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Biodegradation of Textile Dyes by Radish Peroxidase (*Raphanus sativus* L.) Immobilized on Coconut Fiber

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Abstract: The importance of biodegradation in the context of dye removal is presented as a function of the need to use enzyme immobilization as a key technology for effluent treatment. Immobilization is based on the need to improve the stability and recyclability of the biocatalyst in relation to the free enzyme, in addition to a lower risk of product contamination as an advantage. The present study aims to evaluate the biodegradation of textile dyes using radish peroxidase immobilized by physical adsorption method on coconut fiber, as well as its characterization in relation to pH, temperature, and infrared spectrum (FTIR). The discoloration efficiency (DE) of free radish peroxidase (FRP) and immobilized (IRP) on the dyes methylene blue (MB) and navy blue (NB) was determined by biodegradation assays conducted at 200 rpm for 1 h at 25°C. The evaluated parameters were: effect of pH, amount of biocatalyst (FRP for MB and NB; IRP for MB and NB), molar ratio dye: H_2O_2 (hydrogen peroxide) and contact time. Also, operational stability was analyzed. Under optimized conditions [pH 8.0 (MB) and pH 5.0 (NB), amount of biocatalyst to MB: 40.40 U (FRP) and 3.25×10^{-3} U (IRP), and to NB: 13.45 U (FRP) and 26.25×10^{-3} U (IRP), and molar ratio dye: H_2O_2 to IRP: MB (1:0.5 mmol/L) and NB (1:10 mmol/L)], the maximum DE for IRP was 86% for MB and 61% for NB. IRP can be reused ten times for MB and two times for NB. Thus, the results confirm the potential use of peroxidase of radish immobilized on coconut fiber in the biodegradation of dyes.

Keywords: Radish, Coconut Fiber, Immobilization, Biodegradation, Textile Dyes

1. Introduction

Oxidoreductases are the most studied class of enzymes for wastewater treatment. These enzymes catalyze the redox biodegradation of many harmful organic pollutants [1]. Within this class, there are peroxidases, that dynamize a complexity of reactions such as reduction of peroxides, and such as oxidation of a multiplicity of inorganic and organic compounds [2, 3]. In this class are included vegetable peroxidases, classified as heme-peroxidases. Vegetable peroxidases are heme proteins enfold in the oxidation of a diversity of inorganic and organic substrates by organic peroxides as the terminal oxidants [4]. Among vegetable peroxidases, the horseradish peroxidase extracted from *Armoracia rusticana* is the most studied enzyme [4, 5]. However, the high cost of horseradish peroxidase has

encouraged the search for alternative sources for plant peroxidases and their applications. In this sense, peroxidase from radish (*Raphanus sativus* L.) may be an option to horseradish peroxidase. Also belonging to the *Brassicaceae* family, radish peroxidase presents a high degree of similarity to horseradish peroxidase (HRP) [6]. Radish peroxidase has 70% similarity to the primary amino acid sequence of HRP [7]. Moreover, radish is one of the most widely cultivated vegetables in the world, especially in East Asia [8, 9], and has favorable agronomic characteristics, such as high resistance to climatic conditions, short cultivation time, and small acreage [7]. However, there is a growing body of literature. This shows that peroxidase is promising for the biodegradation of organic pollutants. Rathner et al., [10] showed the ability of radish peroxidase to degrade more than 90% of 17α-ethynylestradiol (EE2). Studies report the use of vegetable peroxidases, such as: turnip peroxidase for analytical and constituent of diagnostic kits [11, 12] and fuel cells [13], peroxidase from guinea grass leaves as a biosensor construct [14, 15], enzyme immunoassays [16], potato cellulose and mesquite (*Prosopis juliflora*) peroxidases for bioremediation of phenolic compounds [17-19], radish and turnip peroxidases in organic synthesis [7], potential therapeutics for bladder cancer cells [20], and bioremediation and treatment of wastewater using dyes in their reactions [1, 7, 21-23].

Some synthetic dyes are widely used in the textile, cosmetic, paper, drug farming, plastics and food processing industries. Among these, textile dyeing and finishing industries are one of the main polluting industries that consumes a large amount of water [24]. Most dyes are carcinogenic, harmful (to human health), and hard to be biodegraded due to their complex chemical structures and toxic breakdown products). Some of these dyes, such as anthraquinones, are aromatic compounds. Aromatic compounds, including aromatic phenols and amines, are among the new and persistent pollutants [25]. The discharge of dye-containing wastewater into water bodies can cause the reduction in water transparence and oxygen transfer rate into water, adversely influencing photosynthesis by plants in the aquatic systems [8] that can cause an environmental impact related to eutrophication [26, 27]. Enzymatic treatment in industry has been suggested by several researchers because most aromatic compounds in wastewater are toxic and need to be modified/removed before disposal into the environment [25].

Enzymes have the ability to degrade complex structures and a large number of toxic and persistent substances, such as textile dyes [9, 27]. However, a considerable obstacle is the high cost of enzymes. That way, efficient enzyme extraction and immobilization on appropriate supports thus enable their reuse after a series of reaction cycles, reducing costs in industrial applications and allowing process control, becoming an alternative to conventional wastewater treatment processes [28, 29].

Enzyme immobilization can provide stability to the biocatalyst, and advantages of long-term operational, easy recovery and reuse in industrial applications which enhance the process performance and lower the overall cost [23, 7]. One way to achieve this goal is to use a carrier with favorable physical and chemical properties (active groups for enzymatic interaction, pore size, specific area, and internal morphology) to enhance protein-carrier interactions. A successful process of enzyme immobilization involves the maintenance or conformational change of the enzyme structure to a more active form. In this way, catalytic activity must be maintained or increased [7, 30, 31]. Enzyme immobilization can be conducted by encapsulation, covalent bonding, adsorption (chemical or physical) and others [32-35]. Physical adsorption (PA) is one of the simplest methods to immobilize enzymes and not easily change the active site. The binding of the enzyme on the surface of particles involving only weak interactions such as hydrogen bonding, hydrophobic bonding and van der Waals forces [34, 36]. When choosing a carrier for enzyme immobilization, aspects of physicochemical stability, sustainability, and cost-effectiveness must also be considered [37-40]. The literature reports several studies using the method of physical adsorption for immobilization of enzymes in organic, inorganic and some in hybrids [7, 9, 20, 32, 41-43].

