/L did not significantly improve phenol removal. According to Kennedy et al. (2002), the addition of PEG affected the performance of soybean peroxidase (SBP) in the bioconversion of 2,4-dichlorophenol by factors of 10-fold (PEG 3,350) and 50-fold (PEG 8,000) positively. Additionally, Wu et al. (1997) demonstrated that the use of PEG 3,350 significantly reduced the dose of HRP required to remove phenolic compounds from aqueous solutions. The same behaviour was observed for SBP (Caza et al., 1999). Differently, Singh et al. (2017) observed that the addition of PEG decreased the removal of phenol from a synthetic effluent using *Prosopis juliflora* peroxidase.

3.6. Storage stability

There was a noticeable reduction in enzyme activity during the first days for solutions stored at room temperature (21.31 ± 1.22 °C), as shown in Fig. 9. However, at the end of the two weeks, the YPP solution retained 13.25% of activity while HRP accounted for 1.19%. In this case, the storage of some solutions such as buffers and inorganic salts at room temperature, although stable, may lead to contamination and microbial growth. Consequently, there are some protein degradation or inactivity (Bisswanger, 2014).

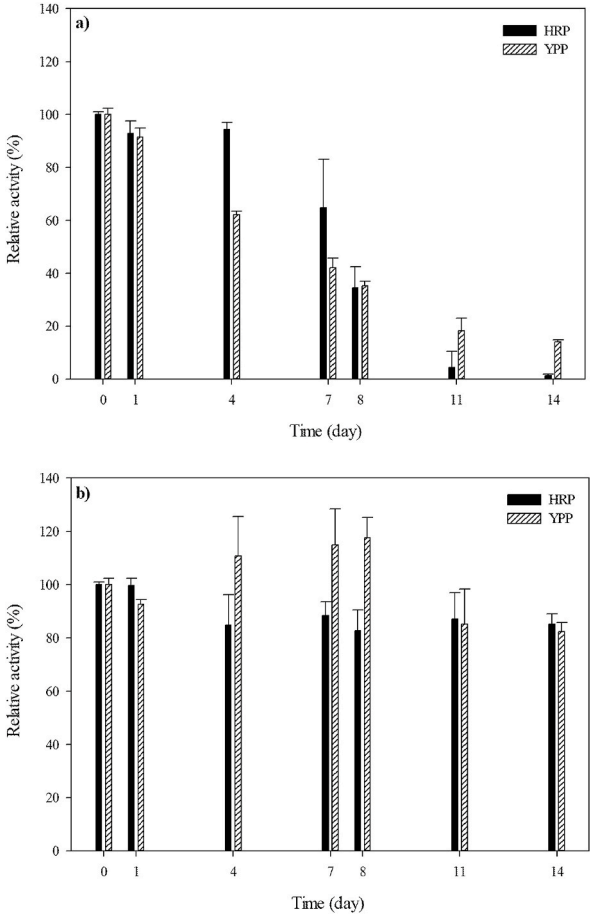
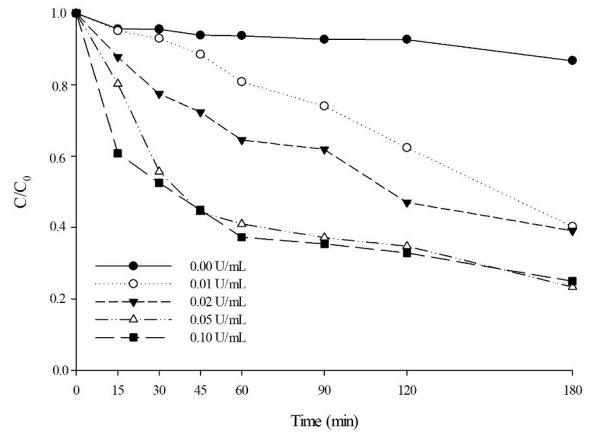


Fig. 9. Remaining activity of HRP and YPP (1 U/mL) as a function of temperature: **a)** room temperature (21.31 ± 1.22 °C) and **b)** refrigeration (−1.22 ± 0.74 °C). Enzyme activity assay: temperature 20 °C; pH 6; [H_2O_2] 7.73 mM; [Pyrogallol] 41.6 mM; contact time 20 s.



Figs. 10. 2,4-DCP bioconversion catalyzed by various YPP concentrations. Enzyme activity assay: [2,4-DCP] 50 mM; temperature 30 °C; pH 7; [H_2O_2] 0.3 mM.

Under refrigeration (−1.22 ± 0.74 °C), no reduction in enzyme activity was observed. Importantly, we detected that the enzyme solution of partially purified YPP showed similar stability compared to commercial HRP for this condition. For this reason, the obtained results are impressive regarding the enzyme application as the YPP requires simple refrigeration to maintain its activity.

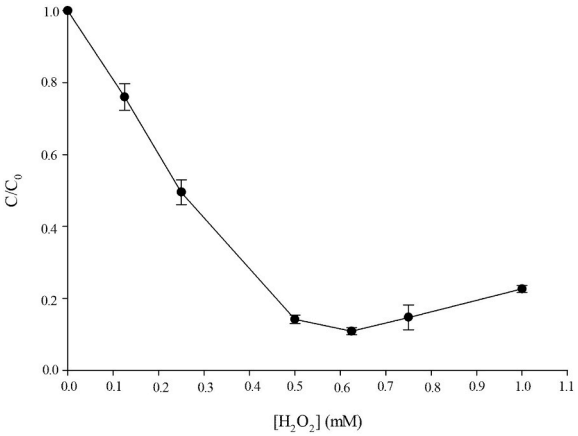


Fig. 11. Hydrogen peroxide effect on the bioconversion of 2,4 DCP catalyzed by YPP. Enzyme activity assay: [2,4-DCP] 50 mM; YPP 0.05 U/mL; temperature 30 °C; pH 7; contact time 180 min.

3.7. Application of YPP on 2,4-DCP bioconversion in synthetic wastewater

We observed that the best results obtained were using YPP with 0.05 and 0.1 U/mL activity, with bioconversion indexes of 76.67 and 75.06%, respectively (Fig. 10). Furthermore, although H_2O_2 concentrations between 0.25 and 0.3 mM have provided better levels of enzymatic activity, in terms of initial velocity, they seemed insufficient to complete the 2,4-DCP bioconversion when applied in prolonged treatment. Thus, in a more detailed study, it was possible to observe that 0.63 mM resulted in a better bioconversion efficiency (89.15%) (Fig. 11). This fact suggests that although this concentration has inhibitory effects on the initial rates, it can be quickly consumed in the reaction without negatively impacting the final bioconversion. However, slight variations result in loss of efficiency, which demonstrates the importance of controlling this parameter in an actual plant.

About the enzyme suicide mechanism, we highlighted that no significant differences ($p < 0.05$) between control and treatments T1 and T2, with a controlled dosage of enzyme and hydrogen peroxide, were observed on 2,4-DCP final concentration (Control: 6.22 ± 0.25 mg/L, T1 6.72 ± 0.46 mg/L and T2 7.09 ± 0.43 mg/L). This result indicates that, for the concentration of H_2O_2 under study, there is no inhibition of the enzyme during treatment. Despite this, it is noteworthy that this is a technique of great importance in optimizing the treatment of phenolic effluents using peroxidase enzymes and can bring gains in the final efficiency of over 20% (Kennedy et al., 2002). Moreover, simulated contaminated water containing 50 mg/L of 2,4-DCP (measured concentration of 48.07 ± 1,20 mg/L) was used to evaluate the application of YPP in potential environmental remediation. The treated water by YPP and HRP showed remaining 2,4-DCP concentrations of 6.34 ± 0.19 and 14.06 ± 0.11 mg/L, respectively. Thus, the results indicate an average bioconversion of 86.82% for YPP and 70.76% for HRP under the same reaction conditions. Similarly, Singh et al. (2017) extracted peroxidase from *Prosopis juliflora* and found superior removal efficiency of phenols and chlorophenols for this enzyme compared to HRP. Other authors also obtained effective removals of 2,4-DCP using alternative peroxidases. Kurnik et al. (2017) studied potato pulp residues as a source of peroxidase enzymes to remove 2,4-dichlorophenol from water and obtained results between 95 and 98%. Lakshmi et al. (2018) evaluated peroxidases extracted from corn production residues as an alternative to remediation of phenolic effluents and obtained efficiency close to 90%.

As was previously stated, the use of many residues for peroxidase extraction has been considered by many authors. Our study demonstrated that yacon peels must be considered a significant source of peroxidase. Further studies may consider the YPP in diverse potential biotechnology applications.

4. Conclusion

In conclusion, we demonstrated that the most advantageous extraction and partial purification of yacon peroxidase were via the ATPS process. In addition, the enzyme was characterised, and the reaction mechanism was demonstrated. Another critical point is the high sensitivity of the enzyme activity in terms of hydrogen peroxide concentration. Also, we verified that YPP showed stability similar to the commercial Horseradish peroxidase. Overall, peroxidase from yacon is an alternative to be explored in bioprocesses. However, further studies must address particular potential applications using this enzyme.

Declaration of interests

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

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Research article

Application of glycerol as carbon source for continuous drinking water denitrification using microorganism from natural biomass

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ABSTRACT

The contamination of water resources by nitrate is a global problem. Indeed, traditional treatment technologies are not able to remove this ion from water. Alternatively, biological denitrification is a useful technique for natural water nitrate removal. This study aimed to evaluate the use of glycerol as a carbon source for drinking water nitrate removal via denitrification in a reactor using microorganisms from natural biomass. The experiment was carried out in a continuous fixed bed reactor using immobilised microorganisms from the vegetal *Phyllostachys aurea*. The tests were started in batch mode to provide cells growth and further immobilisation on the support. Then, the treatment experiments were accomplished in an up-flow continuous reactor. Ethanol was used as the primary carbon source, and it was gradually replaced by glycerol. The C:N (carbon to nitrogen) ratio and the hydraulic residence time (HRT) were evaluated. It was possible to remove 98.14% of nitrate using a C:N ratio and HRT of 3:1 and 1.51 days, respectively. The results have demonstrated that glycerol is a potential carbon source for denitrification in a continuous reactor using immobilised cells from natural biomass.

1. Introduction

Despite its availability, groundwater quality has been affected by the increased concentration of the nitrate ion. Several factors contribute to this problem, including the final disposal of domestic sewage in septic tanks followed by septic drain field and the indiscriminate use of fertilisers in agriculture (Liu et al., 2012; Ravnjak et al., 2013). Nitrate contamination of groundwater is a worldwide problem (Almasri, 2007). Apart from the anthropogenic activities, other reasons such as geogenic factors may be related to the presence of nitrate in nature (Shukla and Saxena, 2018). High concentrations of nitrate have been detected not only in groundwater but also in surface waters supplies (World Health Organization, 2016). Besides, the water contamination by nitrate covers large areas around the world, for instance: Bulgarian rural areas (Gatseva and Argirova, 2008), Morocco (Sadeq et al., 2008), France (Rogers et al., 2012), Mexico (Rojas Fabro et al., 2015), Indonesia (Sadler et al., 2016) and Poland (Czekaj et al., 2016). Therefore, the importance of the study and development of effective methods of treatment for these problems have been increasing. Particularly in Brazil, the municipality of Natal (RN) is an example, where 40% of drilled wells are contaminated by this ion due to the poor sanitation conditions of collection and domestic sewage treatment (Cabral et al., 2009). Drinking water containing high concentrations of nitrate and nitrite may be related to the incidence of severe health issues, for instance, the methemoglobinemia or "the blue baby syndrome". Furthermore, the daily intake of high nitrate doses may be related to the development of gastric cancer, caused by the formation of carcinogenic nitrosamines (Bryan et al., 2012; Espejo-Herrera et al., 2015; Li et al., 2015; Shrimali and Singh, 2001).

Based on this scenario, the reduction of nitrate concentration in the drinking water up to safe values is of great importance. However, due to the high stability and solubility of this ion, the removal via precipitation is impractical. Consequently, nitrate removal using conventional water treatment technologies is considered a challenge (Gibert et al., 2008). Several treatment processes have been studied for nitrate removals, such as adsorption, membrane separation and electrochemical processes (Gao et al., 2019; Gouran-Orimi et al., 2018; Kalaruban et al., 2017). However, the main drawbacks of these processes include the generation of undesirable by-products and a high cost, what makes the biological

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nitrate removal process via denitrification a promising alternative (Mohseni-Bandpi et al., 2013; Shrimali and Singh, 2001). This process is a selective method and is generally more cost-effective than physico-chemical methods and has typically demonstrated high efficiency in the treatment (Cyplik et al., 2013; Liu et al., 2012). Additionally, denitrification has also been performed by inorganic catalysts and showed promising results (Guo et al., 2018; Li et al., 2019).

In this paper, we focused on biological denitrification. Although nitrate removal from the water via biological denitrification is a well-known process, studies using natural biomass as a source of microorganisms are still scarce. Most studies report the use of sewage sludge as inoculum for this process (Buttiglieri et al., 2005; Ergas and Rheinheimer, 2004; Ravnjak et al., 2013; Zhang et al., 2009; Zhao et al., 2012). However, when it comes to natural biomass, one of the first works is described by Bucco et al. (2014). The authors used bamboo as a source of microorganisms for denitrification. The clean origin of biomass was considered as the main advantage of its use, an essential aspect in the treatment of drinking water. In this same sense, Chu and Wang (2016) evaluated the mixtures of different biopolymers as carbon source and biofilm carriers. The authors concluded that the use of the bamboo-based materials allowed a faster start-up of the bioreactor, as well as increased nitrate removal efficiency. Recently, the use of bamboo is also reported in the study by Costa et al. (2018). The authors have observed complete removal of nitrate and nitrite in 48 h and concluded that the microorganism *P. mirabilis* played a significant role in the denitrification process.

The bacteria responsible for denitrification are mostly heterotrophic. Consequently, it is necessary the addition of one external carbon source for bacterial growth and energy supplying to convert nitrate into gaseous nitrogen (Mohseni-Bandpi et al., 2013; Salem et al., 2007; Vasiliadou et al., 2009). Methanol is the most commonly used chemical for this purpose (Ergas and Rheinheimer, 2004; Zhao et al., 2012). Nonetheless, this use has as main disadvantage its toxicity potential (Waters et al., 2018) and, for this reason, the use of less toxic carbon sources becomes attractive. Bucco et al. (2014), Ravnjak et al. (2013) and Buttiglieri et al. (2005) have applied ethanol as carbon source and obtained a high percentage of denitrification. Zhang et al. (2009) reported the use of glucose and showed satisfactory results as well. Costa et al. (2018) used ethanol as a carbon source only to start up the process for microorganism's development. However, the cost related to the use of these sources may be a drawback for large-scale application (Modin et al., 2007).

In the last decades, the glycerol has been being a common by-product of the biodiesel manufacturing process. This compound is considered a promising source of carbon, given its low cost (Hájek and Skopal, 2010). Cyplik et al. (2013) and Costa et al. (2018) proposed the use of glycerol and observed high denitrification rates. Although some denitrification studies applied glycerol as the carbon source, they were carried out in batch mode. On the other hand, the use of continuous flow bioreactors, as reported by Cyplik et al. (2013), was applied for wastewater treatment. Therefore, the study of continuous processes consists of a fundamental approach to evaluate the applicability of this technology for real systems. Studies reporting the use of glycerol as a carbon source in continuous systems containing microorganisms obtained from natural biomass for water treatment are inexistent.

In this context, the objective of this study was to evaluate the use of glycerol as a carbon source for denitrification of water contaminated with nitrates, in a fixed bed reactor, using immobilised microorganisms from natural biomass.

2. Material and methods

2.1. Source of denitrifying microorganisms

As a source of denitrifying microorganisms, the vegetal *Phyllostachys aurea* obtained in the municipality of Lages (Brazil) (27°47′28.6″S and 50°18′23.2″W) was used. This municipality is characterised by a climate type Cfb - Temperate (wet mesothermic with mild summers) (Wahrlich et al., 2018). The average annual temperature is around 16 °C and annual average precipitation 1555.7 mm (Brazilian Agricultural Research Corporation - Embrapa, 2012).

The plant species used in this study was the *Phyllostachys aurea*, previously studied by Costa et al. (2018). Additionally, the authors considered the microorganism *Proteus mirabilis* as responsible for the denitrification process.

2.2. Inoculation of microorganisms and reactor start-up

For inoculation of the microorganisms, the vegetable stalks were cut and added to tap water at a ratio of 30% (weight basis) according to previous studies (Bucco et al., 2014; Costa et al., 2018). The tap water was contaminated with 25 mgN-NO_3/L and, ethanol was added as a carbon source at a C:N ratio of 1:1. Previous results demonstrated that these values provided a significant denitrification efficiency comparable to that obtained using higher organic compounds concentration (Costa et al., 2018). First, denitrifying bacteria developed in the biomass and eventually migrated to the water. So, the stalks were removed under stirring, and the microorganisms remaining in the solution were considered for the next steps. The microorganism's growth in the solution and were afterwards exposed to contact with a high-density polyethylene support medium for immobilisation (type BWTXTM) (Fig. S1 Supplementary Fig. S1). This Biofilm Support was kindly provided by Memphis LTDA (Brazil). The immobilisation process occurred through its growth adhered to the polyethylene support.

After complete denitrification, the support medium containing the immobilised microorganisms was transferred to the reactor. It consisted of a glass cylinder operated under the continuous up-flow regime (Fig. 1). The inner diameter and height of the reactor were 80 mm and 400 mm, respectively. The volume of the reactor was 2.01 L. The reactor was surrounded by a jacket for heated water circulation to maintain the internal temperature. In the study of Costa et al. (2018), the impact of different temperatures on the denitrification rate presented by microorganisms was evaluated. The best results were obtained with temperatures close to 30 °C. Thus, in this work was set the temperature of 28 °C for the experiments.

Contaminated water was introduced into the reactor using a peristaltic pump with a maximum flow rate of 1.2 L/h. The water contaminated with both nitrate and ethanol was stored in an external 20 L container. The experiment was monitored by analysing nitrate, nitrite and turbidity. Analytical determination of the first two parameters was performed on a PHARO 300 (Merck) spectrophotometer. The effluent turbidity was determined by an Alfakit portable turbidimeter. The standard methodology for nitrite determination (4500-NO2-B) and turbidity (2130 B) was followed (APHA, 2005). Nitrate was measured according to the methodology established by the German Institute for Standardization (Deutsches Institut Für Normung, 2011). Nitrate and nitrite concentrations were compared with the guidelines values for drinking-water quality (WHO, 2017). In this case, the maximum nitrate concentration is 50 mgNO_3/L, equivalent to 10 mgN-NO_3/L, and the maximum nitrite concentration is 3 mgNO_2/L, equivalent to 1 mgN-NO_2/L.

2.3. Variation of the ratio C:N, carbon source and HRT

Initially, ethanol was used as a carbon source, since it presented better denitrification results, according to the study by Costa et al. (2018). The carbon concentration introduced varied according to each condition tested. After achieving high (above 90%) denitrification efficiency, glycerol was partially fed as a carbon source. A portion of the carbon supplied to the system was due to both ethanol and glycerol, in different proportions (Table 1). This procedure was performed in order to gradually adapt the microorganisms to the new carbon source until

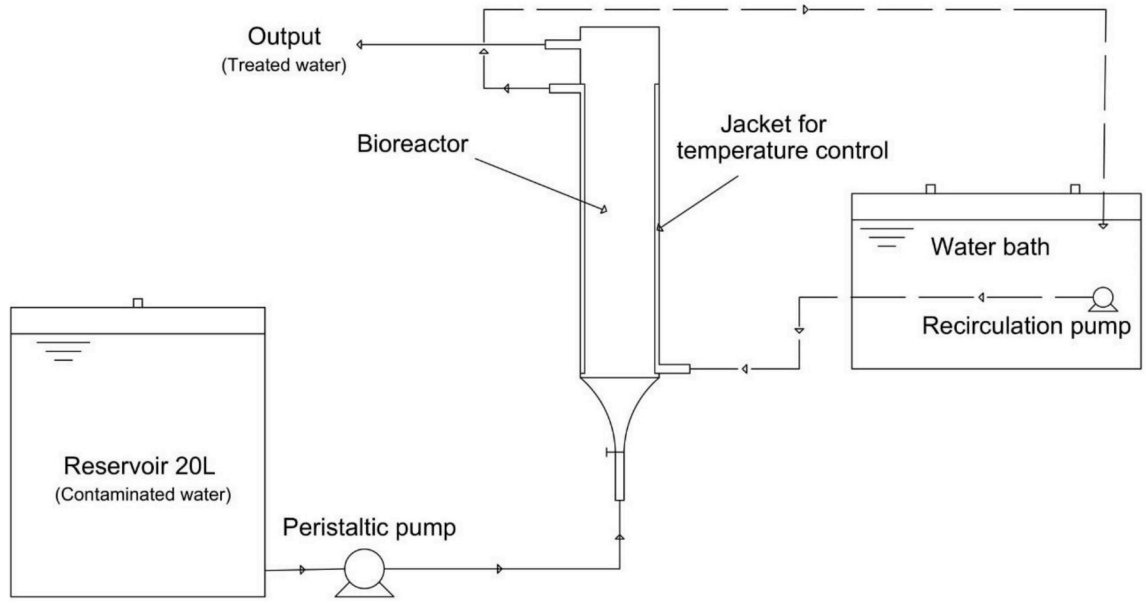

Fig. 1. - Experimental assembly used to perform the experiments in continuous flow. Dashed line: recirculation of water for heating; continuous line: reactor feed and effluent.

the complete introduction of glycerol in the system. Different C:N ratios were also tested after the partial introduction of glycerol until denitrification yield reached at least 90%.

All the experiment was performed once. Even though it involves a natural matrix, the reproducibility of this denitrification process was confirmed in previous work (Costa et al., 2018).

2.4. Post-treatment

In order to reduce the turbidity in the treated water, the effluent was subjected to filtration in a portable vacuum filtration system (K15-1500, PES membrane Kasvi®, Brazil). This system has a pore size of 0.22 μm, equivalent to a microfiltration system. Comparatively, the treated water was filtered through a gravity filter to simulate the treatment in a conventional water treatment plant (WTP). To do so, we used a glass column filled with the following materials: 25 cm of stones with 5 different diameters (2.36 mm, 4.75 mm, 6.30 mm, 9.50 mm and 19.00 mm), 15 cm of sand (average particle size of 0.42 mm) and 20 cm of activated charcoal (average size of 0.56 mm). The turbidity of the treated water was measured to determine the efficiency of each process. The method used to determine turbidity was the same as mentioned in item 2.2.

2.5. Identification of microorganisms

The sample containing the immobilised microorganisms was seeded by plaque reduction assay on Blood Agar (0.5% sheep blood) and incubated at 37 °C for 24 h. Then, the characteristics of the colonies were visualised. Gram staining was then performed for further biochemical testing using test tubes and identification of the bacteria. The test tubes were incubated at 37 °C for 24 h and then read, according to Oliveira (2012).

Table 1
Denitrification process conditions.

Condition	Ethanol (%)	Glycerol (%)	HRT (days)	C:N
1	100	0	0.18	1:1
2	100	0	0.42	1:1
3	100	0	0.62	2:1
4	100	0	0.46	1:1
5	75	25	0.63	2:1
6	50	50	0.71	2:1
7	25	75	0.87	2:1
8	13.33	86.66	1.05	3:1
9	0.00	100	1.51	3:1

2.6. Disinfection

As a complementary step to the treatment, a disinfection test was performed using chlorine in different concentrations. The disinfectant residue was eliminated by the addition of sodium thiosulfate for further microorganism determination. The assay was carried out in 250 mL Erlenmeyer containing 50 mL of denitrified water. As a disinfecting agent, commercial sodium hypochlorite solution was used with 2.25%. The tested dosages of chlorine were 0.5, 1.0, 2.5, 5.0, and 10 mg/L and the contact time of 1 h. The efficiency of the disinfection process was evaluated by counting microorganisms in Petri dishes containing brain and heart infusion broth (BHI). The microorganisms were incubated at 37 °C for 48 h.

3. Results and discussions

After the reactor start-up, the denitrification conditions varied according to the HRT, C:N ratio and carbon source used. These variations occurred to reach the optimal operating conditions for the system. The first condition used involved the use of ethanol only as a carbon source, at a C:N ratio of 1:1, and HRT of 0.18 day. The results showed that denitrification efficiency was 14.37% (Fig. 2a-1) and a residual nitrite concentration of 2.38 mg/L (Fig. 2b-1). The low nitrate removal efficiency is due to the short hydraulic retention time. This condition resulted in high nitrite concentrations in the treated water. Costa et al. (2018) obtained nitrate removal efficiency close to 60% in the retention time of 48 h for microorganisms immobilised on biomass. This fact highlighted the need for increased hydraulic detention time. Thus, condition 2 aimed to increase the HRT in order to increase the contact time of the contaminant with microorganisms. The result was an increase in nitrate removal efficiency from 14.37% to 54.90% (Fig. 2a-2). The increase in nitrite concentration from 2.38 mg/L to 5.83 mg/L (Fig. 2b-2) may be justified by the higher nitrate conversion rate under this condition. However, the denitrification process was not complete. In this work, we analysed the concentration of nitrate and nitrite in the

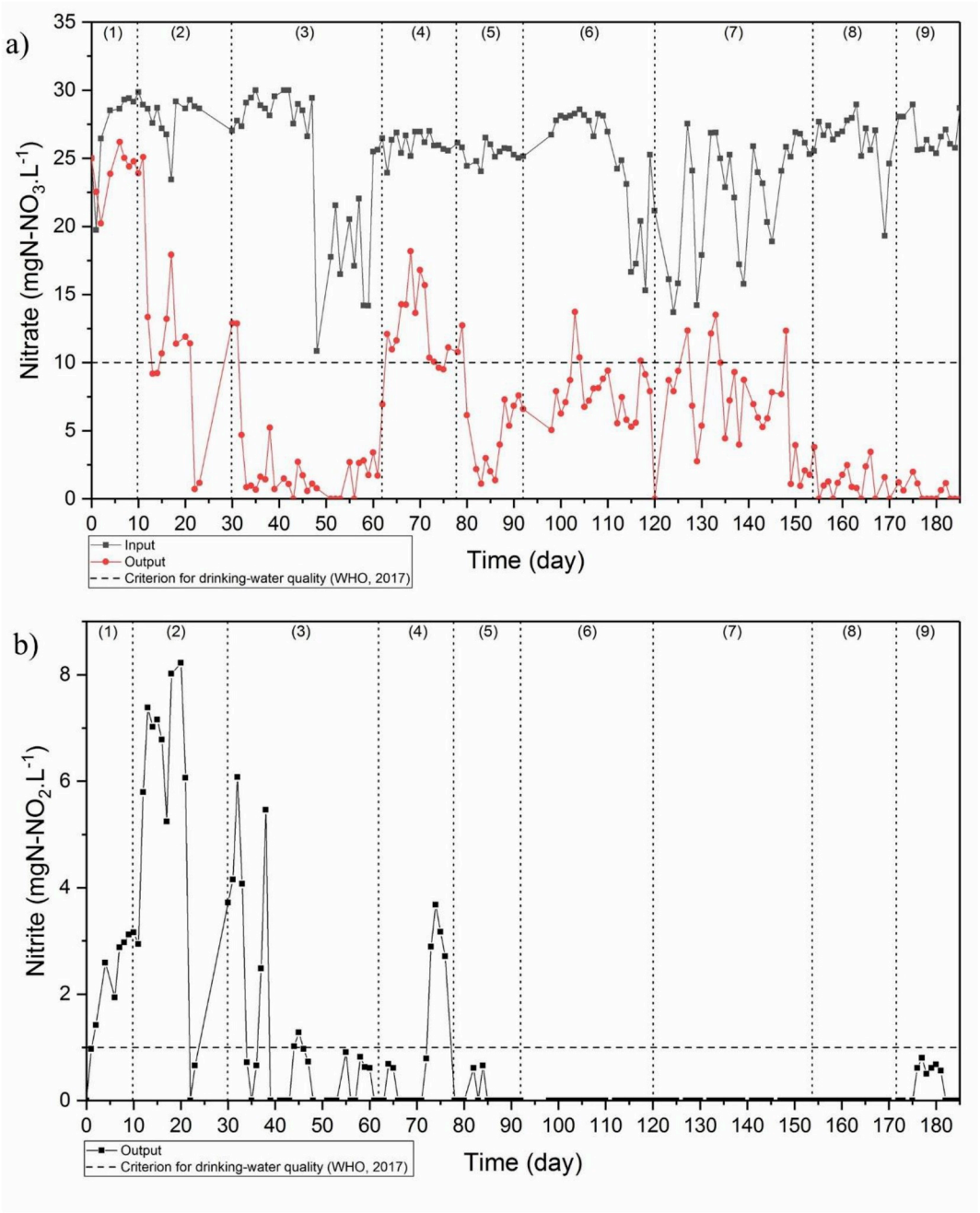

Fig. 2. a) Nitrate concentrations at the reactor inlet and outlet for the studied conditions; (b) Nitrite concentrations at the reactor outlet for the studied conditions. Values in parentheses indicate the conditions specified in Table 1.

water. Therefore, we assume that the enzymatic reduction of nitrate to nitrite and consequently its transformation into nitric oxide (NO), nitrous oxide (N_2O) and nitrogen (N_2), follows the well-known process of denitrification (Averill and Tiedje, 1982).

Denitrification capacity depends on the concentration of biodegradable substrate present, i.e. the C:N ratio (Liu et al., 2010). Therefore, for condition 3, a new C:N ratio was defined in order to increase the growth of the microorganisms and consequently, higher efficiency in the process. This resulted in an increase in terms of nitrate removal from 54.90 to 91.74% (Fig. 2a-3) and a reduction in nitrite concentration from 5.83 mg/L to 0.90 mg/L (Fig. 2b-3), which is in accordance to the Guidelines for drinking-water quality (<1 mgN-NO_2/L) (WHO, 2017).

The results obtained under condition 4 was similar to the ones with condition 2 and aimed to verify the impact of the carbon source reduction and HRT on denitrification yields. It was also motivated by the probable extra growth of the microorganism provided by condition 3. As a result, we observed a lower efficiency in terms of nitrate removal, which went from 91.74 to 51.08% (Fig. 2a-4). There was also an increase in nitrite concentration from 0.90 mg/L to 1.04 mg/L in treated water (Fig. 2b-4), highlighting the influence of HRT and ethanol concentration on the system.

After identifying the optimal C:N ratio for ethanol use, condition 5 aimed to insert glycerol as a carbon source partially. A reduction in efficiency from 91.74 to 78.44% (Fig. 2a-5) was observed concerning condition 3. In contrast, there was a considerable reduction in nitrite concentration from 1.04 mg/L to 0.09 mg/L at the reactor outlet (Figs. 2b–5).

Under conditions 6 and 7, the increased glycerol ratio resulted in a

slight reduction in nitrate removal efficiency to 70.24% (Fig. 2a-6) and 68.43% (Fig. 2a-7), respectively. However, nitrite concentrations were below the detection limit of the method used (Fig. 2b-6 and 2b-7). Conditions 5, 6 and 7 showed a decrease in removal efficiency with the introduction of the new carbon source. This fact might be associated with the effect of the carbon source on the denitrification rate (Mohseni-Bandpi et al., 2013). In other words, the denitrification rate is higher when ethanol is used, considering the same C:N ratio. It is also observed that introducing glycerol as a carbon source resulted in lower residual nitrite concentrations.

In this context, condition 8 aimed to increase nitrate removal efficiency. To do so, a new C:N ratio (3:1) was defined. It resulted in an increase in nitrate removal efficiency from 68.43 to 95.99% (Fig. 2a-8), with nitrite concentration lower than the detection limit of the method used (Fig. 2b-8). Finally, condition 9 aimed the complete replacement of the carbon source of the system with glycerol. Nitrate removal from the water was almost complete, with increased efficiency from 95.99 to 98.14% (Fig. 2a-9). In this condition, the average nitrite concentration (0.29 mg/L) also remained below the guidelines for drinking water quality (Fig. 2b-9). These results are similar to those presented by Cyplik et al. (2013). The authors observed that a C:N ratio of 3:1 resulted in complete removal of nitrate from wastewater, also using glycerol as a carbon source. Furthermore, they reported that superior relationships showed alternative metabolic pathways, forming organic acids and propane-1,3-diol, which may inhibit the activity of denitrifying microorganisms.

The HRT in condition 9 was 1.51 days. Comparatively, the study conducted by Cyplik et al. (2013) reported a retention time of 6.81 days for complete removal of nitrate and nitrite. Gibert et al. (2008), obtained similar results using wood as an organic substrate, and a 6.6-day HRT provided nitrate removal efficiency over 96%. However, there was nitrite accumulation and ammonia generation due to the high detention time. Bucco et al. (2014) used ethanol as a carbon source and similar biomass as a source of microorganisms (*Bambusa tuldóides*). The authors obtained 94% nitrate removal efficiency with an HRT of 10 h. Nitrite concentrations remained close to zero, indicating that microorganisms were able to metabolise this ion. No ammonia concentrations were found throughout the experiment.

Apart from the study carried out by Cyplik et al. (2013), the use of glycerol as a carbon source in the denitrification process was also proposed by Bodík et al. (2009). However, it was applied for wastewater treatment purposes. Bodík et al. (2009) applied the process to a real sewage treatment plant. Alternatively, Cyplik et al. (2013) used this carbon source for continuous flow denitrification of effluents from explosive production, but on a laboratory scale. Thus, there are limited studies using glycerol for drinking water treatment similar to the one accomplished by Costa et al. (2018), which was only performed in batch mode.

The results obtained in this study showed a considerable denitrifying capacity of microorganisms with the introduction of glycerol as a carbon source. This compound is a by-product in biodiesel production which is an advantage in terms of availability and acquisition. The increasing trend in biofuel production must also be considered, which leads to higher glycerol production that commonly does not have an attractive value (Costa et al., 2018). Thus, alternatives for its use must be developed. Bodík et al. (2009) showed denitrification potential using glycerol and highlighted its low cost compared to other sources and the eco-friendly characteristics.

Additionally, the use of natural biomass in the denitrification process as a source of microorganisms is of importance due to its clean origin, minimising the impacts of the process. The use of natural biomass as bamboo has the advantage of being a fast-growing plant with tolerance to diverse weather conditions (Yuen et al., 2017).

Temperature also influences the denitrification process, affecting the growth rate of microorganisms, which may occur between 7 and 40 °C. However, some authors report that the optimal range is between 25 and 35 °C (Gibert et al., 2008; Tchobanoglous et al., 2003). Costa et al. (2018) studied different temperatures for the denitrification process and obtained greater efficiency at temperatures close to 30 °C. Thus, the temperature of 28 °C used in this work was very favourable to the process.

3.1. Identification of microorganisms

It was possible to observe a change in the biofilm colouration, turned to pink, during the experiments. The identified microorganisms were described as Gram-positive bacillus, bacteria commonly found in the air. However, it was not possible to identify its gender.

The pink colouration of the microorganisms leads us to believe in the presence of *Rhodotorula* sp., which may be yellow, orange or red and is known as carcinogenic yeast or pink yeast due to carotenoid production (Marova et al., 2011). The species of the genus *Rhodotorula* are widely distributed in nature and are located mainly in dust and air, from where they drop to the food (Wirth and Goldani, 2012). These organisms can survive in extreme environmental conditions and are commonly used for bioremediation (Nguyen et al., 2001). Furthermore, they are tolerant of temperatures between 25 °C and 37 °C, and some genera can assimilate nitrogenous compounds (Harrison et al., 1991).

3.2. Post-treatment

It was noticed that, sometimes, treated water presented turbidity above the guideline value (Fig. 3) defined as 5 NTU (Nephelometric Turbidity Unit), according to the Guidelines for drinking-water quality (WHO, 2017). The observed turbidity was likely associated with the organic matter released due to the microorganisms growing, which could be released from the support.

In the last condition studied, the average turbidity value was 0.93 NTU, with a maximum of 2.67 NTU. These values were probably obtained due to the slower velocity in the reactor, which may have reduced the drag of microorganisms since it was applied the highest HRT. Bucco et al. (2014) also evaluated a continuous flow system and obtained turbidity values close to 10 NTU. However, the authors stated that bioreactor effluent could be easily treated by conventional methods before sending to the supply system. On the other hand, the assessment of turbidity was not considered in the studies performed by Cyplik et al. (2013) and Gibert et al. (2008).

The results obtained in this study showed lower turbidity than that found in the study by Costa et al. (2018), which averaged 85 NTU, using microorganisms immobilised on the original biomass. As the authors performed the tests under the batch mode, this fact might explain the difference between the results. Thus, the post-treatment becomes simplified for a continuous process, which facilitates its applicability.

The post-treatment was performed by conventional filter and microfiltration, which obtained efficiencies of 87.48 and 94.59%, respectively. The higher efficiency obtained in the microfiltration process is justified by the pore size (0.22 μm). Both methods resulted in turbidity values according to guidelines for drinking water quality.