Coconut fiber is an abundant product that can be considered as a carrier for the immobilization of enzymes, since derivatives with excellent properties have been produced. It is an important bioresidue in the global agricultural industry [44, 45]. The use for such a purpose increases the product value and helps to improve the cost-benefit ratio of the enzyme immobilization. In this sense, immobilized enzymes offer advantages for industrial applications, such as improved pH and thermal stability, ease of product separation, and reuse of the derivative for multiple cycles [46]. Coconut fiber is a lignocellulosic material with high strength and durability. This is mainly due to its high lignin content compared to other natural fibers [47]. However, coconut fiber pretreatment is recommended for further use as a support for immobilization of enzymes.

The use of different types of biomasses with chemical pretreatment as a support for the immobilization of enzymes and cells has a purpose of industrial application [48, 49]. The alkaline pretreatment of the fiber compounds such as sodium hydroxide (NaOH) causes volumetric expansion of biomass, improving the internal surface favoring the reactions that reduce the degree of polymerization and crystallinity of cellulose, exposing hydroxyl groups transformed into alkoxides [49, 50]. According to Almeida et al., [51] and Arsyad et al., [52], coconut fiber content after the alkaline treatment resulted, in terms extractives of 3.1%, mineral material (ash) 6.8%, and lignocellulosic composition (20.3% of hemicellulose, 32.2% lignin and 37.3% cellulose). As support in the immobilization of enzymes, this represents an increase in the accessibility of the enzymes to the reactive groups present in the lignocellulosic material [53], due to the availability of functional groups for the interaction of the enzyme to the support in the immobilization methods [54].

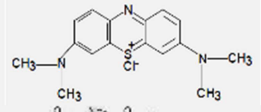
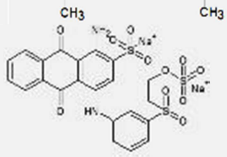
Therefore, the present study aims to evaluate the textile dye biodegradation process using enzyme peroxidase from radish (*Raphanus sativus* L.) immobilized by physical adsorption in support organic of coconut fiber.

2. Methodology

2.1. Materials

The radish was obtained with degree of maturity in the local market (Aracaju-SE). The vegetable was sanitized, peeled, fractionated in 250 g portions, and conditioned at −10°C. Coconut fiber was provided by the Brazilian Company of Agropecuária (EMBRAPA, Coastal Tabuleiros) of the state of Sergipe/Brazil produced and grown in the South Coast Region of the State. Navy blue dye was supplied by Santa Monica Textile Industry, city of Estância/Sergipe, and methylene blue dye was purchased from Vetec Brazil. The chemical structure of the dyes is shown in Table 1. All reagents used in this study were of analytical grade and commercially available.

Table 1. *Chemical structures of methylene blue (MB) and navy blue (NB) dyes.*

Dye	Structural Formula	Molecular Formula	Molecular Weight (g/mol)
Methylene Blue (Cationic) – MB		$C_{16}H_{18}N_3SCl$	319.85
Navy Blue (Reactive) – NB		$C_{22}H_{16}N_2Na_2O_{11}S_3$	626.55

2.2. Materials

2.2.1. Peroxidase Crude Extract Preparation

The peroxidase extraction was executed out according to the proceedings described in the literature by Fricks [55]. To obtain the crude extract (CE), radish samples (250 g) were homogenized in an industrial blender with 100 mL of anhydrous dibasic sodium phosphate buffer (Na_2HPO_4), 100 mM, pH 6.5. The extract was then filtered and centrifuged at 6500 rpm for 30 min at 4°C. The supernatant, the crude extract containing peroxidase, was kept at 4°C for later use. The total protein concentrate in solution was stated by the Bradford method utilize bovine serum albumin as standard [56].

2.2.2. Coconut Fiber Alkaline Pretreatment

The pretreatment of the organic support was carried out according to the methodology proposed by Santos et al., [57]. 5 g of the dry support, pre-crushed in knife mill, were transferred to Erlenmeyer flasks containing 250 mL of NaOH (0.5 M) and kept under rotary stirring at 120 rpm for 24 h, then were washed with distilled water and dried at 100°C to constant weight. The pretreated fibers were sieved through a standard sieve to obtain particle size between $42 < x \leq 60$ mesh.

2.2.3. Immobilization of Radish Peroxidase (Raphanus sativus L.)

Radish peroxidase (RP) was immobilized by the physical adsorption method (PA) on pretreated coconut fiber according to the methodology proposed by the research laboratory group. The support (1 g) was suspended in anhydrous dibasic sodium phosphate buffer (Na_2HPO_4) 100 mM, pH 8.0, and kept under mechanical agitation for 15 minutes. Crude extract (1 mL) and buffer were added to the final volume of 10 mL. The system was kept under stirring for 3 hours at 25°C and later stored at 4°C for 24 h. The immobilized biocatalyst (IB) was filtered and washed with buffer to remove non-adsorbed enzyme, and the filtrate was reserved for quantifying the enzymatic activity remaining. To select the best loading for immobilization, a study was conducted by varying the loading from 2.6–6.5 mg of protein per gram of support.

2.2.4. Determination of Peroxidase Activity and Immobilization Efficiency (IE)

The enzymatic activity of radish peroxidase was determined by a colorimetric method based on the change in absorbance at 470 nm due to the formation of tetraguaiacol, the oxidation product of guaiacol, during three minutes ($\varepsilon_{tetraguaiacol}$: 26.6 mM^{-1} cm^{-1}) [58]. One enzyme unit (U) refers to the amount of enzyme capable of forming 1 μmol of product in one minute at 25°C and pH 6.0 [59]. Immobilization efficiency (IE) (%) was analyzed using equation (1).

$$IE\ (\%) = \frac{U_f - U_o}{U_f} \times 100 \quad (1)$$

where U_o is units of peroxidase activity offered and U_f is units of enzyme remaining.

2.2.5. Determination of pH and Temperature Effects on the Peroxidase Activity (Free and Immobilized Forms)

To determine the effect of pH on the peroxidase activity, assays were performed as described in the previous item in pH interval of 3.0–9.0, at 25°C. The buffers used were: citric acid ($C_6H_8O_7$)/sodium citrate dihydrate ($C_6H_5Na_3{\cdot}O_72{\cdot}H_2O$) 0.1 M for pH 3.0 and 5.0; sodium phosphate dibasic anhydrous (Na_2HPO_4) 0.1 M for pH 6.0–8.0; and borax ($NaB_4O_710{\cdot}H_2O$)/sodium hydroxide (NaOH) 0.1 M for pH 9.0.