3.3. Influence of temperature on experiment start-up

The denitrification efficiency varied with inoculation of microorganisms removed from *Phyllostachys aurea*, although it was obtained at the same seasons during 2017 and 2018. This fact may be related mainly to the temperature variations observed in the periods (Fig. 4). Denitrifying microorganisms taken from *Phyllostachys aurea* belong to the genus *Proteus mirabilis*, a Gram-negative bacterium of the *Enterobacteriaceae* family (Pattanayak et al., 2018). This genus is classified as a mesophilic bacterium because it grows and multiplies at temperatures of 20 °C and 45 °C. However, they can slowly grow at temperatures below 10 °C (Normand et al., 2011). Although the bamboo species, *Phyllostachys aurea*, originates from East Asia (Wigzell et al., 2016), and is

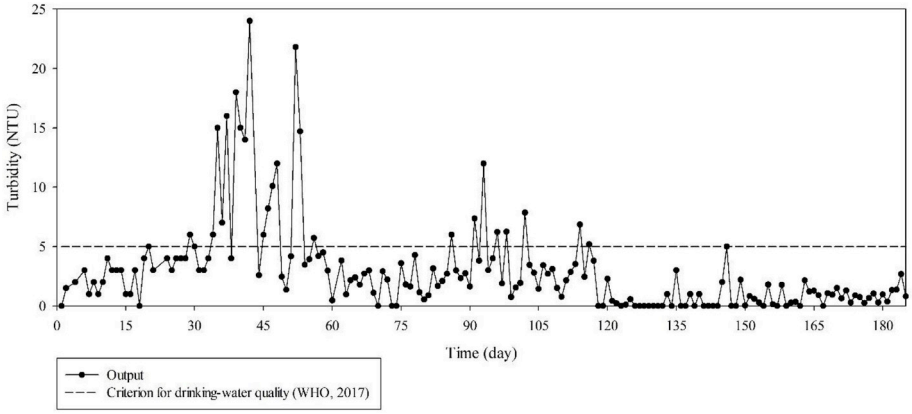

Fig. 3. Turbidity of treated water in the reactor outlet over time.

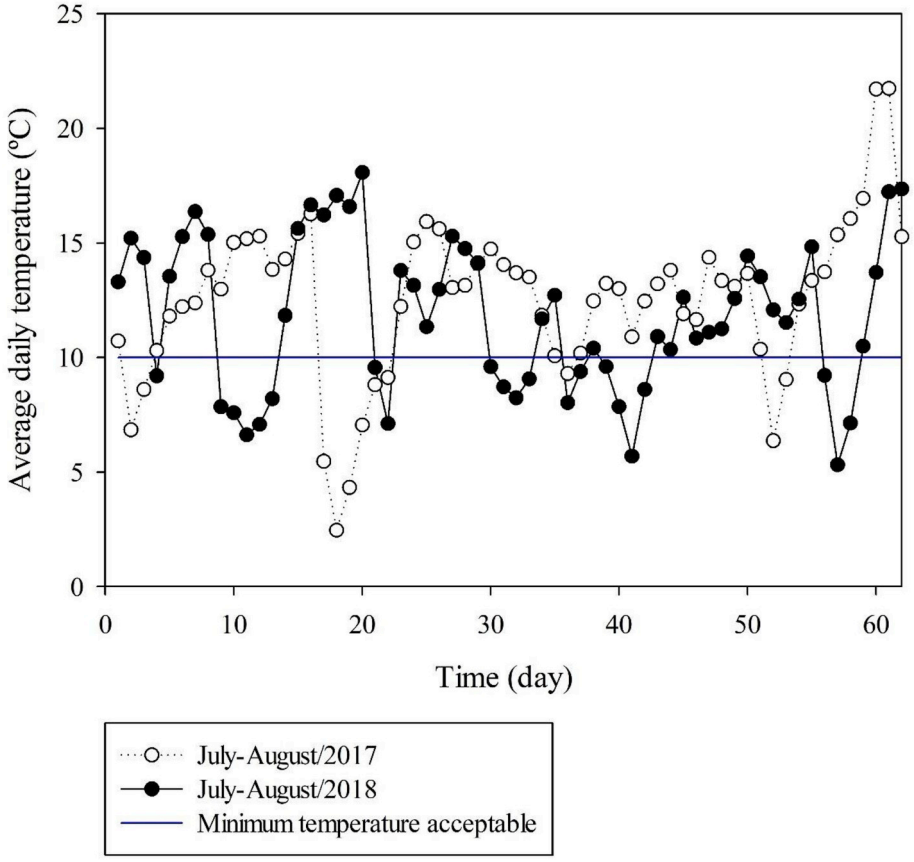

Fig. 4. Comparison of temperature behaviour during biomass harvesting periods.

common in places with cold and temperate climates.

The microorganisms inoculated from *Phyllostachys aurea* collected between July and August 2017 showed better performance in the reactor start-up. The temperatures of this period, although low compared to the optimum range, were higher than the minimum acceptable conditions for bacterial growth on most days. In the second collection period (July–August/2018), lower daily average temperatures were recorded, which may have inhibited the development of microorganisms. Thus, the removal of bamboo in mild periods seems to be of high relevance to the quick start of the system and to obtain relevant results in a shorter period.

3.4. Disinfection

It was observed that the sodium hypochlorite concentration of 2.5 mg/L was sufficient for the complete removal of microorganisms from water (Fig. 5). It is related to the high biocidal power of this disinfecting agent. Lazarova et al. (1999) stated as typical chlorine concentrations for disinfection of effluent treatment plants ranging from 5 to 20 mg/L and 30–60 min of contact time.

The brain and heart infusion broth is a culture medium for growth and isolation. It is suitable for growing large varieties of aerobic and anaerobic microorganisms including fungi and yeast. Thus, the hypothesis of the presence of *Rhodotorula* yeast can again be considered. Also, different characteristics can be observed in the colonies formed

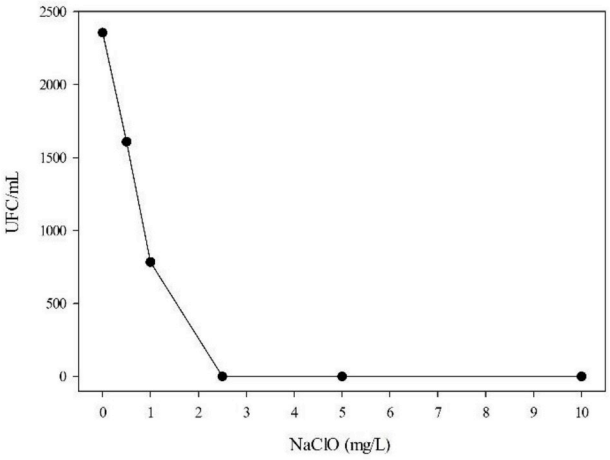

Fig. 5. Disinfection using different concentrations of sodium hypochlorite.

concerning colour and shape. It highlights the presence of more than one species of microorganisms present in the treated water.

4. Conclusion

The use of immobilised microorganisms in a support medium in the temperature-controlled continuous up-flow reactor was efficient to remove nitrate without accumulation of nitrite. Glycerol is an excellent source of carbon in place of ethanol. Thus, the denitrification process is presented as an alternative for the use of biodiesel production by-product. The C:N ratio of 3:1 was critical for the almost complete removal of nitrate from the system, as was the 1.51-day HRT. Further studies should be conducted in order to study a better condition to decrease the added carbon concentration. Besides, it is crucial to evaluate its effect on treated water in terms of disinfection by-products that can be generated and the presence of resistant microorganisms.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jenvman.2019.109964.

Credit author statement

Aline Schroeder: Conceptualization, Methodology, Formal analysis, Investigation and Writing - Original Draft. **Diego Hoefling Souza:** Methodology, Investigation and Formal analysis, **Mylena Fernandes**: Methodology. Formal analysis and Writing - Review & Editing, **Eduardo Bello Rodrigues**: Resources, **Viviane Trevisan**: Validation, Supervision, Writing - Review & Editing and **Everton Skoronski**: Supervision, Project administration, Writing - Review & Editing and Funding acquisition.

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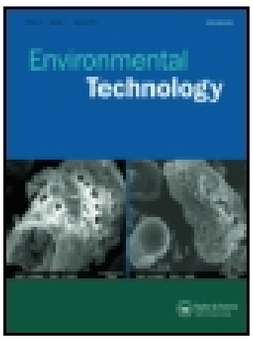

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Enhanced removal of phenol from biorefinery wastewater treatment using enzymatic and Fenton process

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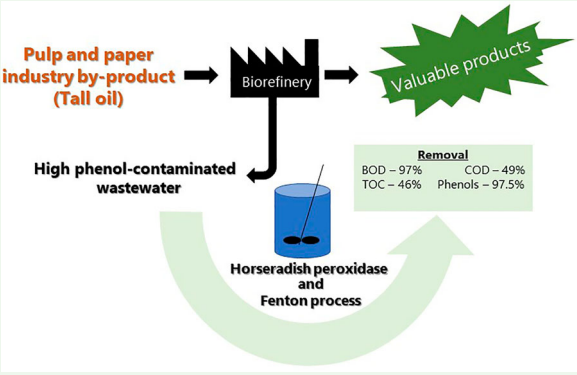
ABSTRACT

The valorisation of biomass has been commonly carried out in biorefineries. The environmental concerns about these processes have not been intensely considered, demanding further investigations. Particularly, phenols are founded in high concentrations in biorefinery wastewater and are considered compounds of major concern. In this study, we evaluated the bioconversion of phenols by enzymatic treatment using the enzyme Horseradish peroxidase (HRP) and the Fenton process. The results showed an enzymatic phenol conversion of 97.5% at pH 7.0, enzyme activity of 0.8 U/mL and hydrogen peroxide concentration of 1.61 g/L. So as to enhance the treatment, we evaluate the Fenton reaction as a complementary process for further remaining phenol conversion. The best conditions for Fenton process were achieved using a hydrogen peroxide concentration and $[H_2O_2]$:[Fe] ratio of 3.90 g/L and 74, respectively, and the obtained phenol concentration in the treated wastewater was 0.11 mg/L. Chromatography analysis showed that 2-methoxyphenol was the majority compound in the original wastewater, which was subsequently precipitated by the enzymatic treatment. Furthermore, many physicochemical parameters were modified due to the treatment, such as biochemical oxygen demand, chemical oxygen demand and total organic carbon, with removal efficiencies of around 97, 49 and 46%, respectively. HRP combined with Fenton can be considered as an alternative methodology for the biorefinery wastewater treatment, especially regarding the phenols conversion.



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Horseradish peroxidase; phenol; biorefinery; Fenton; enzyme

1. Introduction

Wastewater containing high phenol concentration is widely generated in traditional industrial processes such as pulp and paper production, petrochemical, textile, among others [1]. These compounds are highly toxic and hazardous to living organisms. Even at low concentrations, phenols can be harmful to human health since they act as substrate inhibitors in some metabolic pathways [2,3]. In the last decades, many conventional methods for phenolic wastewater treatment have been proposed, such as extraction, adsorption, chemical, and biochemical oxidation processes and electrochemical processes [4]. However, these processes present significant disadvantages regarding costs, incomplete removal and by-product formation [5,6].

Alternatively, oxidative enzymes are shown as a process that can be applied in the removal of phenol from industrial wastewater and among them,

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peroxidases have been successfully used to phenol bioconversion. Peroxidases (E.C.1.11.1.7 – hydrogen peroxide oxidoreductases) are predominantly heme proteins. It uses hydrogen peroxide (H_2O_2) as an electron acceptor to oxidise many organic and inorganic compounds [7]. Oxidation products are highly reactive radicals which play an essential role in polymer reactions. These high molecular weight polymer products have low water solubility and can, therefore, be easily removed from the solution by physicochemical processes such as settling or filtration [8]. Among the peroxidase groups, the Horseradish peroxidase (HRP) has been efficiently used in the biotransformation of phenols in several industrial wastewaters such as pulp and paper industry [9], petrochemical industry [10], textile processes [11] as well as biorefineries [12].

In this work, we studied the removal of phenol in effluents generated from a biorefinery of valorisation of Tall Oil, a significant by-product of the pulp and paper industry. Since the number of biorefineries has increased in recent years, environmental aspects must be considered to provide the sustainability of these industrial plants. Although HRP is a widely studied enzyme in phenol removal, few works have been reported on its use in the treatment of biorefinery effluents. In work published by Ely and coworkers [12], the HRP application did not produce a treated biorefinery effluent under required Brazilian environmental legislation (phenols < 0.5 mg/L), [13].

In this context, we evaluated the possibility of combining enzymatic treatment by applying the HRP enzyme with the Fenton process to enhance the conversion of phenols in biorefinery wastewater. The Fenton reaction is considered a complementary alternative due to its high oxidising power, generating hydroxyl radicals able to oxidise the remaining compounds [14].

2. Material and methods

2.1. Industrial wastewater

The wastewater samples studied in this work were collected in a Biorefinery located in Southern Brazil and maintained refrigerated until used. Phenol concentration in wastewater ranged from 250 to 500 mg/L.

2.2. Enzymatic process

The horseradish peroxidase (HRP) purchased by Toyobo (Brazil) was used in the experiments. The tests were conducted in Erlenmeyer, using 50 mL of effluent, under shaker orbital at 110 rpm for 35 min at 28 °C. The pH of the effluent was adjusted with 1.0 M KOH solution. As the desired temperature was reached, the enzyme and hydrogen peroxide solution was added. At specified times, the samples were collected and immediately centrifuged (5,000 rpm for 5 min), and the supernatant was used for phenol determination.

The optimum condition for phenol removal was determined using a 2^3 factorial design – Central Composite Rotatable Design (CCRD) – including the triplicate of the central point. Studied variables were pH, enzyme and H_2O_2 concentration. The levels of these factors are presented in Table 1. The values considered for the levels were chosen based on preliminary tests [12]. The analysis of the surface response was used to identify the optimal conditions for the reaction. This analysis was accomplished by the software Statistica 10.

2.3. Fenton sequence process

After the enzyme application, the samples containing phenol concentration at 5.33 mg/L were centrifuged at 7,500 rpm, for 10 min, and the supernatant was used for the Fenton reaction. Experiments were carried out at pH 3.0, adjusted with an acid solution (H_2SO_4 96–98% PA). An aqueous solution of $FeSO_4.7H_2O$ to provide the required amount of Fe^{2+} and H_2O_2 was added according to the established in the CCRD. Then samples were kept in an environment-controlled incubator shaker at the temperature of 28 °C and speed of 110 rpm for 24 h. The reactions were stopped by raising the pH to 7.0–8.0 using 1.5 M NaOH solution.

A 2^2 factorial CCRD design was used to determine the effects of the parameters concentrations of Fe^{2+} and H_2O_2 on the oxidation of the chemical compounds present in the enzymatic effluent. We included the

Table 1. Yield for the bioconversion of phenols via the enzymatic process.

Run	A_1	B_1	C_1	Phenol removal (%)
1	5.03 (−1)	0.33 (−1)	1.07 (−1)	96.93
2	5.03 (−1)	0.33 (−1)	2.55 (+1)	95.25
3	5.03 (−1)	0.94 (+1)	1.07 (−1)	98.85
4	5.03 (−1)	0.94 (+1)	2.55 (+1)	98.58
5	7.54 (+1)	0.33 (−1)	1.07 (−1)	97.72
6	7.54 (+1)	0.33 (−1)	2.55 (+1)	97.82
7	7.54 (+1)	0.94 (+1)	1.07 (−1)	97.82
8	7.54 (+1)	0.94 (+1)	2.55 (+1)	98.56
9	3.77 (−2)	0.63 (0)	1.81 (0)	73.60
10	8.80 (+2)	0.63 (0)	1.81 (0)	98.29
11	6.29 (0)	0.03 (−2)	1.81 (0)	21.99
12	6.29 (0)	1.24 (+2)	1.81 (0)	98.76
13	6.29 (0)	0.63 (0)	0.33 (−2)	98.03
14	6.29 (0)	0.63 (0)	3.29 (+2)	99.02
15	6.29 (0)	0.63 (0)	1.81 (0)	98.33
16	6.29 (0)	0.63 (0)	1.81 (0)	98.12
17	6.29 (0)	0.63(0)	1.81 (0)	98.00

Note: The abbreviations A_1, B_1 and C_1 refer to the factors pH, enzyme activity (U/mL) and hydrogen peroxide concentration (g/L), respectively. The numbers in parentheses are the levels used in the experimental design 2^3.

triplicate of the central point and the levels of these factors are presented in Table 2. The values for the levels were chosen based on preliminary tests. The removal efficiency was calculated based on the phenol concentration of 5.33 mg/L.

2.4. Analysis of the effluent

2.4.1. Physical–chemical parameters

Parameters such as pH, Biochemical Oxygen Demand (BOD), Chemical Oxygen Demand (COD), Total Organic Carbon (TOC), colour, Total Solids (TS), Total Volatile Solids (TVS), Total Fixed Solids (TFS), total iron and phenol for the untreated and treated samples were evaluated. All analyses were conducted according to Standard Methods [15].

2.4.2. Identification of chemical compounds

The GC-MS analysis was used to identify the phenolic compounds present in the untreated and treated effluent. For these determinations, the samples were prepared using previously optimised and validated procedures. Briefly, 30 mL of the sample was extracted using 30 mL of methyl *tert*-butyl ether (MTBE). The obtained extract was evaporated to the volume of 1 mL and transferred onto silica gel/anhydrous sodium sulfate clean-up cartridge. Before cleaning up, the cartridge was preconditioned with 5 mL of methanol. Analytes were eluted using MTBE (10 mL), the eluate was evaporated under the gentle nitrogen stream and reconstituted with to the volume of 0.5 mL. The obtained extract was dried under a gentle N_2 stream and derivatised using N, O-bis(trimethylsilyl)-trifluoroacetamide (BSTFA) in acetonitrile to form TMS-ethers (Trimethylsilyl ethers) at 70 °C for 1 h before instrumental measurements.

A Zebron ZB-5MSi GC column – Phenomenex (USA.) was employed in these analyses. Helium was used as the carrier gas and a Dani Master TOF-MS (Italy) Instrument. 1.5 µL sample was injected; and the analyses conditions with initial oven temperature 50 °C, equilibration time 1.0 min; temperature programming was at 15 C/min to 295 °C, then with a final isothermal period of 3.0 min. Compounds were tentatively identified by the standard spectral library (NIST02L) and the reported literature.

3. Results and discussion

3.1. Enzymatic process for phenol removal

Samples contained phenol at a concentration of 303.68 mg/L. Table 1 shows the yield of phenol bioconversion. The best results showed bioconversions above 98%, demonstrating the ability of HRP in the oxidation of phenols. Experimental data analysis utilised a quadratic model with the individual linear effect and the linear combinations of these effects. The statistical significance of the parameters was determined by the analysis of variance (ANOVA), and it is shown in Table 3.