To determine the effect of temperature on the peroxidase activity, tests were performed under optimal pH at temperatures of 25, 30, 40, 50 and 60°C. Results were expressed as relative activity, using as reference 100% the highest obtained activity.

2.2.6. Biodegradation Test of Textile Dyes

Stock solutions of the dyes methylene blue (MB) and

navy blue (NB) were prepared in distilled water at the standard concentration of 5000 ppm. For the biodegradation assays, dye solutions were used at concentrations of 20 mg/L for MB [60] and 100 mg/L for NB [61]. The dye (MB-cationic and NB-reactive) biodegradation experiments were conducted in the presence of radish peroxidase (RP) in free and immobilized form (FRP and IRP) in a thermostatic bath with orbital stirring at 200 rpm, with duration of 1 h at 25°C and total volume of 10 mL. All experiments were produced in triplicate. For starting control, in each experimental condition the biodegradation tests were conducted in the absence of enzyme (FRP) or only in the presence of the pretreated coconut fiber (for biodegradation catalyzed by IRP).

The discoloration of the solution was monitored by UV-Vis spectrophotometry with Biochrom Libra S22 equipment. The discoloration efficiency (DE) was determined by equation 2. The wavelengths adopted for discoloration efficiency determination were 660 and 625 nm respectively, for MB and NB, according to the literature Celebi et al., [61] and Pereira et al., [60].

$$\text{DE}\ (\%) = \frac{A_0 - A_f}{A_0} \times 100 \quad (2)$$

where A_o is initial absorbance (control) and A_f is absorbance after treatment.

(i). Effect of pH

The effect of pH was investigated in the range of pH 3.0–9.0 for MB and NB at 25°C, at 200 rpm with duration of 1 h, molar ratio dye: H_2O_2 (1:0.5). The dye solutions (50 mmol/L) were prepared in aqueous solution. The buffers used were: citric acid ($C_6H_8O_7$)/sodium citrate dihydrate ($C_6H_5Na_3{\cdot}O_72{\cdot}H_2O$) for pH 3.0, 4.0 and 5.0; sodium phosphate dibasic anhydrous (Na_2HPO_4) for pH 6.0, 7.0 and 8.0; and borax ($NaB_4O_710{\cdot}H_2O$)/sodium hydroxide (NaOH) for pH 9.0.

(ii). Amount of Biocatalyst

The dye biodegradation was studied with different amounts of biocatalyst. For MB biodegradation the ranges were: FRP — 0.202–60.60 U and IRP — 0.65–5.20 × 10^{-3} U. For NB: FRP — 0.224–33.72 U and IRP — 5.25–42.00 × 10^{-3} U.

(iii). Effect of Molar Ratio Dye: Hydrogen Peroxide (H_2O_2) for Immobilized Biocatalysts (IRP)

The molar ratio (MB and NB) dye: hydrogen peroxide was determined from the best condition in the previous items (subsection i and ii) for IRP at 25°C for 1 h of reaction at 200 rpm. From the initial condition, the dye: H_2O_2 concentration varied in the range 1:0.1–1:100 mmol/L for MB and NB.

(iv). Contact Time for Immobilized Biocatalysts (IRP)

The immobilized radish peroxidase biocatalysts were incubated at 25°C in a thermostatic bath with 200 rpm orbital shaking, under optimized conditions from previous items (subsections i – iii). Samples were withdrawn at different time intervals (0–12 h) to determine the discoloration efficiency of MB and NB.

(v). Analysis by Fourier Transform Infrared Spectroscopy (FTIR)

The samples were submitted to FTIR (Agilent Cary 630 FTIR spectrophotometer), and spectra were obtained in the range of 4000 to 650 cm^{-1}, to identify the compounds present in the support and immobilized biocatalysts.

2.2.7. Operational Stability

The experiments were performed repeatedly using the same IRP sample for 1 h under optimized conditions in previous items (subsections i – iii). Once the reaction is complete, the IRP is separated from the mixture, washed with buffer of the best pH condition and used again in another biodegradation reaction. The discoloration of the dye was monitored by UV-Vis spectrophotometer in the colorant wavelength used.

2.2.8. Statistical Analysis

All experiments were performed in triplicate, and experimental results were expressed as standard error of the mean (mean ± S.E.) and presented as error bars in the figures using Origin 2022 software. Data were analyzed for statistical significance using analysis of variance (ANOVA). The significance level was evaluated $p < 0.05$, considering that lower values would correspond to the existence of significant differences. The statistical software used was Minitab (version 2021).

3. Results and Discussions

3.1. Obtaining of Radish Peroxidase and Determination of Peroxidase Activity

The results of obtaining the crude radish extract and determination of the peroxidase activity are shown in Table 2. The activity of radish peroxidase was determined in anhydrous dibasic sodium phosphate buffer (Na_2HPO_4), 0.1 M and pH 6.5 as described by Fricks et al., [55]. The concentration of protein determined in the crude extract was 0.65 mg/mL, which was twice as high as that obtained by Fricks et al., [62] (0.33 mg/mL), and total protein (mg) of crude extract containing the peroxidase was 113.75 mg, indicating a satisfactory result compared to 21.6 mg total protein determined by Melo et al., [5]. Possibly due to the edaphoclimatic conditions of each region, there was a significant difference in the results described by Dos Santos et al., [63], Fricks et al., [62], and Melo et al., [5].

Table 2. *Determined parameters of the crude extract of radish prepared with anhydrous dibasic sodium phosphate buffer.*

Parameters	Crude Extract
Volume (mL)	175
Concentration Protein (mg/mL)	0.65
Total Protein (mg)	113.75
Enzymatic Activity (U/mL)	0.63
Specific Activity (U/mg)	0.97

3.2. Immobilization of the Radish Peroxidase (RP) on Coconut Fiber by Physical Adsorption

The effect of protein loading on a carrier for the purpose of enzyme immobilization should be evaluated, especially when different carriers and techniques are studied. For the immobilization of radish peroxidase in coconut fiber by physical adsorption, the effect of the loading of the crude extract of 2.6–6.5 mg protein per g support was studied, the results of which are presented in Figure 1. All immobilization efficiencies were statistically significant ($p < 0.05$).

As observed (Figure 1), the maximum immobilization efficiency was around 70 ± 3.8% for the loading of 5.2 mg protein per g support. The result of immobilization of crude radish peroxidase on coconut fiber support by physical adsorption was promising compared to the literature. Barbosa et al., [7], worked with immobilized enzyme when presented a 48.15% in physical adsorption using coconut fiber as organic support. Appropriate physical immobilization and adsorption techniques can optimize the catalytic performance of immobilized enzymes. For higher loadings, a decrease in the immobilization efficiency was observed, probably due to saturation of the enzyme on the support [64]. In addition, excessive protein loading (including the enzyme to be immobilized and other protein molecules) may block substrate access to the enzyme active site and reduce the activity of the immobilized system [31].

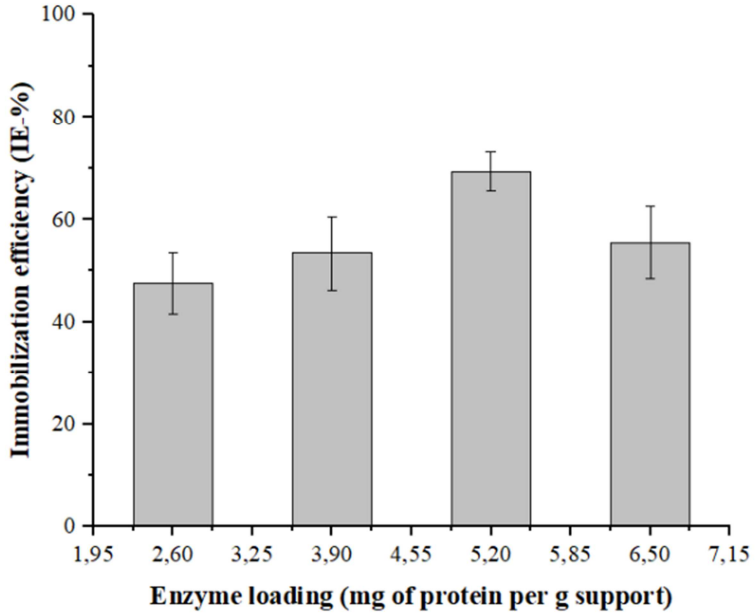


Figure 1. *Immobilization efficiency of radish peroxidase on coconut fiber support by physical adsorption as a function of enzymatic loading.*

3.3. pH and Temperature Effects on the Peroxidase Activity

The catalytic activity of an enzyme is affected by pH because the enzyme-substrate interaction can be altered. The influence of pH on immobilized enzymes needs to be evaluated with respect to possible application on an industrial scale [65]. The effect of pH in the range of 3.0–9.0 was evaluated on the activity of the free enzyme extracted from the radish and immobilized by physical adsorption, as shown in Figure 2. All relative activities for pH were statistically significant ($p < 0.05$). Both free and immobilized biocatalysts exhibited a greater activity at pH 7.0, maintained activity above to 95%. This result indicates the similarity of biochemical properties and stability of these enzymes. Moreover, according to the literature, most peroxidases show optimum activity at acidic pH [66]. At acidic pH, radish peroxidase showed a decrease in activity, possibly due to ionic alteration of the enzyme, and consequently its active site. The reduction of activity can also be observed at pH 8.0 and 9.0. Mohamed et al., [64] describe that considering plant peroxidases, the optimal pH recurrently increases, and horseradish immobilized in wool showed an optimal pH at 7.0. According to Chagas et al, [59], the microenvironment of the immobilized enzyme and the buffer solution usually have an uneven distribution of H^+ and OH concentrations due to electrostatic interactions with the matrix, which often leads to shifts in the pH activity profile. Similar results were reported by Malani et al., [67] to verify the optimum pH of horseradish peroxidase in the free and immobilized forms through polyethylene glycol, obtaining a pH at 7 for both. The reduction in enzyme activity observed at extreme pH values may be due to denaturation of enzymes by ionization [68].

The relative activity as a function of temperature for free and immobilized radish peroxidase was determined in a range of 25–60°C as shown in Figure 2. The maximum activity occurred in the range of 25–40°C for the immobilized biocatalyst, while the free enzyme reached the highest activity at 50°C. All relative activities for temperature were statistically significant ($p < 0.05$). In general, the immobilization allowed action of horseradish peroxidase in a wider temperature range (25–40°C). Immobilization of an enzyme can increase its thermal stability and preferably exhibit high catalytic activity over a wide

temperature range [7]. However, elevated temperatures within the experimental range studied (50–60°C) cause a sudden decrease in enzyme activity. Contrary results were reported by Jamal et al., [69] to verify the catalytic activity of the peroxidase obtained from cauliflower (*Brassica oleracea*) in free form and immobilized on alginate gel and pectin, obtaining the highest activity at 40°C. Mohamed et al., [70] worked in optimum temperature for free radish peroxidase in 40°C, with horseradish peroxidase.

Possibly, the effect of higher temperatures (above 60°C) leads to almost 80% loss of enzyme activity, indicating that these temperatures cause denaturation of the enzyme, resulting in irreversible conformational changes that affect its activity [71].

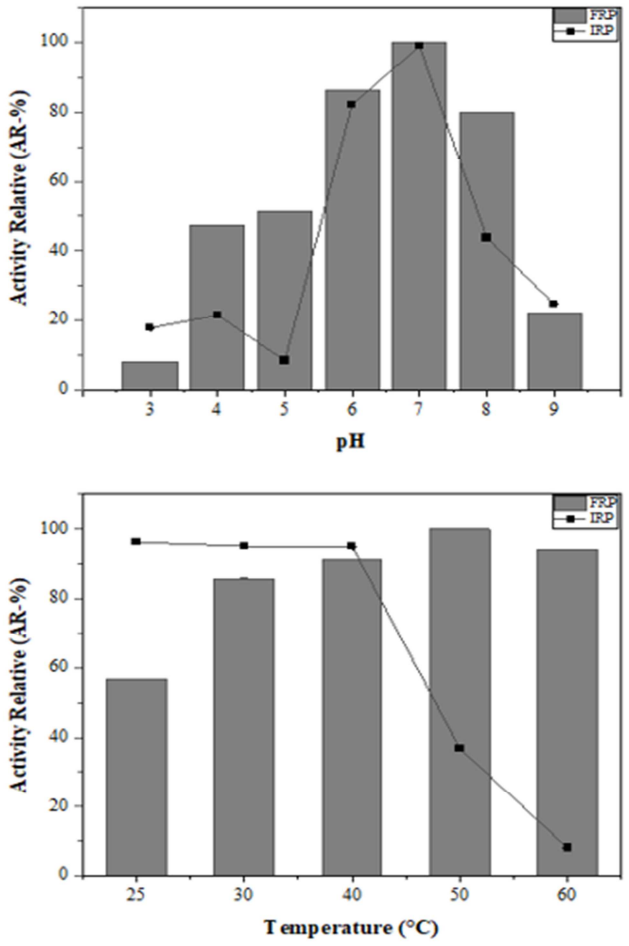


Figure 2. *Effect of pH and Temperature in the activity of free (FRP) and immobilized (IRP) radish peroxidase.*