Analysis of the data obtained from ANOVA demonstrated linear effects of pH and enzyme concentration leading to the most significant influence on the bioconversion yield, which means these variables, especially enzyme concentration, are crucial to phenol removal in the studied effluent. Also, hydrogen peroxide (H_2O_2) concentration and the interactions with the enzyme were not statistically significant at a significance level of 5% ($p < 0.05$). The generated model cannot be considered valid within the 95% confidence interval, because it did not have a considerable fit to the experimental data, due to reason aforementioned ($R^2 = 0.64$). It is also far from the maximum explainable variation ($R_{adj} = 0.17$). The p-value for lack of fit <0.001 confirmed this statement. Thus, alternative models would be considered to improve the mathematical description of the studied process.

However, the results were also transformed into response surfaces, to allow the visualisation of the

Table 2. Results for the phenol removal after the Fenton reaction. Initial phenol concentration of 5.33 mg/L.

Run	A_2	B_2	$[Fe^{2+}]$ (mg/L)	Phenol (mg/L)	Removal (%)
1	37.00 (−1)	1.34 (−1)	36.4	0.37	93.06
2	37.00 (−1)	3.90 (+1)	105	0.43	91.93
3	74.00 (+1)	1.34 (−1)	18.2	0.59	88.93
4	74.00 (+1)	3.90 (+1)	52.5	0.11	97.94
5	55.50 (0)	0.81 (−1.41)	14.7	0.95	82.18
6	55.50 (0)	4.43 (+1.41)	79.1	0.17	96.81
7	29.41 (−1.41)	2.62 (0)	88.9	0.63	88.18
8	81.86 (+1.41)	2.62 (0)	32.2	0.23	95.68
9(C)	55.50 (0)	2.62 (0)	46.9	0.27	94.93
10(C)	55.50 (0)	2.62 (0)	46.9	0.18	96.62
11(C)	55.50 (0)	2.62 (0)	46.9	0.3	94.37

Note: The abbreviations A_2 and B_2 refer to the factors $[H_2O_2]$:[Fe] ratio and hydrogen peroxide concentration (g/L), respectively. The values B_2 are referred to the new addition of H_2O_2, as they do not consider the residual concentration after the enzymatic process. The numbers in parentheses are the levels used in the experimental design 2^2.

Table 3. Analysis of variance (ANOVA) for the enzymatic process.

Source	SS[a]	df[b]	MS[c]	Coefficient	*F*-value	*p*-value[d]
		9		102.14		
A_1	166.47	1	166.47	3.22	6,113.99	<0.001
A_1^2	121.60	1	121.60	−2.49	4,466.04	<0.001
B_1	1,599.49	1	1,599.49	9.99	58,745.38	<0.001
B_1^2	1,533.37	1	1,533.37	−8.87	56,316.95	<0.001
C_1	0.40	1	0.405	0.16	14.86	0.061
C_1^2	9.37	1	9.37	0.72	344.16	0.003
$A_1 \times B_1$	2.12	1	2.12	−0.51	77.99	0.013
$A_1 \times C_1$	0.72	1	0.72	0.30	26.57	0.036
$B_1 \times C_1$	0.078	1	0.078	0.099	2.88	0.23
Lack of Fit	2,093.57	5	418.71		15,378.33	<0.001
Pure Error	0.054	2	0.027			
Total SS	5,792.13	16				

$R^2 = 0.64$; $R_{adj} = 0.17$; [a]Sum of squares; [b]Degree of freedom; [c]Mean square; [d]*p*-value <0.05 indicates statistical significance.

influence of the variables on the phenol removal (Figure 1) and a tendency of the optimal conditions. The results indicated that greater enzymatic phenol bioconversion was observed at pH 7.0, enzyme concentration of 0.8 U/mL and H_2O_2 concentration of 1.61 g/L. These conditions were obtained from the response surface plot as the optimised condition. Therefore, we defined this condition for the further steps of our study. However, tests involving lower H_2O_2 concentration (for instance, 0.33 g/L) were also responsible for significant phenol removal efficiency (Table 1). These conditions could also be explored for cost–benefit analysis. In the previous study, the authors [12] found optimal conditions pH 6.32, enzyme concentration of 1.13 U/mL and H_2O_2 concentration of 1.24 g/L for the same effluent, but for another sample. These differences are expected due to the wide variety and concentration of chemical compounds present in the industrial

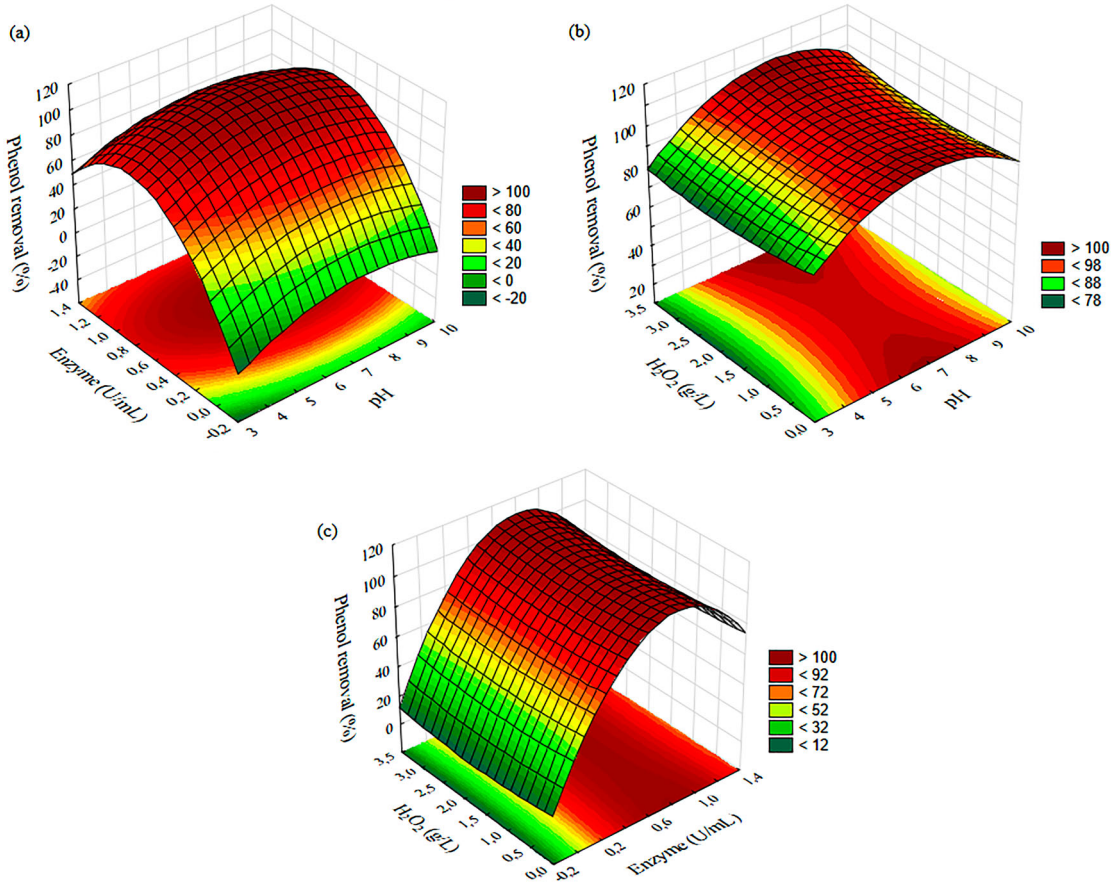

Figure 1. Response surfaces for the phenol removal in the enzymatic process.

effluent. Incomplete removal should be associated with the complex composition of phenol species present in the effluent, especially the substrates which HRP has a lower affinity. Aiming to enhance the conversion of phenols, the Fenton's reagents was chosen as a complementary step to the treatment process. The phenol concentration decreased to 5.33 mg/L after the enzymatic process.

3.2. Fenton reactions

The results obtained after the Fenton reaction are shown in Table 2. Overall, the tests reduced the phenol concentration to values below 1.0 mg/L. Eight tests presented concentrations lower than 0.5 mg/L (Conama 430/2011), and only three tests presented concentration lower than 0.2 mg/L (Santa Catarina State Law 14.675/2009).

The ANOVA was also applied to understand the effects of Fenton reaction in the phenols conversion. The quadratic model was presented as the most appropriate. The statistical significance of the parameters determined by the ANOVA is shown in Table 4. It is observed that the linear and quadratic effects of the H_2O_2 concentration did not present a significant influence on the phenol bioconversion. On the other hand, when associated and combined with $[H_2O_2]$:[Fe] ratio, these effects shown significant influence. It means the importance of the presence of the H_2O_2 in the Fenton process. The model can be considered valid within the 95% confidence interval. Additionally, the $MS_{lackfit}/MS_{erro}$ was 9.79, lower than the F_{critic} (3, 2) which was 19.16. Also, the mathematical model fitted to the experimental data. The lack of fit showed that the model fit appropriately to the data with the determination coefficient of 0.81. The result was transformed into a response surface (Figure 2), in order to allow the visualisation of the influence of the variables on phenol removal and a tendency of the optimal conditions of the process.

The results indicate that the optimum H_2O_2 concentration and H_2O_2:Fe ratio is above the values studied.

Table 4. Analysis of variance (ANOVA) for the Fenton process.

Source	SS[a]	df[b]	MS[c]	Coefficient	F-value	*p*-value[d]
		5		95.30		
A_2	19.67	1	19.67	1.578	14.34	0.063
A_2^2	7.11	1	7.11	−1.12	5.18	0.151
B_2	102.02	1	102.02	3.57	74.36	0.013
B_2^2	30.91	1	30.91	−2.34	22.52	0.042
$A_2 \times B_2$	25.70	1	25.70	2.53	18.73	0.049
Lack of Fit	40.30	3	13.43		9.79	0.0940
Pure Error	2.74	2	1.37			
Total SS	222.32	10				

$R^2 = 0.81$; $R_{adj} = 0.61$; [a]Sum of squares; [b]Degree of freedom; [c]Mean square; [d]*p*-value <0.05 indicates statistical significance.

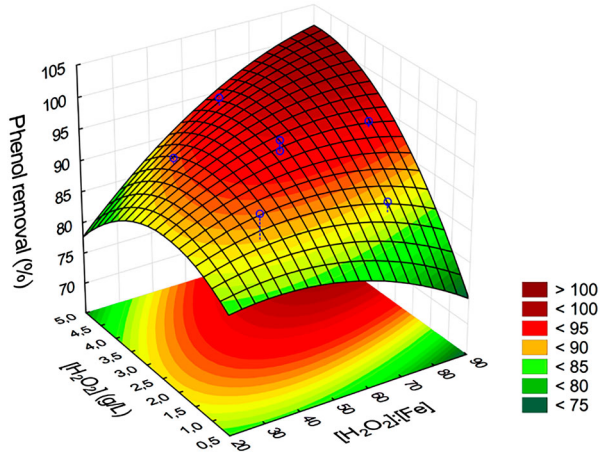

Figure 2. Response surface showing the influence of the variables in the Fenton process on the phenol removal.

However, as phenol concentrations below 0.2 mg/L were obtained in some experiments, it was chosen to work with the H_2O_2 concentrations (3.9 g/L) and Fe^{2+} (52.5 mg/L) (Run 4) which showed the maximum phenol removal during this study. Several studies have shown the best $[H_2O_2]$:[Fe] ratios for the removal of phenol in synthetic aqueous solutions [16,17]. At concentrations between 95 and 100 mg/L, the Fenton process presented higher efficiency on the phenol removal when compared to others advanced oxidative process, using the optimum concentration of 363.8 mg/L H_2O_2 and an $[H_2O_2]$:[Fe] ratio equal to 120:1 [18]. Kavitha and Palanivelu [16] found an optimum peroxide concentration of 1,020 mg/L and an $[H_2O_2]$:[Fe] ratio of approximately 23:1 for the 82% of phenol removal from aqueous solution with a concentration of 200 mg/L.

3.3. Effluent analysis

3.2.1. Physical–chemical parameters

In addition to the total phenol concentration, other physicochemical parameters were also investigated using previously described the best conditions. The results for the treated and untreated effluent are shown in Table 5. The pH of the raw effluent is extremely acidic due to the acidification process with H_2SO_4 in the industrial plant. For the enzymatic treatment, the pH of the effluent was adjusted to the optimal condition (7.0), and it was observed that, after the process, the pH decreased to the value of 5.32. Because of this neutralisation, the TFS (total fixed solids) is increased from 0.46 to 1.74 g/L. The higher value in the TVS (total volatile solids) of the treated effluent should be associated with some residual of H_2O_2 or some low molecular weight compound generated during side reactions. These increases in the TFS

Table 5. Physicochemical analysis for the treated and untreated effluent.

			Treated	
Parameter	Unit	Untreated	HRP	Combined process
pH	–	3.76	5.32	6.86
Total phenol	mg/L	284.03	3.76	0.20
BOD	mg/L	500	7.0	15
COD	mg/L	5,198	3,658	2,605
TOC	mg/L	833	597	448
Color	uH	583	1,664	918
TS	g/L	0.82	2.26	4.51
TFS	g/L	0.12	0.58	2.30
TVS	g/L	0.63	1.64	2.28
H_2O_2	g/L	–	0.82	0.02
Total iron	mg/L	2.6	–	26.5

and TVS affect directly the TS, which raised from 1.17 to 2.86 g/L. For BOD, COD, and TOC, the removal efficiencies were 97, 49 and 46%, respectively.

After the Fenton process, it was added 1.0 mL of catalase enzyme (Sigma-Aldrich) for each 1.0 mL of effluent, in order to degrade the remaining H_2O_2 that could interfere in the COD analysis [17]. Even after catalase addition, the post-Fenton effluent had an H_2O_2 concentration of 20 mg/L. However, it was lower than the 1.45 g/L concentration obtained before the addition of the enzyme. The pH is close to neutrality, obtained by adjusting required to quench the reaction. The solids content increased in comparison to the crude effluent due to the formation of insoluble polymers. Consequently, the colour also increased. Regarding total iron, there was the consumption of 49.52%, with remaining concentration (26.5 mg/L), above the limit allowed by Conama Resolution 430/2011 (15 mg/L). This limitation may be associated with the complexation of the metal with organic matter [19]. In order to avoid the need for iron removal, which would increase the cost, the use of heterogeneous catalysts, which are used as media carriers for iron [14], might be an alternative.

Some studies were published using the Fenton reaction for phenol conversion. Resin manufacturing real effluent with COD and phenol of 2,904 and 1,215 mg/L, respectively, was treated and the effective removal of phenol was achieved in 5 min with COD removal efficiency of 82% [17]. In effluents from textile industries, chemical, metal, and food, containing 1,750–3,323 mg/L COD, apart from heavy metals and non-biodegradable and toxic organic pollutants, such as dyes, oxidation by Fenton reached up to 100% colour removal and more than 90% reduction of COD [20]. In another textile effluent with 1,200 mg/L BOD_5, 8,100 mg/L COD and 3,010 mg/L TOC, Fenton process reduced about 45, 60, 40 and 71.5% values of COD, BOD_5, COT and colour, respectively [21].

3.3.2. Identification of chemical compounds

The GC-MS analysis confirmed the complexity of the chemical composition of the studied effluent. In the untreated sample was found different compounds including phenols, volatile acids, and their esters. Among the phenols, the most abundant substance was the 2-methoxyphenol and phenolic compounds such as 2,6-dimethoxyphenol and 2-ethyl-2-phenyl-1,3-benzodioxole were detected in lower quantities. Furthermore, fatty acids such as heptanoic, propanoic, octanoic, benzoic and butanoic acid were present in the sample.

After HRP treatment, fatty acids remained in the effluent, and some other phenols were formed, such as *m*-cresol and 2,4-bis (1-methyl-1-phenyl ethyl) phenol. The phenolic compounds found in the raw effluent precipitated, as they were not detected in the sample. However, higher molecular weight phenols remained in the effluent. It is contrasting another study that used synthetic phenol solution, and no soluble product was present after 3 h HRP treatment [22]. This comparison confirms the limitation of bioconversion provided by HRP in real effluents, due to the non-affinity of the enzyme for all substrates present.

In the sample treated with Fenton's reagents, the presence of molecular weight high fatty acids, such as hexadecanoic, tetradecanoic and octadecanoic acid, and acids such as pentanoic and propanoic was observed. An intermediate phenolic compound formed was 2,6-bis (1,1-dimethylethyl)-4-methylphenol. In the degradation of an H-acid dye, the authors found that short-chain fatty acids were produced as the Fenton oxidation proceeded [23]. The phenol degradation by heterogeneous Fenton, through immobilised iron oxides, led to the generation of carboxylic acids as by-products. These acids, especially oxalic acids, improved the dissolution of iron oxides and, consequently, effectively catalysed the degradation of phenol [24]. Fenton oxidation almost eliminated the phenolic compounds present in the olive processing effluent [25]. Kang, Lee, and Yoon [26] indicated that the availability of Fe^{2+} and its interaction with the organic intermediate is a crucial factor in improving the phenolic compounds decomposition performance.

4. Conclusion

The combined process involving the Horseradish peroxidase (HRP) and Fenton reaction showed that it is possible to reduce the phenol concentration in the wastewater. Moreover, other parameters that are crucial for treatment evaluations also demonstrated the reliability of the suggested methodology. Also, the reduction in the iron concentration in the wastewater after the Fenton

process must be appropriately addressed. Overall, the association of the processes could be an interesting alternative for a complex biorefinery wastewater treatment, improving the pollution control in this economic activity.

Disclosure statement

No potential conflict of interest was reported by the authors.

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Obtaining soybean peroxidase from soybean hulls and its application for detoxification of 2,4-dichlorophenol contaminated water

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ABSTRACT

This work aimed to evaluate the performance of soybean peroxidase on 2,4-dichlorophenol bioconversion. Soybean peroxidase was obtained from transgenic cultivar and purified to homogeneity followed by enzymatic characterisation using pyrogallol as standard substrate and applied to 2,4-dichlorophenol bioconversion. The results demonstrated that soybean peroxidase optimal conditions of pH and temperature were 6.5 and 71.6°C using pyrogallol as substrate. Regarding the 2,4-dichlorophenol bioconversion, removal of 80 % and 96 % was achieved in 1 and 24 h reaction time, respectively, using the purified enzyme. In comparison, the use of soybean hulls bioconverted only 30 % 2,4-dichlorophenol in 24 h. The toxicity effect of the treated water was evaluated using *Lactuca sativa L.* var. *Buttercrunch* as bioindicator. The decreasing in the toxicity after the treatment leads to 100 % germination and 54 % seed root elongation assessments. Overall, we conclude that the studied enzyme and the presented methodology is of interest for soybean hulls lower-value commodity valorisation from a transgenic cultivar.