3.4. Biodegradation of Methylene Blue and Navy Blue Dyes by Free Radish Peroxidase (FRP) and Immobilized (IRP)

3.4.1. Effect of pH on the Discoloration Efficiency by FRP and IRP

The effect of pH was investigated in the pH range (3.0–9.0) to MB and NB at 200 rpm for 1 h at 25°C, molar ratio dye: H_2O_2 (1:0.5 mmol/L) and the amount of biocatalyst to MB: FRP (20.20 U) and IRP (3.25×10^{-3} U), and to NB: FRP (22.48 U) and IRP (26.25×10^{-3} U). The effect of pH on the discoloration efficiency (DE —%) of MB and NB is shown in Figure 3. To methylene blue (Figure 3A), the free radish peroxidase (FRP) presented low values of discoloration efficiency for the pH effect between 3.0–5.0. In this pH range, the discoloration efficiency was minimal when compared to the immobilized radish peroxidase (IRP) in coconut fiber. For FRP and IRP, the highest discoloration efficiency was obtained in the pH range of 7.0 to 9.0. For the navy blue, the values shown in Figure 3B showed a significant increase in values from 3.0 to 5.0 for both forms. For higher values of the pH range (6.0–9.0), the biodegradation of the reactive dye remained almost constant.

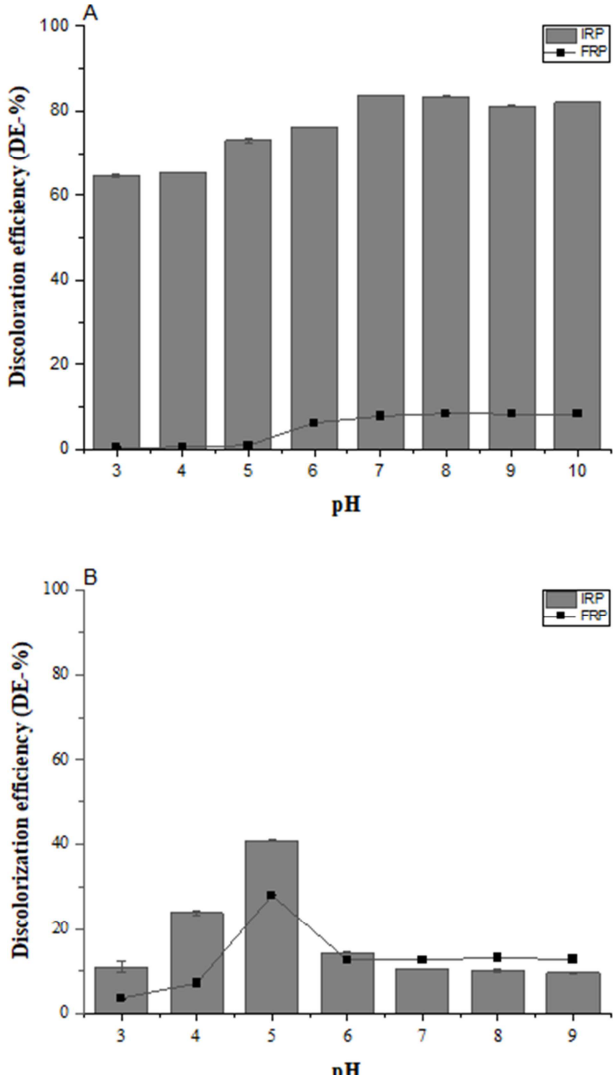


Figure 3. *Effect of pH on the discoloration efficiency of methylene blue (A), and navy blue (B) by free radish peroxidase (FRP) and immobilized (IRP).*

According to CONAMA [72], the Brazilian legislation currently in force regulates the conditions and standards for effluent releases. The effluents from any polluting source can only be released directly into the receiving body when complying with the conditions and standards set forth in article 16, section II (Effluent Release Conditions and Standards), where the pH limit is in the range 5.0 to 9.0, and

with other applicable requirements, such as temperature, sedimentary materials, oils and greases and BOD. The results show maximum degradation of dyes occurred at pH 8.0 for methylene blue with discoloration efficiency of 8.5% (FRP) and 83% (IRP), and at pH 5.0 for navy blue with discoloration efficiency of 28% (FRP) and 40% (IRP).

According to Pereira et al., [60], pH has a greater influence on the degree of ionization of existing groups of amino acids in the enzyme or even in its catalytic action. The pH can influence both the enzymatic structure and the substrate itself that can be strongly affected by the concentration of H^+ ions, depending on the pK of each substrate grouping, which may result in a greater or lesser interaction with the enzyme.

The discoloration efficiency for methylene blue catalyzed by FRP and IRP was higher at pH above 5.0. Radish peroxidase presents isoelectric point (pI) between 4.7–5.1 [6, 73]. Thus, at pH greater than 5.0 the anionic fraction of the enzyme is favored, therefore with greater potential to oxidize MB that presents cationic characteristics [61]. The reactions that make up the catalytic cycle of vegetable peroxidase involve oxidation of the heme prosthetic group of peroxidase by hydrogen peroxide by two electrons and the formation of an intermediate compound with positive characteristics (oxyferryl — $O=Fe_5^+$). Possibly, this cation induces biodegradation of the cationic dye [74].

IRP showed higher MB and NB biodegradation power compared to FRP due to the presence of hydroxyl groups in the immobilized biocatalyst. The hydroxyl groups collaborate in the orientation of the substrate in the interaction with the active site, which justifies the better IRP performance in the biodegradation of MB and NB [75].

According to Ali et al., [76] where the ginger peroxidase was polypyrrole-zirconium (IV) selenoiodate cation exchanger nanocomposite, the pH effect on the cation type dye degradation process may be added to changes in the immediate microenvironment of the immobilized enzyme, which depends on the surface and residual charges of the immobilization support.

Although more modest results in terms of DE were obtained with NB, the maximum biodegradation of NB by FRP and IRP occurred at pH 5.0. NB is an anthraquinone anionic dye [61]. Regarding biodegradation of NB, Dehvari et al., [77] describes that the presence of hydroxyl groups at high pH values increase the negative charge of the dye, thus decreasing the removal efficiency. As previously mentioned, at pH 5.0 the anionic form of the radish peroxidase is favored. However, the interactions with the anionic dye are not favored in these conditions, thus reducing the degradation power of the enzyme. Similar results were mentioned in the literature. Celebi et al., [61] studied the biodegradation of reactive blue 19 (navy blue) dye catalyzed by horseradish peroxidase in the free form. The enzyme was inhibited at pH values (6.0–9.0) and showed maximum removal at pH 5.0. Champagne and Ramsay [78] studied the degradation of the reactive blue 19 dye by laccase immobilized on silica, and the best result was obtained at pH 5.0. Mahmoodi et al., [33] reported that discoloration using laccase immobilized on nanoparticles of manganese ferrite immobilized on discoloration dye acid blue 92 with anionic characteristics showed a degradation at pH 5.0.

3.4.2. Effect of the Amount of Biocatalyst on the Discoloration Efficiency by FRP and IRP

The effect of the amount of biocatalyst on the discoloration efficiency (ED —%) of methylene blue and navy blue (Table 3) was investigated in the range of: 0.202–60.60 U (FRP) and 0.65–5.20 × 10^{-3} U (IRP) for MB, and 0.224–33.72 U (FRP) and 5.25–42.00 × 10^{-3} U (IRP) for NB, in 200 rpm for 1 h at 25°C, pH 8.0 (MB) and pH 5.0 (NB) and molar ratio dye: H_2O_2 (1:0.5 mmol/L) to both.

Table 3. *Effect of the amount of the biocatalyst on the discoloration efficiency of methylene blue and navy blue by free and immobilized radish peroxidase.*

Methylene blue – MB			
FRP (U)	**DE (%)**	**IRP (U×10^{-3})**	**DE (%)**
0.202	1.30±0.30	0.65	41.65±0.10
0.404	2.26±0.15	1.30	67.64±0.11
20.20	7.01±0.48	1.95	82.37±0.09
24.20	9.18±0.45	2.60	86.13±0.30
28.28	10.27±0.24	3.25	87.36±0.05
32.32	12.57±0.01	3.90	87.10±0.06
36.36	13.22±0.09	4.55	88.73±0.07
40.40	26.27±0.13	5.20	88.88±0.05
60.60	17.04±0.06		

Navy blue – NB			
FRP (U)	**DE (%)**	**IRP (U×10^{-3})**	**DE (%)**
0.224	39.77±0.03	5.35	47.81±0.02
1.12	56.75±0.08	10.50	55.14±0.19
2.24	58.52±0.13	15.75	57.72±0.05
4.49	58.80±0.01	21.00	60.15±0.27
5.99	59.32±0.38	26.25	61.65±0.28
13.45	62.15±0.28	31.50	57.29±0.38
17.98	61.34±0.07	36.75	54.13±0.70
22.48	61.81±0.12	42.00	47.92±0.76
33.72	60.50±0.52		

The amount of biocatalyst for the methylene blue dye presented in its free form a discoloration efficiency (26%) with 40.40 U of the biocatalyst, and in the immobilized form the highest DE (87%) was reached with only 3.25 × 10^{-3} U of IRP.

For the navy blue dye (NB), we can verify that the effect of the amount of the FRP obtained a maximum discoloration efficiency of 62% with 13.48 U of biocatalyst, remaining almost constant from that point, whereas for IRP the maximum DE was similar (61%) with 26.25 × 10^{-3} U of biocatalyst, observing a slight decrease from that point.

MB-FRP, the low biodegradability of the dye may be related to the specificity of the enzyme in relation to factors that influence the rate of reaction, such as enzyme concentration, molar ratio dye: H_2O_2 and/or the presence of mediators [74]. While for MB-IRP, good biodegradability can be attributed to the reduction of convection diffusion limitation due to structural changes often caused by the immobilization process [79]. Similar results were found in Boucherit et al., [80] using peroxidase from zucchini (*Cucurbita pepo*) immobilized by entrapment in calcium

alginate beads on the degradation of direct azo dye, finding a removal of 76%.

Forgiarini and Souza [81] reported similar results to NB-FRP when using free horseradish peroxidase in the degradation of textile dyes in the range of 2.985 to 29.85 U, achieving the highest efficiency (58%) with 14.99 U of biocatalyst, according to the authors. Similar results to NB-IRP were described by Satar and Husain [82], ranging from 0.1 to 1.0 U of white radish peroxidase immobilized on Celite in the degradation of reactive blue red 120 and 171 dyes, obtaining an efficiency of around 60%.

The optimization of the amount of enzyme was carried out with the objective of high efficiency of discoloration with less amount of enzyme used in the process (IRP). From these results, it was found that often, using a higher enzyme concentration (in the case of FRP), the staining of the dye was not significantly influenced. Thus, we can verify that with a minimum amount of enzyme immobilized on a support (IRP), we can obtain practically the same discoloration efficiency as with a much larger amount of enzyme in its free form (FRP). Consequently, this reduces process costs and achieves reuse through immobilization.

3.4.3. Effect of Molar Ratio Dye: Hydrogen Peroxide (H_2O_2) for Immobilized Biocatalyst (IRP)

Hydrogen peroxide (H_2O_2) acts as a co-substrate in reactions catalyzed by peroxidase [83] in the oxidation of proton donor substrates in the presence of H_2O_2 solution [84]. H_2O_2 is environmentally friendly, as its degradation products are only water and oxygen, with no hazardous residues [85]. Thus, the effect of molar ratio dye: H_2O_2 was investigated in the range (1:0.1–1:100 mmol/L) at 200 rpm for 1 h at 25°C, to methylene blue: pH 8.0 and amount of biocatalyst IRP (3.25×10^{-3} U), and to navy blue: pH 5.0 and amount of biocatalyst IRP (26.25×10^{-3} U), as described in Figure 4.