1. Introduction

The herbicide 2,4-D (2,4-dichlorophenoxyacetic acid) is one of the most widespread compounds used in agriculture for weed control in conventional farm systems [1]. Although its use is of great economic importance, there are concerns about its environmental impact, as well as association with human health problems due to chronic exposure [2]. Microbial degradation of 2,4-D produces 2,4-dichlorophenol (2,4-DCP) during the first metabolic step. 2,4-DCP toxicity has been extensively studied in the last decades [3], and the World Health Organization (WHO) has classified 2,4-DCP as a possible carcinogen compound classified as 2B [4].

Based on this concern, several methods for 2,4-DCP remediation/removal have been studied, including adsorption on solid matrices [5,6], chemical catalytic oxidation [7–9] and enzymatic/photocatalytic oxidation [10]. Apart from these techniques, an enormous effort has been applied to the use of enzymes for environmental management of 2,4-DCP [11]. Fungal laccase [12–14] and horseradish peroxidase (HRP) [15–21] are the two classes of enzymes which have been frequently studied.

Other natural sources of enzymes have been explored for 2,4-DCP removal, including *Brassica napus* hairy roots [22], potato pulp [23], hairy tomato roots [24,25]. Although the studies aforementioned are interesting, the respective natural sources are not available as waste in substantial amounts for its economic viability, limiting their application in 2,4-DCP bioremediation.

On the other hand, soybean peroxidase (SBP) has been largely studied [20,26,27], and its use is considered very attractive since the enzyme is present in significant amounts in the soybean hulls, which is a byproduct of the soybean processing industry. Efforts to convert this lower-value commodity into a value-added product are of substantial economic and environmental impact mainly due to the dramatic soybean production worldwide. The United States and Brazil are the two largest soybean producers harvesting between 119,518 and 116,996 million tons, respectively, in 2017/2018 [28].

Although studies have been performed using SBP for 2,4-DCP

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removal [26,29], utilising either seed hulls or a purified commercial SBP, fewer studies have been performed using agricultural waste extracted and purified soybean peroxidase for 2,4-DCP removal [29,30]. We obtained and purified soybean peroxidase from transgenic cultivar hulls and utilised this enzyme for 2,4-DCP bioconversion followed by an assessment of residual toxicity.

2. Material and methods

2.1. Materials

Soybean hulls were collected in Xanxerê, Santa Catarina (Brazil). The respective soil is classified as dystrophic Red Latosol (Oxisoil), and the climate classified as Cfb. The hulls were obtained from the Glyphosate-Resistant soybean cultivar NA 5909 RG (RR) (Nidera Sementes, Brazil) which is a transgenic lineage, with superprecoce growth habit and, the estimated plant density of 400–520,000 plants per hectare.

All of the other chemicals used in this study were of analytical degree.

2.2. Peroxidases extraction and purification

The soybean hulls were mixed with potassium phosphate buffer solution (pH = 6.0) at 1:10 (m/v) ratio and ground in a blender until a slurry was obtained. The mixture was subjected to mechanical stirring, 200 rpm, (Edutec; Model EEQ-9034) for 2 h. After that, the mixture was filtered using cheesecloth, to separate the crude extract, and then centrifuged at 4 °C, 15,000 *g* for 15 min (Eppendorf AG centrifuge; Model: 22331).

After centrifugation, the supernatant was subjected to acetone precipitation using four times the sample volume of cold (−20 °C) acetone to the tube. Afterwards, the protein precipitate was resuspended with 20 mM TRIS − HCl buffer pH 8.0, and the solution was concentrated by diafiltration using 30 kDa regenerated cellulose ultrafiltration membrane (Merck-Millipore; Amicon Ultra-15).

The protein concentrate was injected in a Fast Protein Liquid Chromatography (FPLC), AKTA system, (GE Healthcare; Model: AKTA Pure 150 L) for enzyme purification. The system operated at room temperature and 0.22 MPa total system pressure. A 5 mL ion-exchange column type HiTrap DEAE FF was used. Protein was loaded into 5 mL HiTrap DEAE FF column washed with buffer A (TRIS − HCl 20 mM pH 8.0) and eluted in a linear gradient with buffer B (TRIS − HCl 20 mM + NaCl 1 M, pH 8.0). Active fractions were pooled, concentrated and stored at -20 °C until use.

The total protein concentration was determined by the Bradford reagent method [31] using bovine albumin (BSA) as the standard protein. The measurements were carried out at 595 nm in a UV–vis spectrophotometer (Merck; Model: Pharo 300). The concentration was expressed in mg mL^{-1}. The molecular weight of the peroxidase and purification assessment was estimated using peroxidase concentrated solution applied to denature gel electrophoresis (SDS-PAGE) with 12 % polyacrylamide gel using Coomassie blue G250 such as staining.

2.3. Peroxidase characterisation

2.3.1. Optimal pH and temperature

Based on the preliminary results of pH and temperature, a Response Surface Methodology-based Central Composite Design on two variables was used as experimental design, with 2 levels, 8 tests (4 factorials and 4 rotations) and 3 repetitions of the central point, totalling 11 tests, according to Table 1. Software Stat Soft Statistica 10 was used to analysis.

2.3.2. Enzymatic activity assay

The enzyme activity was determined using the reaction with pyrogallol for the formation of purpurogallin in the presence of peroxidase as a catalyst (Eq. 1). The methodology used was based on [32]. According to this protocol, a Unit (U) is the amount of enzyme which converts 1 mg of pyrogallol in purpurogallin in 20 s at 20 °C and pH 6.0. The measurements were made at 420 nm in UV–vis spectrophotometer (Merck; Model: Pharo 300), and the enzymatic activity was expressed in U mL^{-1}.

$$H_2O_2 + \text{pyrogallol} \xrightarrow{\text{peroxidase}} \text{purpurogallin} + 2H_2O \quad (1)$$

Table 1
Levels of the factors for the central composite design used for the optimisation of pH and temperature.

Factors	Levels				
	−1.41	−1	0	+1	+1.41
pH	2	4	6	8	9
Temperature (°C)	10	30	50	70	90

2.4. Application in the 2,4-DCP bioconversion and measurement of 2,4-DCP

The experiment was conducted using an SBP stock solution (0.21 mg mL^{-1}), and optimal reaction conditions determined in previous tests. The pH of the simulated effluent was adjusted to 6.5 using phosphate buffer, and the temperature was kept in the range of 23 − 24 °C. The simulated effluent was prepared with the addition of 2,4 DCP (100 mg L^{-1}, 0.6 mM). The bioconversion initiated by the addition of different concentrations of hydrogen peroxide (0.6, 1.8 and 3.0 mM) and enzyme (28.75, 57.5 and 86.25 U L^{-1}). Also, control experiments were performed using tests using soybean hulls (3.11 g L^{-1}) as the catalyst, which was calculated based on Liu [33] and Gillikin and Graham [34] studies. The experiments were performed in 125 mL flasks, in triplicate, under orbital shaking at room temperature. Aliquots of 200 µL were collected at 60 and 1440 min to determine the concentration of remaining 2,4-DCP. For each aliquot collected, the reaction was interrupted by the addition of 10 µL of 0.1 M sulphuric acid. After done this assay, the residual hydrogen peroxide in the solution was neutralised with the use of the catalase enzyme. After that, the treated effluent was conducted for the total organic carbon (TOC) analysis and toxicity tests.

The concentration of 2,4-DCP was determined according to the method 5530 D [35], based on the colourimetric analysis of total phenols, using 4-aminoantipyrine as a reagent. The analyses were performed in a UV–vis spectrophotometer (Merck; Model: Pharo 300) at 420 nm. Total organic carbon was measured using a TOC analyser (Shimadzu; Model: TOC-L), provided with an auto-sampler and using glucose solution as the calibration standard.

2.5. Toxicity assessment

Lettuce seeds (*Lactuca sativa L.* var. *Buttercrunch*) with 100 % germination, 99.9 % purity, from Feltrin® Sementes, were used for testing. The bioassay was carried out according to adaptation from Wang [36] and Sobrero and Ronco [37] as follows: lettuce seeds were soaked for 20 min in a bleach solution and washed five times with distilled water to kill the fungal spores. After that, 2 ml of the prepared solution was added on a petri dish with a paper filter. It was used 5 seeds spaced evenly on each petri dish, in a total of 25 seeds for a test run. The Petri dishes were covered with a plastic bag, to avoid water evaporation, and incubated in the dark at 22 °C for 120 h. The seeds germinated were counted and the root length of each measured to the nearest mm. The effect of the germination (AG) was determined by Eq. 2 and the relative root growth inhibition (RRGI) by Eq. 3, where MCRG is the mean

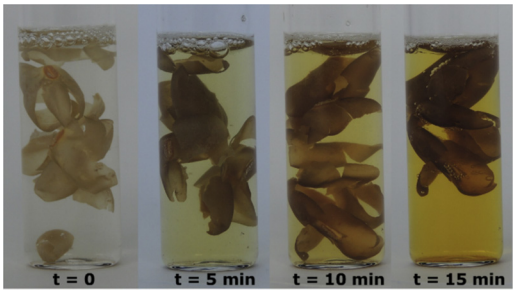

Fig. 1. Test of absence/presence of peroxidase in soybean hulls.

control root growth and MSRG is the mean sample root growth. The means of the treatments were compared by Tukey's test, using software Stat Soft Statistica 10. It was studied the behaviour of the control/water (1), concentrated 2,4-DCP solution (2), enzymatic treatment/pure enzyme (3), enzymatic treatment/hulls (4), H_2O_2 treatment (5) and enzymatic treatment/pure enzyme in absence from catalase (6).

$$\%AG = \left(\frac{number\ of\ germinated\ seeds}{total\ number\ of\ seeds}\right) \times 100 \quad (2)$$

$$\%RRGI = \left(\frac{MCRG - MSRG}{MCRG}\right) \times 100 \quad (3)$$

3. Results and discussion

3.1. Peroxidases extraction and purification

Soybean Peroxidase (SBP) is located in the hulls of some cultivars, being absent in others [38]. Therefore, it was necessary to perform a preliminary test to investigate peroxidase activity on soybean hulls of different cultivars used in this study. Fig. 1 shows the positive result for peroxidase using pyrogallol as substrate (Eq. 1), by changing the colour of the medium from clear to yellow.

The crude extract was subjected through two purification steps, as aforementioned, until complete homogeneity to proceed with 2,4-DCP bioconversion. Soybean hull extracts several present proteins, including trypsin inhibitors, hydrophobic protein, proline-rich proteins, class I chitinase and SC24, as mentioned by Al-Ansari and co-workers [39]. After the purification steps described in this paper, we obtained pure SBP (Fig. 2), with high specific activity (Table 2).

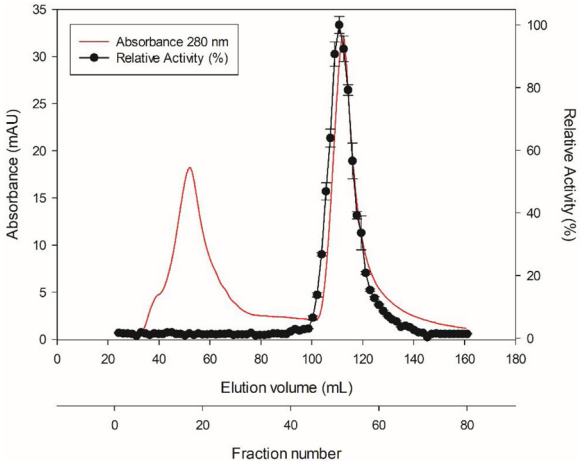

Fig. 2. Purification chromatogram of peroxidase SBP.

3.2. Peroxidase characterisation

SDS polyacrylamide gel electrophoresis analysis (Fig. 3) allowed to estimate SBP Molecular mass of around 44 kDa, in accordance with previous studies [38,40,41]. According to Fig. 3, it is possible to observe the purification along with the stages, in which a significant part of the enzymes that are not the object of the study was eliminated. It is also observed that in the crude extract of the soybean SBP is not the major enzyme.

The maximum enzymatic activity of the purified enzyme in terms of pH and temperature factors was determined by the Response Surface Methodology-based Central Composite Design. The activity results are shown in Table 3 in terms of relative activity as a function of pH and temperature levels. Through the linear, quadratic and combined interaction of the factors, it was possible to obtain models that represent the activity data of the SBP, according to Eq. 4.

$$\text{Relative Activity (\%)} = = -154.8 + 53.03.\ \mathbf{pH} - 5.48.\ \mathbf{pH}^2 + 1.60.\ \mathbf{Temperature} - 0.02.\ \mathbf{Temperature}^2 + 0.22.\ \mathbf{pH}.\ \mathbf{Temperature} \quad (4)$$

In order to evaluate if the quadratic model obtained by the planning is significant, analysis of variance (ANOVA) was performed, as shown in Table 4. Table 4 shows that the linear pH effect for the SBP enzyme is not statistically significant since it presented p-value greater than 0.05. These effects can be better visualised in the Pareto chart in Fig. 4. In that analysis of variance, the lack of fit was significant (p-value < 0.05), according to Table 4. It means that the proposed model can not adequately represent the data predicted, so a new model should be analysed. The non-representativeness of the model is depicted through the low coefficient of determination presented by the model, 0.7591. It means that is required additional terms in the model so as to enhance its fitting to experimental data.

Although the presented model does not adequately adjust the data, through the response and contour surfaces (Fig. 5), it is possible to verify the region of maximum enzymatic activity for both enzymes. It is observed that these maximised regions were within limits previously defined through the planning levels. Thus, the optimal conditions for the SBP enzyme were pH equal to 6.57 and temperature of 71.64 °C.

3.3. Application in the 2,4-DCP bioconversion

After enzymatic biocatalyst characterisation, its application on bioconversion was carried out. Purified SBP was applied to the bioconversion of 2,4-dichlorophenol. The effect of pH, hydrogen peroxide concentration and enzyme concentration on the enzyme performance was assessed and is presented in Figs. 6–8, respectively.

Concerning the effect of hydrogen peroxide concentration, three values, 0.6, 1.8 and 3.0 mM, corresponding to stoichiometric concentrations, 3- and 5-fold excess were tested. The results showed that the increase in the concentration of this electron receptor decreases the removal of 2,4-DCP from 52.5–39.3 % within the range studied (Fig. 6). Indeed, the excess peroxide is known to play an essential role on the enzymatic activity of peroxidases as a function of favouring the formation of non-active forms of the enzyme (compound III), which decreases its performance [42,43].

In the case of pH, the optimum value found in this work for the removal of 2,4-DCP was 6.5, being close to pH 6.57, considered with the condition of maximum activity using pyrogallol as a substrate (Fig. 7). Moreover, it is worth to note that the removal performance at pH 4.0 and 6.5 was similar. Differently, [29] found the value of 8.2 for the optimum pH of the commercial soybean peroxidase used in its work. Thus, it is probable that the plant source of PBS could have influenced the characteristics of the enzyme, leading to different behaviours as a function of pH. Similarly, Talano et al. [25] studied the application of tobacco hairy root cultures in the bioconversion of 2,4-

Table 2
Purification table of peroxidase SBP.

Fraction	Volume (mL)	Total Protein (mg mL^{-1})	Total Activity (U mL^{-1})	Specific Activity (U mg^{-1})	Purification Folds	Yield* (%)
Crude Extract	1000	0.35	0.045	0.13	1	100
HiTrap DEAE FF	14	0.21	2.88	13.7	100	3.56

* The yield is the amount of target (either total target protein or total activity) at that step divided by the amount of target in the first step (defined as 100 %).

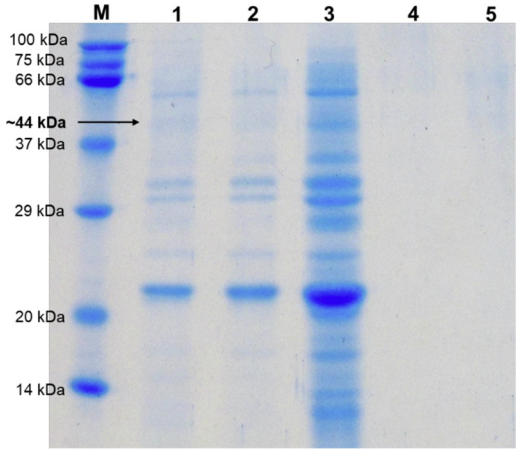

Fig. 3. Polyacrylamide gel electrophoresis of the enzymes SBP. M: marker; 1: crude extract; 2: acetone precipitation; 3: diafiltration; 4: fraction collected from the chromatography and 5: diafiltration after FPLC.

Table 3
Central Composite Rotatable Design for pH and temperature optimisation.

Run	Factors		Relative Activity (%)	Predicted (%)
	pH	Temperature (°C)		
1	−1 (4)	−1 (30)	13.2 ± 2.29	13.2
2	−1 (4)	1 (70)	34.3 ± 7.59	34.3
3	1 (8)	−1 (30)	43.2 ± 12.4	43.2
4	1 (8)	1 (70)	100 ± 13.0	100.0
5	−1.41 (2)	0 (50)	1.73 ± 0.19	1.73
6	1.41 (9)	0 (50)	0.00 ± 0.00	0.00
7	0 (6)	−1.41 (10)	14.7 ± 5.84	14.7
8	0 (6)	1.41 (90)	71.3 ± 6.64	71.3
9 (C)	0 (6)	0 (50)	85.6 ± 10.2	81.8 ± 3.37
10 (C)	0 (6)	0 (50)	79.1 ± 7.27	
11 (C)	0 (6)	0 (50)	80.7 ± 7.42	

Table 4
Variance analysis (ANOVA) of the optimisation of pH and temperature.

Factors	Df	SS	MS	F-value	p-value
pH **(L)**	1	75.29	75.29	6.61	0.1238
pH **(Q)**	1	5617.13	5617.13	493.39	0.0020
Temperature (°C)**(L)**	1	3049.51	3049.51	267.86	0.0037
Temperature (°C)**(Q)**	1	1414.72	1414.72	124.26	0.0079
pH **(L)** x Temperature (°C)**(L)**	1	317.62	317.62	27.90	0.0340
Lack of fit	3	3271.73	1090.57	95.79	0.0103
Pure Error	2	22.77	11.38		
Total SS	10	13675.69			
R^2		0.7591			
R^2 adjusted		0.5182			

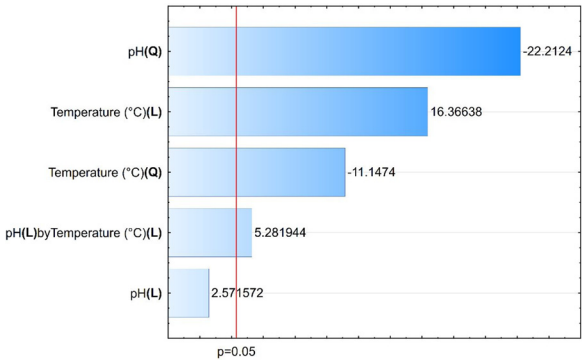

Fig. 4. Pareto chart of effects.