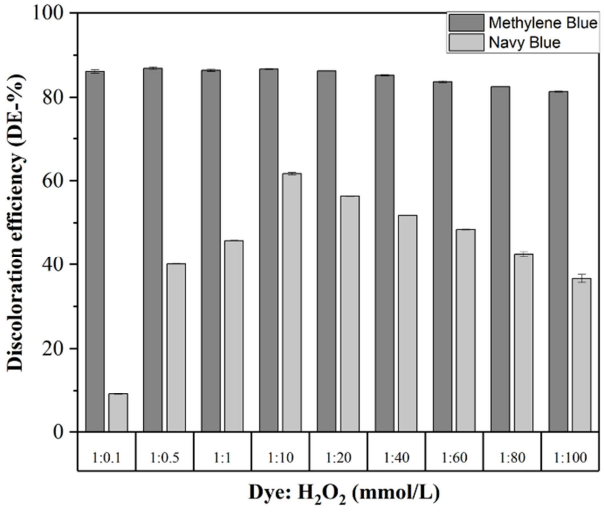


Figure 4. *Effect of molar ratio dye: H_2O_2 on the discoloration efficiency by immobilized radish peroxidase of methylene blue and navy blue.*

In Figure 4 for the MB dye, we can observe that the increase in hydrogen peroxide concentration did not significantly influence the biodegradation process. Therefore, the molar ratio MB: hydrogen peroxide, 1:0.5 mmol/L, corresponding to 86.8% discoloration efficiency, was selected.

For the NB dye, the discoloration efficiency increases with the addition of H_2O_2 from 0.1 to 10 mmol/L, with a decrease in its efficiency thereafter. Thus, the molar ratio selected was 1:10 mmol/L for IRP, with a discoloration efficiency of 61%.

For Boucherit et al., [80], the high concentration (values greater than 1:10) of H_2O_2 acts as an irreversible inhibitor of the peroxidase activity of the ferric group of the oxidant enzyme. Thus, immobilization has the advantage of protecting the enzyme from high concentrations of peroxide. Hydrogen peroxide function as a co-substrate to intensify the enzymatic action of the radical.

3.4.4. Effect of the Contact Time

The effect of the contact time to immobilized biocatalyst (IRP) was investigated in the range (0–12 h) at 200 rpm at 25°C and in defined conditions in previous items (subsection 3.4.1–3.4.3), as shown in Figure 5 for both dyes.

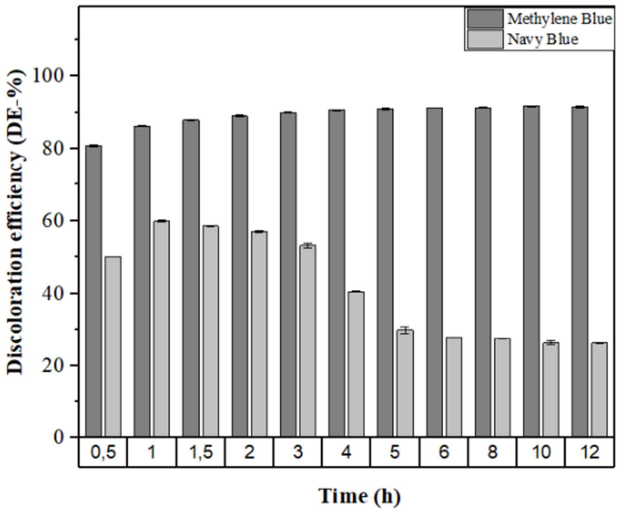


Figure 5. *Effect of the contact time on the discoloration efficiency by immobilized biocatalysts of methylene blue and navy blue.*

The immobilized biocatalyst used in the degradation of cationic and reactive dyes showed different behaviors with contact time. For the cationic dye (MB), the influence was not significant for the discoloration efficiency with increasing contact time. For the reactive dye (NB), the highest discoloration efficiency was achieved with 1 h of contact (61%), with a decrease in efficiency with increasing contact time. Therefore, for both dyes reaction time was set as 1h.

The results obtained for the methylene blue dye were similar to those obtained by Mohan et al., [86], who reported 90% ED in 1 h for diazo dye of BX black acid by strong root peroxidase immobilized on acrylamide gel support.

For the navy blue dye, the literature discusses in Sun et al., [87] that when horseradish peroxidase was immobilized on the calcium alginate beads, significantly high reaction time (240 min) was required to degrade the acid blue 113.

Normally, it is expected that the enzyme immobilization causes a stabilizing effect restricting the unfolding process of

the protein as a result of random intermolecular forces [86]. An investigation of reaction time is always necessary because it will be helpful to determine the shortest time necessary for obtaining the highest discoloration and so enhancing cost-effectiveness [87].

3.5. FTIR Spectra Analysis

The assay Fourier transform infrared spectroscopy (FTIR) is one of the most commonly used methods for the chemical characterization of natural fibers. It allows to identify absorption bands characteristic of functional groups. The FTIR spectra of the free radish peroxidase, treated coconut fiber, immobilized biocatalyst (IB), IB after treatment of methylene blue and navy blue, and the pure dye methylene blue and navy blue are shown in Figure 6.