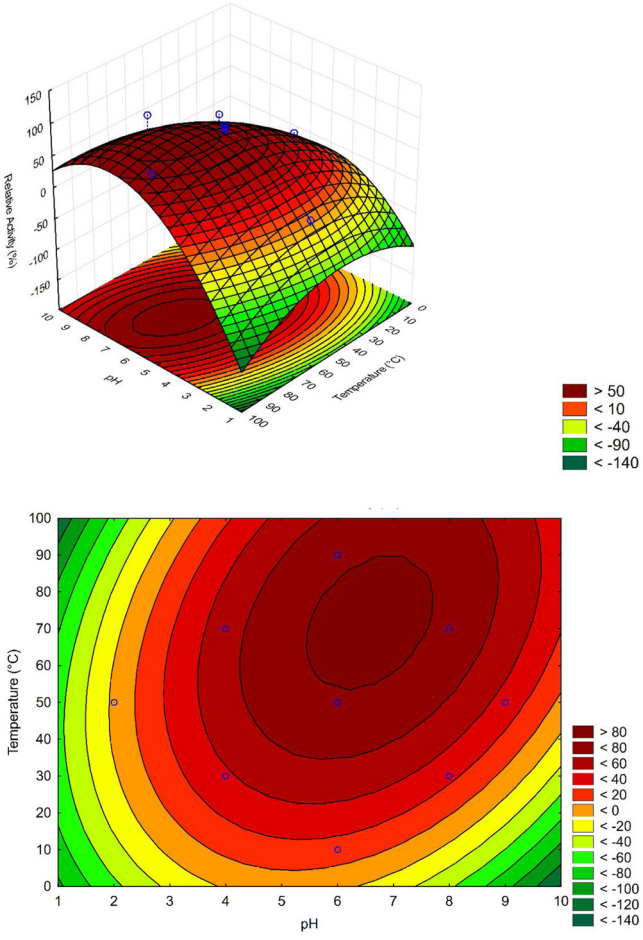

Fig. 5. Response and contour surface.

DCP and performed their experiment at pH 6.5. In another study, purified HRP enzyme showed an optimal pH of 6.0 for the 2,4-DCP removal [21].

Regarding the concentration of enzymes added to the reaction, an increase in the bioconversion of 2,4-DCP was observed as a function of the increase in the amount of biocatalyst added. When the concentration increased from 28.75–86.25 U L^{-1} within 1 h, the reaction yield increased from 31.5–85.7 %. Thus, the increase in enzyme concentration had a positive effect on the studied range. The same behaviour was observed by Kennedy et al. [29] using a commercial SBP.

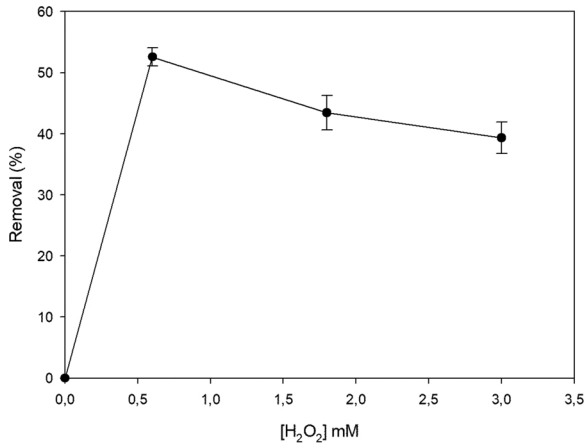

Fig. 6. Effect of hydrogen peroxide on the 2,4-DCP removal within 1 h, at pH 6.5 and enzyme concentration of 57.5 U L^{-1}.

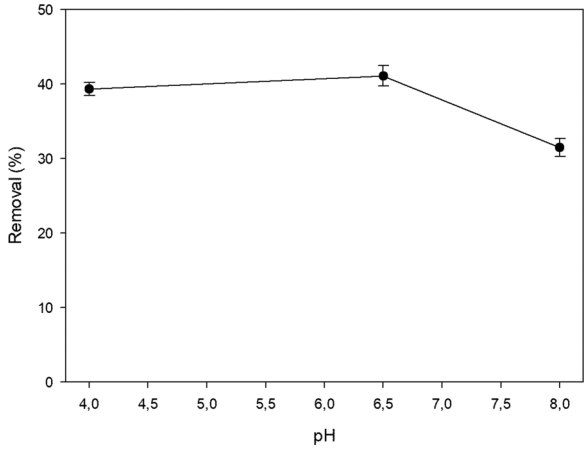

Fig. 7. Effect of pH on the 2,4-DCP removal within 1 h, at hydrogen peroxide concentration of 1.8 mM and enzyme concentration of 57.5 U L^{-1}.

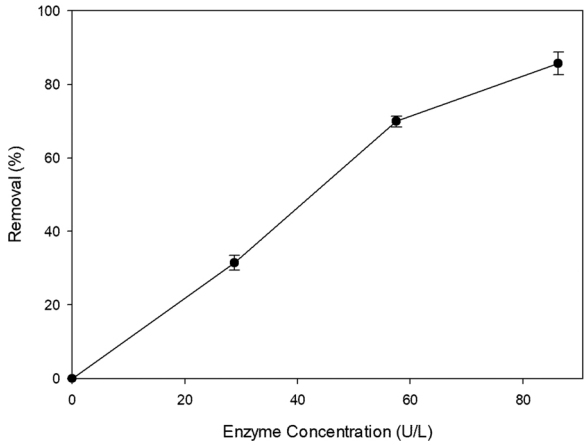

Fig. 8. Effect of enzyme concentration on 2,4-DCP removal at pH 6.5 and hydrogen peroxide of 1.8 mM.

Table 5
Effect of enzyme concentration in the bioconversion of 2,4-DCP 50 mg L^{-1} at room temperature, pH 6.5 and hydrogen peroxide 3.0 mM.

Enzyme concentration	After 1 hour	After 24 hours
Purified extract (1500 u L 50 m L^{-1})	84.61 ± 2.08	96.65 ± 0.52
Soybean hulls (3.1 g L^{-1})	11.33 ± 4.07	39.03 ± 10.47

Additionally, the purified enzyme and the equivalent amount of soybean hulls with the same enzyme activity observed in the free form were applied for the 2,4-DCP bioconversion. The reactions took place at pH 6.5 and an enzyme concentration of 86.25 U L^{-1}. The results are shown in Table 5.

It was possible to see that most of the bioconversion occurred during the first hour once 84.6 ± 2.08 % of the 2,4-DCP was bio converted within this period and the reaction reached 96.6 ± 0.52 % after one day. Kennedy et al., [29] applied soybean peroxidase from Sigma Chemicals for 2,4-DCP removal and stated that it is possible to obtain a complete removal in 1 min applying 1000 U L^{-1}. However, this is a concentration much higher than that used in this work. On the other hand, the same authors achieved approximately 80 % conversion with an enzyme concentration of 60 U L^{-1}, in three hours of reaction. These data are more compatible with those obtained in the present paper and, therefore, it is possible to affirm that the enzymes have compatible performance regarding the removal of 2,4-DCP.

Moreover, as the soybean hulls are a solid matrix, the effect of adsorption of 2,4-DCP on their surface was as well as assessed. The results showed that after 24 h occurred a decreasing in the 2,4-DCP concentration in the solution of 10.3 ± 0.45 %, suggesting that this effect should be due to the adsorption process. Meanwhile, the overall reduction in 2,4-DCP during the bioconversion was 39.03 % in average using a soybean seed dose of 3.1 g L^{-1}, which means that this result is associated with the biocatalytic activity of the enzyme released by the soybean seed hulls alongside with its adsorption capacity. Bassi and coworkers [26] applied soybean hulls harvested in the United States of America on the removal of 2,4-DCP. The authors used a dose of soybean hulls at 4 g 50 m L^{-1} (80 g L^{-1}) and obtained a bioconversion of approximately 50 % in 30 min, which seemed to be constant until the addition of more hydrogen peroxide. Thus, it is observed that the transgenic soybean used for the extraction of enzymes provided biocatalysts with a very attractive performance. However, the use of the enzyme in its free form involves better transport logistics, as well as the bioconversion efficiency of 2,4-DCP is better when compared to peel is applied as a source of enzymes.

3.4. Toxicity assessment

In order to determine whether the enzymatic treatment was sufficient for 2,4-DCP removal, it was carried out a toxicity test. Plant seeds have been used to evaluate the degree of toxicity by many studies, such as shown in Table 6, which is common use lettuce seeds. Through environmental testing samples with lettuce seeds, for example, it was possible to evaluate the phytotoxic effects of the pure or complex compounds by germination and elongation seeds in firsts grown days. During the test, the lettuce seeds were maintained in constant temperature at 22 °C. Table 6 shows the effects observed for germination and elongation seeds, through AG and RRGI percentage. In ended up, 120 h was considered germinated the seeds that were the visible detected appearance of the radicle. According to Table 6, 80 mg L^{-1} of the 2,4-DCP solution shows elevated level inhibition of the grown media. Statistically, the same result was observed in enzymatic treatment/hulls, which can be related to inhibition from other enzyme or has fewer hulls than necessary to bioconvert this effluent properly. However, it was observed that the enzymatic treatment decreases the toxicity of this phenolic compound. Using pure enzyme during the treatment was possible to reach 100 % germination and 54.5 % of the elongation when compared to control. It means that the residual concentrations of the 2,4-DCP did not block the germination, just the development of seeds, as claimed by Sobrero and Ronco [37]. According to these authors, this sort of bioassay allows considering the toxic effects of the soluble compounds in low levels that are not effective to inhibit the germination but could retard or inhibit the development of the elongation, depending on the action of the compounds into seeds. Despite the residual concentration of 2,4-DCP, the treatment with pure

Table 6
Effects observed for germination and elongation seeds. Control/water (1), concentrated 2,4-DCP solution (2), enzymatic treatment/pure enzyme (3), enzymatic treatment/hulls (4), H_2O_2 treatment (5) and enzymatic treatment/pure enzyme in absence from catalase (6).

Test Run	(1)	(2)	(3)	(4)	(5)	(6)
Germination (%)	100	0^{a}	$100^{b} \pm 0$	$18.7^{a} \pm 27.74$	$100^{b} \pm 0$	$96^{b} \pm 8.94$
Inhibition (%)	0	100^{A}	$45.5^{C} \pm 8.30$	$94.2^{A} \pm 8.72$	$62.5^{B} \pm 1.73$	$70^{B} \pm 3.33$

Tukey test with interval confidence with 95 %.

Table 7
Effects of various treatments on the toxicity of the treated effluent.

Treatment	Effluent	Specie	Reduction toxicity	Reference
galvanic and hydrogen peroxide	Industrial	*L. Sativa*	NO	[45]
peroxidases from *Prosopis juliflora*	4-CP and 2,4-DCP	*Vigna mungo* e *Allium cepa*	YES	[46]
peroxidase from potato pulp	2,4-DCP	*Lepidium sativum*	YES	[23]
extract from hairy roots	2,4-DCP	*L. Sativa*	YES	[44]
Electrooxidation	wastewaters from cashew-nut	*L. Sativa*	NO	[47]
Ozonation	phenolic compounds	*L. Sativa*	NO	[48]
peroxidase from soybean (pure)	2,4-DCP	*L. Sativa*	YES	*This study*
peroxidase from soybean (hulls)	2,4-DCP	*L. Sativa*	NO	*This study*

and enzymes in the hulls could decrease the toxicity degree from initial concentration, such as shown in Table 7. Angelini and co-workers [44] obtained similar results with the reduction of toxicity using hairy roots extract and Kurnick and co-workers [23] using peroxidase from potato pulp to treatment evaluating the toxicity in *Lepidium sativum*.

Before testing the toxicity of the samples of pure (3) and with hulls treatment (4), the solutions were pre-treated with catalase to remove the residual hydrogen peroxide. Table 6 shows the influence of H_2O_2 in the bioassay. In the presence of H_2O_2 in the media allowed the inhibition of the elongation, even after the enzymatic treatment or pure H_2O_2. Second Tukey's test for both tests, pure and enzymatic treatment, there were no significant differences between them. However, when the H_2O_2 was removed, it was observed differences at the elongation stage, showing the inhibition effect of H_2O_2.

The TOC reduced 36 % after the enzymatic treatment, from 26.80 ± 0.23 mg/L to 17.15 ± 0.84 mg/L. This effect might be associated with the synthesis of volatile or insoluble products. Although no studies have reported the TOC removal after 2,4-DCP oxidation using SBP, some investigations showed this response when using HRP. Huang and co-workers [49], for instance, used immobilised HRP in graphene oxide/magnetite to remove 2,4-DCP and obtained 49 % of TOC removal. In another study, Tatsumi and colleagues [50] demonstrated around 90 % of removal when using immobilised HRP on magnetite. The authors conducted the TOC analysis after polymer sediments removal by centrifugation. Additionally, the same authors observed in another study that the amount of enzyme was directly associated with the nature of the products [51]. In other words, the enzyme concentration may influence the precipitation of the compounds and facilitates its removal [50]. Following this, the reaction products obtained from the enzymatic oxidation of 2,4-DCP has been studied for years. Vali and Gold [52] identified 2-chloro-1,4-benzoquinone as the molecule associated with the 2,4-DCP oxidation via lignin peroxidase (LiP) or manganese peroxidase (MnP). In another study, Laurenti and co-workers [53] investigated the reaction mechanism and kinetics of 2,4-DCP oxidation catalysed by HRP and concluded that 2-chloro-6-(2,4-dichlorophenoxy)-1,4-benzoquinone was the final product. More recently, Chang et al. [54] reported two possible degradation routes for 2,4-DCP oxidation using HRP. The authors stated that, apart from 2-chloro-1,4-benzoquinone, insoluble polymers would also be observed. Despite this interest, no one as far as we know has studied the oxidation products obtained using SBP. In this case, although benzoquinones seem to be a common substance associated with the oxidation catalysed by peroxidases, there is the need to carry out a specific study using SBP.

4. Conclusion

In this work, we have extracted peroxidase from soybean hulls and used this protein for 2,4-dichlorophenol bioconversion in water. The results evidenced that the use of the extracted enzyme showed advantages compared to the use of hull seeds, which justify the extraction process. The bioconversion of the 2,4-dichlorophenol was achieved with performance compared to that one obtained with commercial soybean peroxidases. The ecotoxicological tests showed that there is a noticeable decrease in the toxicity of the treated effluent using the peroxidase. The use of soybean peroxidase extracted from transgenic seeds is a potential alternative to eliminate the harmful effects of 2,4-dichlorophenol in water and wastewaters.

CRediT authorship contribution statement

Mylena Fernandes: Conceptualization, Methodology, Formal analysis, Investigation, Writing - original draft. **Diego Hoefling Souza:** Investigation, Formal analysis. **Rosana Oliveira Henriques:** Writing - review & editing. **Mauricio Vicente Alves:** Resources. **Everton Skoronski:** Validation, Supervision, Writing - review & editing. **Agenor Furigo Junior:** Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

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

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jece.2020.103786.

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Use of phosphonium ionic liquids for highly efficient extraction of phenolic compounds from water

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ABSTRACT

Ionic liquids (ILs) are undergoing development as extractants for phenolic compounds from wastewater due to their outstanding properties. Although many ILs have been considered, the use of phosphonium-based ILs has thus far been neglected, despite advantages in viscosity and low water solubility. This work details the application of phosphonium-based ILs for the liquid–liquid extraction of phenolic compounds from water. The selected target contaminants, phenol and 2,4-dichlorophenol, were extracted using two ILs composed of trihexyltetradecylphosphonium cations with decanoate or bis(2,4,4-trymetylpentyl)phosphinate anions. The effect of volume ratio, time of contact, pH, temperature and (inorganic) salt concentration on the extraction efficiency were examined. Additionally, the ILs used in this study were characterised in terms of water solubility and solvatochromic polarity. These ILs are less soluble in water than the others reported in the literature, and even at a volume ratio (water: ionic liquid) of 135, the IL can extract more than 99% and 89% of 2,4-dichlorophenol and phenol, respectively, in just 10 min, with the best extraction performance at lower temperatures and pH lower than the pKa of these phenolic compounds. Overall, these ILs demonstrate high potential for phenol extraction and can be considered as a powerful alternative for wastewater treatment.

1. Introduction

The environmental control of pollution caused by phenolic compounds is of great importance, due to their high toxicity for most living organisms and their persistence and bioaccumulation in the environment [1,2]. Phenolic compounds are widely present in the wastewater of many industrial processes, especially the pulp and paper, textile, petrochemical, plastics, resins, pharmaceutical and pesticide industries, where concentrations range from 0.1 to 17,000 mg/L [3]. It is a significant issue for conventional treatment plants as even at phenol concentrations lower than 200 mg/L this substance is responsible for the inhibition of microbial growth in the biological treatment process [4]. This effect is also observed within the environment. Paisio and coworkers (2009) [5] studied the lethal and teratogenic effects of phenol on *Bufo arenarum* embryos and found that concentrations of 25 mg/L significantly affect this species.

Consequently, the maximum concentrations allowed for phenols in water, according to the regulations, are usually very low to control the quality of freshwaters and to avoid environmental impacts. As an example, the maximum concentration of pentachlorophenol for drinking waters, which is a common phenol-derivate found in the environment, is 5 μg/L [6]. Maintaining these trace concentrations requires highly efficient treatment processes to control the disposal of wastewaters into aquatic media.

The removal of phenolic compounds from water and wastewater is not easy and, historically, there are no settled methodologies. In the last decades, some physical and chemical processes have been developed, such as adsorption, chemical oxidation and membrane separation [7–10]. However, many drawbacks are associated with these techniques, especially high cost, low efficiency and the generation of toxic residues [8]. Hence, there is still a great need for developing alternative methodologies for the removal of recalcitrant organic compounds from wastewater.

The application of ionic liquids (ILs) for liquid–liquid extraction of organic compounds from water is an alternative attracting increasing attention each year [11,12]. ILs are salts with low melting point temperatures, usually under 100 °C; some being liquid even at room temperature. The number of possible ILs has been estimated to be of the order of 10^6, therefore, they exhibit a wide range of properties. The reason why these compounds are known as designer solvents is that

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their properties can be adjusted for a specific task by the selection of the cation and the anion [13]. Generally speaking, they show neglectable volatilities, are less toxic compared to volatile organic solvents, are non-flammable, have high electric conductivity and outstanding thermal and chemical stability [13,14]. Because of their negligible vapour pressure, they are frequently considered green solvents. Nevertheless, some authors are concerned about the toxicity of ionic liquids [15–17]. These ILs are commonly composed of a cation (such as alkylammonium, alkylphosphonium, alkylnitride, *N*,*N*'-dialkylimidazolium, *N*-alkylpyridinium) and an anion (such as $[NO_3]^-$, $[ClO_4]^-$, $[HSO_4]^-$, $[SO_3]^-$, $[CF_3SO_3]^-$, $[(CF_3SO_2)_2N]^-$, $[(CF_3SO_2)_3C]^-$, or halides). The cation is mainly responsible for the physical properties such as viscosity, melting point, density, solubility, while the anion typically dominates the chemical properties [14]. The asymmetry of the cation limits the packing of the IL in a crystal structure, which explains their low melting points.

With regards to wastewater treatment; some researchers have used ILs for the removal of specific harmful substances, such as metals and halides. Some examples includes: chromium(VI) [18–21]; cadmium [18,22,23]; lead [24]; mercury [25]; palladium [26] and fluoride [27]. Additionally, some organic compounds have been extracted by ILs, including textile dyes [28,29]; halogenated hydrocarbons (HHCs) [30] and herbicides [31].

Many authors have also studied phenol extraction. These studies have shown the feasibility of phenol removal by ILs and have motivated new investigations based on this technology. Several IL-based strategies have been applied for phenol removal such as liquid–liquid extraction [32–38], permeation into supported ionic liquid membranes [39–41] and bulk ionic liquid membranes [42]. Among the several phenolic compounds existing in wastewater, the derivates that have been chosen for most studies include phenol, chlorophenol derivates, 4-nitrophenol, and bisphenol-A. 2,4-dichlorophenol (2,4-DCP) has been also considered as target compound [34,36,43–45]. Furthermore, the use of phosphonium-based ILs, which are highly hydrophobic compounds, for phenol removal has also been studied in previous works [39,46–48].

Nevertheless, apart from the study conducted by Pilli et al. (2014) [39], the phosphonium-based ILs have not been considered for phenol water treatment purposes. Their stability in alkaline media and their low solubility in water are some features which make these compounds for water treatment very attractive.

2. Materials and methods

2.1. Materials (Chemicals)

The chemicals used in this work are detailed as follow, and their puity and CAS number are in brackets: trihexyltetradecylphosphonium decanoate $[P_{66614}][C_9H_{19}COO]$ (≥95%, 465527–65-5) and trihexyltetradecylphosphonium bis(2,4,4-trimethylpentyl)phosphinate $[P_{66614}][(i\text{-}C_8)POO]$ (≥90%, 465527–59-7), phenol (>99%, 108–95-2), 2,4-dichlorophenol (99%, 120–83-2), sodium chloride (≥99%, 7647–14-5), ammonium chloride (≥99,5%, 12125–02-9), ammonium hydroxide (aqueous solution 28–30%, 1336–21-6), aminoantipyrine (reagent grade, 83–07-8), sodium persulfate (≥98%, 7775–27-1) and Reichardt's dye (90%, 10081–39-7). They were obtained from Sigma-Aldrich, and used as received.

2.2. Methods

2.2.1. Ionic liquid characterisation

The density used for trihexyltetradecylphosphonium decanoate and trihexyltetradecylphosphonium bis(2,4,4-trimethylpentyl)phosphinate is 0.883 [49] and 0.895 [50] g/mL at 20 °C, respectively. The solubility of the ILs in water was assessed by inductively coupled plasma mass spectrometry (ICP-MS). In this case, the total phosphorus concentration in the aqueous phase was used to estimate the relative IL concentration.

The solvatochromic polarity measurements of the ILs were determined using Reichardt's dye. The IL-dye mixtures were submitted to ultrasonication to complete solute dissolution. Afterwards, the UV–vis spectrum of the solution was measured to determine the Dimroth-Reichardt polarity parameter $E_T(30)$ in kcal/mol according to Eq. (1):

$$E_T(30) = \frac{28591}{\lambda_{max}} \tag{1}$$

where λ_{max} is the maximum absorbance wavelength (Reichardt, 2005) [51].

2.3. Extraction procedure

To 15 mL centrifuge tubes was added 15 mL of a phenol or 2,4-dichlorophenol (2,4-DCP) solution (1000 mg/L) and different amounts of IL (25.0, 50.0, 75.0 and 100.0 mg) weighed in an analytical balance with a precision of 0.1 mg. These values were converted in volume using the available density data for each IL at 20 °C. We did not measure the IL volumes directly because of their high viscosity. The mixture was stirred in a vortex mixer at 2000 rpm for 60 min. The pH was not adjusted and remained between 6.0 and 6.5. An aliquot of 2 mL was removed for centrifugation in Eppendorf tubes at 14,800 rpm for 2 min. A sample of 0.5 mL was carefully taken out from the aqueous phase for the total phenol determination. Similarly, for the kinetics study, a mixture of 15 mL of 1000 mg/L of phenol or 2,4-DCP and 25 mg of the IL was used. Total phenol concentration were determined at a contact time of 1.0, 2.5, 5.0, 7.5, 10, 15, 30 and 60 min. In addition, the salt effect tests were carried using the same conditions and adding 100, 250, 500, 1000 and 2000 mg/L of sodium chloride to the 1000 mg/L phenol or 2,4-DCP solution. Also, the pH of the phenols solutions was modified to 3.0 and 4.0, using HCl (0.01 and 0.1 mol/L) or 7.0, 9.0 and 11.0 using NaOH (0.01 and 0.1 mol/L) to assess the effect of pH on the phase distribution ratio. It was used 25.0 mg of each IL and 15 mL of 1000 mg/L of phenol or 2,4-DCP. Finally, this same concentration of IL and phenol solution was considered to assess the effect of temperature (20, 30, 40 and 50 °C) on the parameter D. For this particular test, we carried out the experiments in a Heidolph magnetic stirrer hotplate with a temperature controller (±1°C).

The phase distribution ratio (D) was calculated by Eq. (2):

$$D = \frac{C_{IL}}{C_W} = \frac{C_W^0 - C_W}{C_W} \cdot \frac{V_W}{V_{IL}} \tag{2}$$

where C_{IL} (mg/L) is the concentration of phenols in the ionic liquid phase, C_W^0 (mg/L) and C_W (mg/L) are the initial and equilibrium concentration of phenols in water, respectively. V_{IL} (mL) and V_W (mL) are the volumes of ionic liquid and aqueous phase, respectively.

The extraction capacity q (mg/g) was calculated by Eq. (3):

$$q = \frac{(C_W^0 - C_w).\ V_w}{V_{IL}.\ \rho_{IL}} \tag{3}$$

where ρ_{IL} (g/mL) is the density of the ionic liquid.

Finally, the extraction efficiency (E) was calculated according to Eq. (4):

$$E(\%) = \frac{(C_W^0 - C_w)}{C_W^0} \cdot 100 \tag{4}$$

2.4. Thermodynamic analysis

The experiments were performed in 15 mL glass vials, and a hotplate provided the heating and stirring. The tested temperatures were 20, 30, 40 and 50 °C. The systems were kept under stirring for 24 h and samples were collected for analysis.

The parameters ΔG (kJ/mol), ΔH (kJ/mol) and $T\Delta S$ (kJ/mol) were calculated based on the D values according to Eqs. (5) and (6). We have

assumed that the values of ΔH and ΔS do not vary significantly over the temperature range considered for this study.

$$\Delta G = -RTlnD \quad (5)$$

$$lnD = \frac{-\Delta H}{RT} + \frac{\Delta S}{R} \quad (6)$$

where R is the gas constant (8.314 J/K mol).

2.5. Analytical procedures

2.5.1. Colourimetric determination of phenols

We measured total phenol concentration using the direct colourimetric method 5530 D based on the phenol and 4-aminoantipyrine (4-AAP) reaction, including some modifications [52]. This approach included the use of 0.21 mol/L sodium persulfate as a catalyst at pH 10 in ammonium buffer. Phenol concentrations were proportional to the absorbance measured at 504 nm.

2.5.2. Determination of total phosphorus by ICP-MS

The analysis of phosphorous in the aqueous phase was performed in an inductively coupled plasma mass spectrometry (Agilent Technologies 7900 ICP-MS). The samples were acidified with nitric acid (70%) to a final acid concentration of 2% (v/v) and then directly measured in the ICP-MS.

3. Results and discussion

3.1. Characterisation of the ionic liquids

The solubility of the ILs in water was determined in order to assess possible contamination of the water by the IL. Indeed, even very low solubility in water could cause aquatic problems [17]. This characteristic may be a limitation for the IL using in liquid–liquid extraction when applied to environmental applications. The solubility at room temperature (20 ± 1 °C) for trihexyltetradecylphosphonium decanoate was determined to be 8.7 ± 0.2 mg/L and for trihexyltetradecylphosphonium bis(2,4,4-trimethylpentyl)phosphinate 12.6 ± 0.3 mg/L. These results agree with the value reported by van den Berg et al. (2009) [47] of 16 mg/L at 30 °C for trihexyltetradecylphosphonium bis (2,4,4-trimethylpentyl)phosphinate (small differences likely ascribed to temperature effects [53]). To the best of our knowledge, these are the lowest aqueous soluble ILs applied to phenols removal reported in the literature. As a comparison, Egorov at al. (2008) [32] applied tetrahexylammonium dihexylsulfosuccinate (THADHSS) and trioctylmethylammonium salicylate (TOMAS) for phenol removal. The solubility of THADHSS and TOMAS in water are 8.6 ± $0.2.10^{-5}$ mol/L (61.8 mg/L) and 2.0 ± $0.2.10^{-5}$ mol/L (168.8 mg/L), respectively. Moreover, Fan et al. (2014) [35] studied several imidazolium-based ILs which presented the potential for phenol extraction from the water. The solubility varied from 60 to 26,000 mg/L, which are significantly higher than the values found in this study. A recent study addressing the solubility of hydrophobic ILs, with the $[NTf_2]^-$ anion, in artificial seawater, concluded that phosphonium ILs have the lowest solubilities in water and the cation $[P_{8888}]^+$ yielded an IL less soluble than $[P_{66614}]^+$ [54]. The $[P_{8888}]^+$ cation would be a potential candidate for improving the performance of the phosphonium based ILs in phenolic compounds extraction.

Reichardt's dye was used to assess the hydrophobicity of ILs via solvatochromism at room temperature. The hydrophobicity of the IL is one of the most important property as it plays a major role in the water solubility and extraction efficiency of phenols. The measurements were performed with the ILs under the same conditions used in the extraction experiments.

Overall, the ionic liquids considered for this work have comparable $E_T(30)$ values, meaning that their hydrophobicity must be similar. The values of λ_{max} and $E_T(30)$ obtained for $[P_{66614}][C_9H_{19}COO]$ and $[P_{66614}][(i\text{-}C_8)POO]$ were 652.5 nm and 43.81 kcal/mol, and 656 nm and 43.58 kcal/mol, respectively. These values are lower than the ones reported for THADHSS and TOMAS of 46.5 and 48.2 kcal/mol, respectively [32]. It is worth mentioning that the lower the $E_T(30)$ value, the less polar the solvent and the extraction performance is expected to be higher [32].

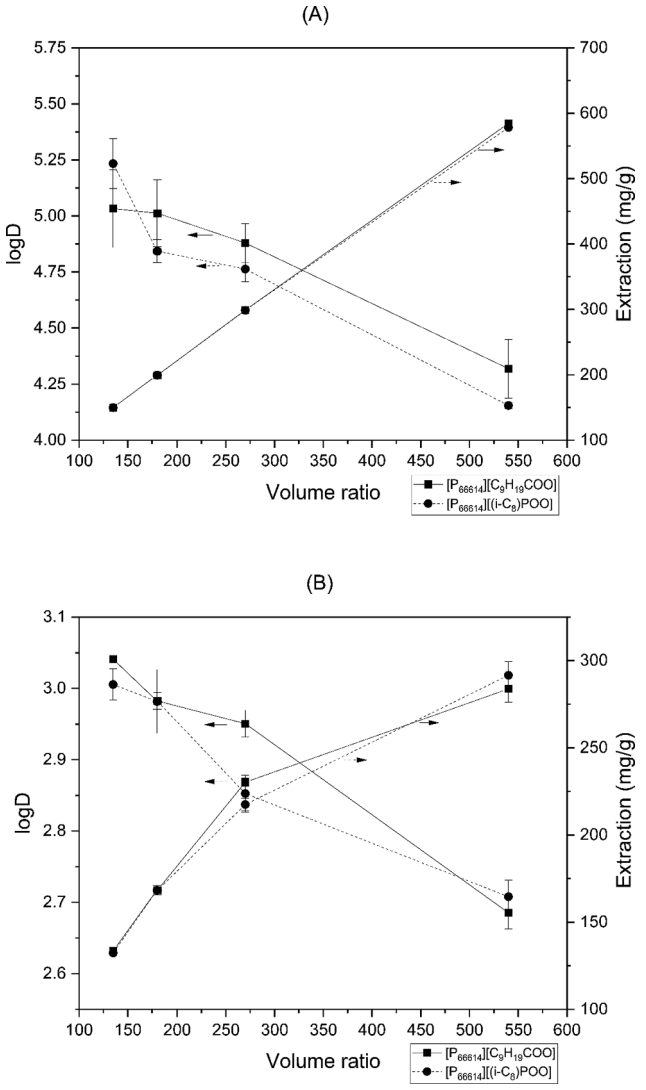

Fig. 1. Effect of phase volume ratio (water/IL) in the extraction of (A) 2,4-dichlorophenol and (B) phenol.

3.2. Effect of volume ratio

The effect of the phase volume ratio between the aqueous solution and the ILs (water/IL) was studied for phenol and 2,4-DCP. The results are presented in Fig. 1. The distribution ratio increased with the reduction in the phase volume ratio for all the systems studied. The values of distribution ratio varied from 21,300 ($logD$ = 4.32) to 107,000 ($logD$ = 5.03) for the extraction of 2,4-DCP using $[P_{66614}][C_9H_{19}COO]$. Similarly, the use of $[P_{66614}][(i\text{-}C_8)POO]$ provided extraction of 2,4-DCP with distribution rations values from 14,300 ($logD$ = 4.15) to 174,000 ($logD$ = 5.23). On the other hand, the distribution ratio values for the extraction of phenol were considerably lower, 1,060 ($logD$ = 3.02) and 890 ($logD$ = 2.94) for the ionic liquids $[P_{66614}][C_9H_{19}COO]$ and $[P_{66614}][(i\text{-}C_8)POO]$, respectively. The difference is likely due to the difference between the solubility of 2,4-DCP and

phenol in water, 4.5 [55] and 82.8 g/L [56], respectively. Additionally, the hydrophobicity has also been considered to explain the distribution ration in some studies [54,55]. The hydrophobicity can be measured by the partition coefficient between octanol and water ($\log K_{OW}$). In this case, the values are 2,4-DCP, and phenol is 3.06 and 1.46, respectively. As a consequence, 2,4-DCP extraction was more favourable as this compound is more hydrophobic (higher $\log K_{OW}$) than phenol. Comparatively, Deng et al. (2011) [55] studied the application of a magnetic phosphonium based IL on phenolic compound removal. They used the same cation as for the ILs considered in our work with tetrachloroferrate (III) as the anion. The authors concluded that liquid–liquid extraction from water is enhanced when the phenolic structures contain more chlorinated substituents. This effect was associated with the greater hydrophobic interactions between the chlorinated phenols and the magnetic phosphonium based IL. Furthermore, the relationship between the *logD* and the phase volume ratio reported by Deng and co-workers (2011) [34] was similar to that observed in our work, i.e. the *logD* increased as the more IL was used for the extraction. Numerically, the *logD* increased from 3.1 to 3.6 when the phase volume ratio decreased from 280 to 120 (water/IL). Values lower than 120 (water/IL) did not change the *logD* results. We have not seen a trend to constant *logD* values above our minimum value of 135 (water/IL) for the phase ratio, as below this we observed emulsification of the system, which caused issues for phase separation. Yang and co-workers (2015) [48] have introduced the potential of using long-chain fatty acid-based phosphonium ILs for phenol extraction. In their study, a volume phase ratio of 1 (water/IL) was applied, and the distribution ratio obtained were 4818 and 735.7 for $[P_{66614}][C_7H_{15}COO]$ and $[P_{66614}][C_{13}H_{27}COO]$, respectively. Although the result obtained for the octanoic acid-derived IL is significant, we are not able to compare this with our data as the volume phase ratio at 1 (water/IL) is significantly lower than the minimum we considered, i.e. 135 (water/IL). Apart from the phosphonium-based ILs, the imidazolium-based IL $[C_nMin][PF_6]$ was also used for phenol removal. In this work, a phase volume ratio from 1 to 50 (water/IL) was used and the distribution ratio varied from 21.6 to 15.9. [44]. Additionally, THADHSS and TOMAS were applied for phenol removal and the distribution ratios obtained were 2.5 and 2.1, respectively, using a phase volume ratio of 20 (water/IL) [32]. These results demonstrate that the phosphonium based ILs are the most highly efficient ILs reported for phenol removal. They present a distribution ratio surprisingly high when compared to the most common ILs reported in the literature.

We also decided to express the results in terms of extraction capacity, similar to the adsorption capacity used for adsorption systems, to compare the amount of pollutant extracted per weight or volume of IL used. As liquid–liquid extraction is an equilibrium process, the concentration of the pollutant in the treated water plays a significant influence on the amount of the same species dissolved in the IL. The extraction capacity for 2,4-DCP and phenol varied with the phase volume ratio range considered in this study. The highest extraction capacities were obtained for the lowest volume ratios, once the highest concentration of a pollutant in equilibrium was reached. It is possible to extract roughly 600 mg of 2,4-DCP using 1 g of any of either of the two ILs. In this case, the equilibrium concentrations were 25.79 and 36.42 mg/L in the aqueous phase, after the extraction using $[P_{66614}][C_9H_{19}COO]$ and $[P_{66614}][(i\text{-}C_8)POO]$, respectively. For phenol, the extraction capacity was lower and the maximum values obtained were 286 and 265 mg/g for $[P_{66614}][C_9H_{19}COO]$ and $[P_{66614}][(i\text{-}C_8)POO]$, respectively. The concentration of phenol in equilibrium at this condition was approximately 550 mg/L. In other work published using the same ILs considered in this study, the authors also verified that the Cyphos 104 (trihexyltetradecylphosphonium bis(2,4,4-trimethylpentyl) phosphinate), showed a better phenol removal than Cyphos 103 (trihexyltetradecylphosphonium decanoate). The authors used a support liquid membrane and obtained an extraction capacity of 53.16 mg of phenol per litre of IL. This study was essential to highlight the outstanding performance of the phosphonium ILs Cyphos 103 and 104 for phenol removal. Additionally, our study revealed that the extraction capacity could be higher when varying the phase volume ratio and using a liquid–liquid extraction process. Overall, our results were essential to valorise this class of ILs for the purposes mentioned earlier.

Table 1
Effect of phase volume ratio on the extraction efficiency of phenol and 2,4-dichlorophenol.

	Phase volume ratio (water/ionic liquid)	Extraction efficiency (%) $[P_{66614}][C_9H_{19}COO]$	$[P_{66614}][(i\text{-}C_8)POO]$
2,4-DCP	135	99.87 ± 0.05	99.92 ± 0.02
	180	99.82 ± 0.06	99.74 ± 0.03
	270	99.64 ± 0.07	99.53 ± 0.06
	540	97.42 ± 0.74	96.35 ± 0.11
Phenol	135	89.05 ± 0.00	88.23 ± 0.51
	180	84.20 ± 0.36	84.16 ± 1.36
	270	76.76 ± 0.77	72.50 ± 1.17
	540	47.30 ± 1.29	48.60 ± 1.32

The values of extraction efficiency considering the lowest phase volume ratio (135:1) were comparable with the other published studies. $[P_{66614}][C_9H_{19}COO]$ extracted 99.92% of 2,4-DCP and 88.73% of phenol (Table 1). Even at a phase volume ratio of 540:1, it was possible to extract 97.42 and 47.30% of 2,4-dichlorophenol and phenol, respectively. Apart from the study involving a hydrophobic magnetic room temperature IL $[P_{66614}][FeCl_4]$, which applied up to 280 vol of water per volume of IL, we reached high extraction removals using considerable less volume of IL per volume of water compared to the reported studies [34]. It is worth noting that $[P_{66614}][FeCl_4]$ is also a phosphonium based IL. 1-hexyl-1-methylpyrrolidinium bis(trifluoromethylsulfonyl)imide $[C_1C_6Pyr][NTf_2]$ and 1-hexyl-3-methylimidazolium bis(trifluoromethylsulfonyl)imide $[C_6C_1Im][NTf_2]$ were applied to the removal of phenol, *o*-cresol and resorcinol. The authors reported a phenol removal between 90 and 95%, in 30 min of contact, using a phase volume ratio (water to IL) of 1.5 [37]. *N*-butylimidazole-derived ILs incorporating hydroxyl, benzyl and dialkyl radicals were used for extraction of phenol. The results showed that the extraction efficiency was raised from 40 to 97.5% when authors reduced the volume phase ratio (water to IL) from 60 to less than 5 (water/IL) [35]. 1-butyl-3-methyl imidazolium tetrafluoroborate $[C_4C_1Im][BF_4]$ dissolved in tributylphosphate was applied for removal of phenol and 2,4-DCP. The efficiency of extraction was 99.74 and 99.18%, for phenol and 2,4-DCP, respectively, at a phase volume ratio of 7. The yield dropped to 50–60% when the authors applied 9 to 11 volumes of water per volume of IL 1-butyl-3-methyl imidazolium $[C_4C_1Im][PF_6]$ dissolved in tributylphosphate extracted 100 and 92.5% of phenol and 2,4-DCP at a volume ratio of 3 [44]. Choline bis(trifluoromethylsulfanyl)imide was able to remove 80% of phenol from water at a volume ratio of 0.5–2 (IL/water) [57]. Recently, phenol were successfully removed (94–98%) from a aqueous solution (15,000 mg/L) by four bis(trifluoromethylsulfonyl) imide based ionic liquids with different cations, namely 1-butylpyridinium ($[C_4Py][NTf_2]$), 4-butyl-1-methylpyridinium ($[C_4C_1Py][NTf_2]$), 4-butyl-1-methylpiperidinium ($[C_4C_1Pip][NTf_2]$) and 4-butyl-1-methylpyrrolidinium ($[C_4C_1Pyr][NTf_2]$), and 4-butyl-1-methylpyrrolidinium bis(fluorosulfonyl) imide $[C_4C_1Pyr][Nf_2]$ at a phase volume ratio between ionic phase and aqueous phase of 1:2 [58].

3.3. Effect of pH

The effect of pH on the phase distribution ratio was studied in the pH range of 3 to 11 (Fig. 2). The extraction performance was favourable under acidic conditions for both target compounds, and ILs tested. There is a trend of reduction in the phase distribution ratio values as the phenol turns from its molecular to an ionic state. For 2,4-DCP, the reduction of the parameter *D* was around 10 times, when the pH was

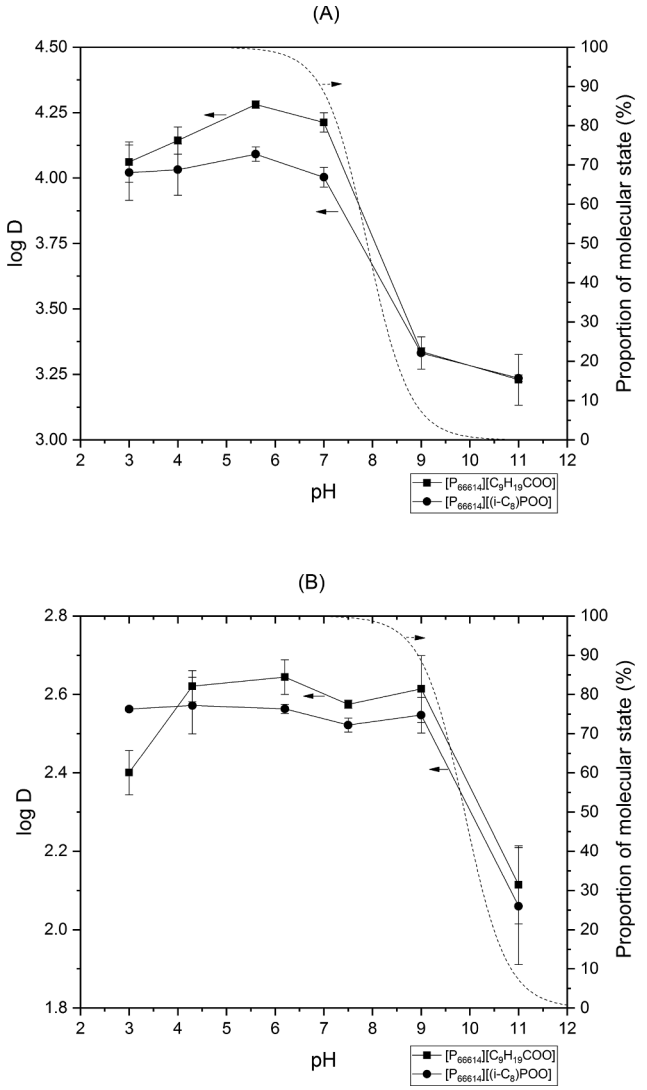

Fig. 2. Effect of pH on the distribution phase ratio on the (A) 2,4-dichlorophenol and (B) phenol extraction. The proportion of molecular state refers to the fraction of protonated phenolic species.

modified from 5.6 to 11. For phenol, the *logD* decreased from 2.6 to 2.1. This effect is in accordance with other studies which also concluded that the extraction is enhanced when the phenolic compounds are in the molecular state. For phenol, the best extraction was reported for pH lower than 7 using imidazolium-based ILs or THADHSS and TOMAS [32,33] or pH of 9 using hydroxyl-, benzyl-, and dialkyl-functionalized ionic liquids [35]. Values of pH lower than 6 were considered fundamental for 2,4-DCP extraction [34,44]. Nevertheless, the reduction in the extraction capacity was lower for the phosphonium ILs we studied here compared to other similar studies. It suggests that apart from the extraction via hydrophobic interactions, an ion-exchange mechanism could be responsible for the extraction under basic conditions [34].

3.4. Effect of contact time

Contact time is one of the essential parameters for practical applications at large scale, particularly for flow systems. Therefore, we studied the effect of contact time on the extraction efficiency, as reported in Fig. 3. The mass transfer of phenols from water to the IL occurred quickly in the first few minutes and equilibrium is reached in approximately 10 min for 2,4-DCP and phenol for both ILs. In this case, the nature of the phenolic compound does not seem to influence the

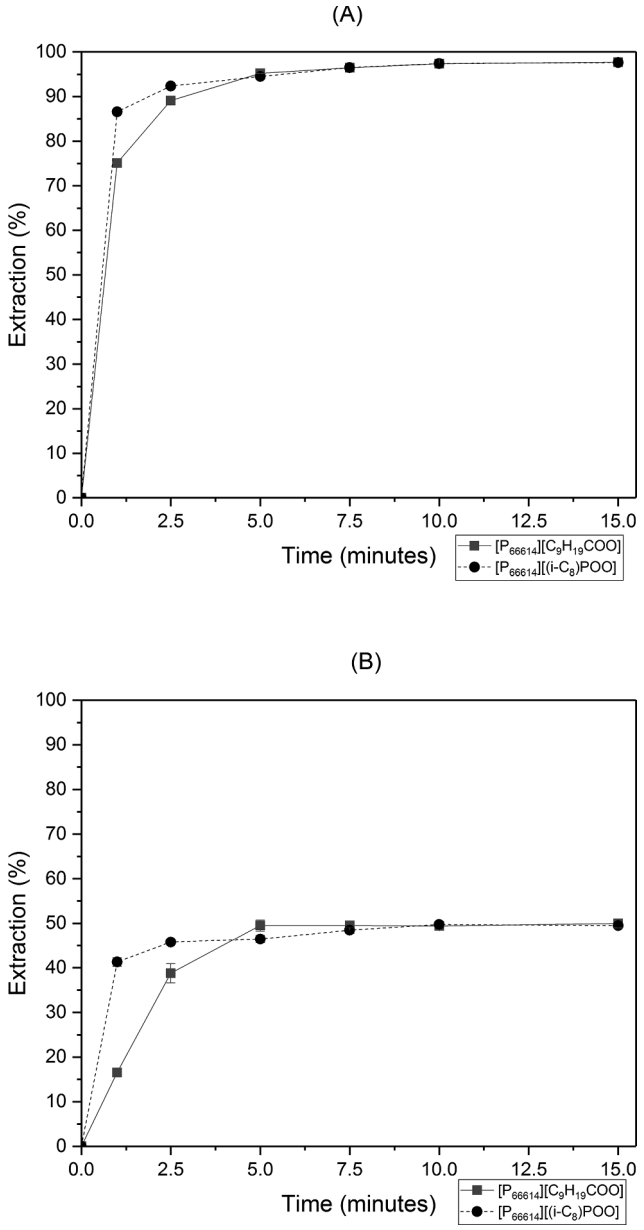

Fig. 3. Effect of contact time on the (A) 2,4-dichlorophenol and (B) phenol extraction.

kinetics of extraction. Other studies involving phenol extraction considered a minimal contact time to reach the equilibrium within 5 to 30 min [34,37,44]. All of these studies applied the methodology of liquid–liquid extraction for phenol extraction. When a supported liquid membrane was used for the tests, the equilibrium was reached only after 24 h. Therefore, liquid–liquid extraction could be considered an advantageous alternative in terms of kinetics.

3.5. Effect of salt concentration

Natural water and industrial wastewaters have many dissolved solids which can play a significant rule in the extraction processes. In our study, we have considered NaCl as a dissolved model solid and a concentration range was applied of 0 to 2000 mg/L. This maximum value is representative for most industrial wastewaters. From the results illustrated in Fig. 4, it is possible to conclude that the NaCl concentration did not influence the extraction performance within the experimental conditions. Some authors reported an improvement in the extraction

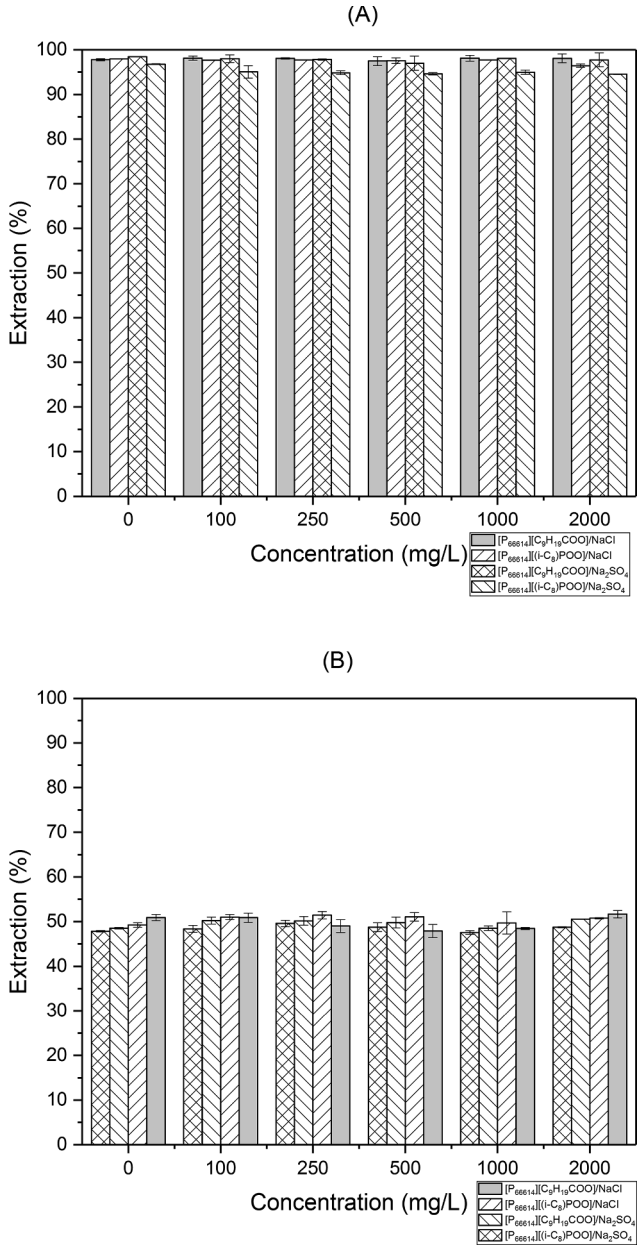

Fig. 4. Effect of salt concentration on the extraction of (A) 2,4-dichlorophenol extraction and (B) phenol.

yields due to the salting-out effect. In this case, the hydration ability is considered an essential property which can enhance the extraction performance. For example, Fan and co-workers (2008) [33] applied $[C_6C_1Im][PF_6]$ for phenol removal from water and did not verify significant effect due to the addition of NaCl up to 0.9 mol/L (52,650 mg/L). The authors attributed this result to the weak hydration ability for NaCl. On the other hand, Fan et al. (2014) [35] observed that the extraction efficiency for phenols could be raised from 87 to 92% when they added 5% of NaCl in water and extracted with the functionalized IL $[C_4C_{11}OHim][BF_4]$. Based on this, the influence of salt seems to be associated with the nature of the IL. Additionally, in both studies, the concentration was approximately 50,000 mg/L; beyond our studied scale and most common environmental matrices.

We have carried out other tests using sodium sulphate (Na_2SO_4) as it is a salt which presents stronger hydration ability. The results are plotted in Fig. 4 and were similar to those found using NaCl as a salt. The influence of Na_2SO_4 on the extraction of phenol or 2,4-DCP by both ILs could be considered negligible. The maximum concentration tested was 2000 mg/L (0.014 mol/L). Differently, Fan et al. (2008) [33] claimed that the presence of Na_2SO_4 increased the phenol extraction by $[C_6C_1Im][PF_6]$. However, the concentration of salt was approximately ten times higher than was considered for this paper. Therefore, we can conclude that the presence of salt does not influence the extraction efficiency of phenol and 2,4-DCP by $[P_{66614}][C_9H_{19}COO]$ and $[P_{66614}][(i\text{-}C_8)POO]$ within the salt concentration of 0 to 2,000 mg/L.

3.6. Thermodynamic analysis

The effect of temperature on the extraction performance was studied, and the results are presented in Table 2. For both phenols, the partition coefficient decreased with temperature; the decrease was more pronounced for 2,4-DCP. The negative values for ΔG indicate that the extraction of both phenols by the phosphonium-based ILs are spontaneous. Additionally, the ΔG data indicate that 2,4-DCP is preferably extracted as they presented lower Gibbs free-energy changes. The extraction process can also be classified as an exothermic according to the $\Delta H < 0$ value. The positive ΔS indicates increased randomness in the system, as during the extraction, there is a dehydration of the extracted molecules, increasing the amount of free water in the media, increasing system entropy. An entropy-driven extraction process is a characteristic of hydrophobic interactions, and this pattern was in agreement with the conclusions from the study of Fan et al. (2014) [35]. Those authors obtained negative values for ΔG and ΔH and positive ΔS for the extraction of phenols by $[C_4C_{11}OHim]BF_4$. The study of González et al. (2018) extracting phenol with imidazolium and pyrrolidinium ionic liquids and also demonstrated that the extraction efficiency decreases with increasing temperature.

4. Conclusion

We have applied two phosphonium-based ionic liquids for the extraction of phenol and 2,4-dichlorophenol. The selected ionic liquids showed an extremely low solubility in water, which may be a unique property when compared to other solvents reported in the literature. However, further studies focused on water toxicity due to the solubilised IL must be considered. Additionally, a high extraction of phenols could be achieved in short contact time and using a minimal amount of ionic liquid over a broad range of pH and temperature. These results can encourage further studies involving diverse methodologies which could take advantage of the phenol extraction properties of phosphonium-based ionic liquids. Also, it must be evaluated their potential to achieve the regulation limits for phenolic compounds in the environment.

CRediT authorship contribution statement

Everton Skoronski: Conceptualization, Methodology, Formal analysis, Investigation, Writing - original draft, Writing - review & editing. **Mylena Fernandes:** Methodology, Investigation, Formal analysis. **Francisco J. Malaret:** Validation, Writing - review & editing. **Jason P. Hallett:** Writing - review & editing, Resources, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

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

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Table 2
Effect of temperature on the 2,4-dichlorophenol and phenol extraction.

Ionic liquid	Contaminant	T(°C)	Log D	ΔG(kJ/mol)	ΔH(kJ/mol)	$T\Delta S$(kJ/mol)
$[P_{66614}][C_9H_{19}COO]$	2,4-DCP	20	4.24	–23.79	−21.66	2.10
		30	4.10	–23.82	−21.66	2.18
		40	3.98	–23.86	−21.66	2.25
		50	3.88	−24.02	−21.66	2.32
	Phenol	20	2.63	−14.78	−4.89	9.93
		30	2.62	−15.21	−4.89	10.27
		40	2.59	−15.54	−4.89	10.61
		50	2.55	−15.79	−4.89	10.94
$[P_{66614}][(i\text{-}C_8)POO]$	2,4-DCP	20	4.16	–23.33	−21.80	1.55
		30	4.05	–23.50	−21.80	1.60
		40	3.89	–23.34	−21.80	1.66
		50	3.81	–23.57	−21.80	1.71
	Phenol	20	2.63	−14.74	−6.06	8.70
		30	2.61	−15.13	−6.06	9.00
		40	2.54	−15.25	−6.06	9.30
		50	2.54	−15.70	−6.06	9.59

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.seppur.2020.117069.

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