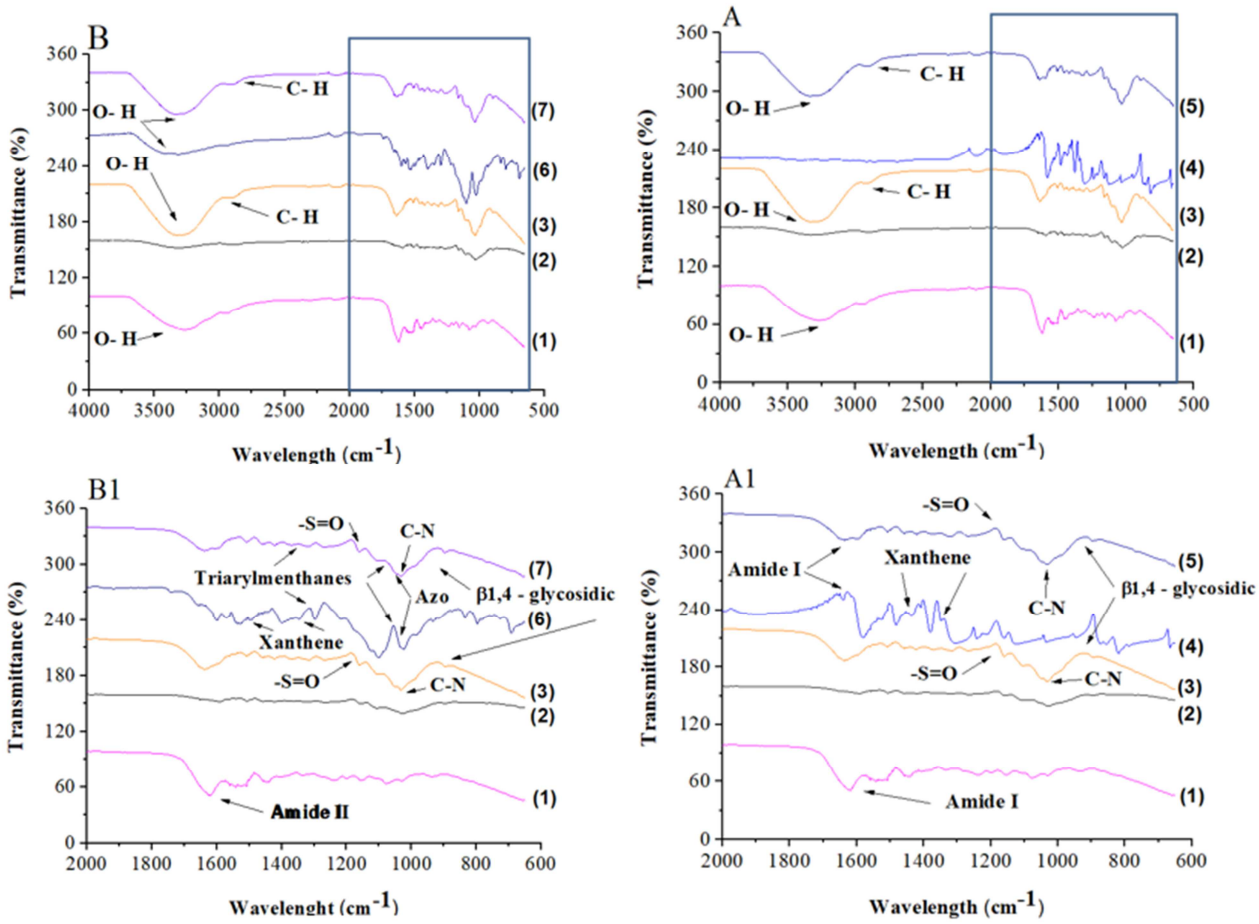


Figure 6. *Infrared spectrum (FTIR) (4000–650 cm^{-1}) for dye biodegradation by radish peroxidase (A) dye methylene blue (MB) and (B) dye navy blue (NB). Free radish peroxidase (1), coconut fiber treated (2), immobilized biocatalyst (IB) (3), MB (4), IB after MB biodegradation (5), NB (6) and IB after NB biodegradation (7). Scaling up of the FTIR (2000–650 cm^{-1}) is shown in the spectra A1 and B1.*

The expressed bands at 3500–3000 cm^{-1} are associated with the hydroxyl group stretching vibrations (O–H) in the composition of the free enzyme (1), immobilized biocatalyst (3), navy blue dye (6), IB after treatment of MB (5) and NB (7) in Figures 6A and B [54]. The bands 3000–2800 cm^{-1} show an elongation of groups of methyl and methylene (C-H) in cellulose, hemicellulose in the samples of the immobilized biocatalyst (3), IB after treatment of MB (5) and NB (7) in Figures 6A, A1, B and B1 [85]. In the 910 cm^{-1} bands, it presents characteristics of ß1,4-glycosidic bonds between the anhydroglucose units in the cellulose present in the immobilized biocatalyst (3), IB after treatment of MB (5) and NB (7) (Figures 6A, A1, B and B1) [88].

The spectrum of free radish peroxidase (1) (Figures 6A, A1, B and B1) showed characteristic absorption bands of the protein, 1617 cm^{-1} amide I related to the α-helix structure [89], 1600 cm^{-1} amide II, corresponding to the N-H bending and C-N stretching attributed to the peptide bond of the enzyme [90].

The spectra of the treated coconut fibers showed typical vibrational bands of the lignocellulose fraction, and an absorption band at 897 cm^{-1} was observed during expansion. This band is attributed to C–O–C stretching, which relates to glycosidic bonds between the sugar units of the cellulose structure [91]. In addition, bands at 1160 cm^{-1} related to asymmetric C-O-C stretching [35] and a more intense band at 1028 cm^{-1} associated with C–OH stretching vibration were observed [91]. These results indicate the efficiency of alkaline treatment of coconut fibers in terms of lignocellulosic material digestion [92]. The absorption bands in the spectrum of immobilized biocatalyst (3) and the IB after treatment of the

dyes (5 and 7) presented a band at 1200 cm^{-1} due to the stretching vibrations (S=O), as well as a stretching vibration at 1045 cm^{-1} for nitrile functional groups (C-N) [93].

The classification between the different dyes is possible due to the specific groups in each molecule whose characteristic bands are identified in the spectra of pure dyes. The bands 1450–1460 cm^{-1} and 1340–1360 cm^{-1} are related to the xanthene molecule, which is an organic compound derived from fluorescein, eosins and rhodamines, which are structures related to the color intensity of the dyes methylene blue (4) and navy blue (6) (Figures 6A, A1, B and B1), respectively. After treatment with the IB of the dyes MB (5) and NB (7), the characteristic bands of the xanthene molecule disappeared (Figures 6A, A1, B and B1), indicating the biodegradation of these dyes. In the bands around 1160–1170 cm^{-1} and 1360–1370 cm^{-1}, triarylmethanes were identified as characteristic of pure NB dye (6) and after degradation with IB of the dye navy blue (7) the disappearance of these bands also was observed (Figures 6B and B1). The results accord to those of Prati et al., [94]. The band at 1100 cm^{-1} corresponds to pure dyes such azo as navy blue (6), and after degradation with IB (7), the decrease in band intensity characteristic of these dyes are observed in Figures 6B and B1. The band at 1649 cm^{-1} indicates the presence of amide I and/or amines of the pure methylene blue dye (4). After degradation with IB, this characteristic band was not observed (5). This fact confirms the efficiency of discoloration of the dyes after the proposed treatment (Figures 6A and A1). Therefore, the use of the infrared spectral analysis (FTIR) of the characteristic bands of dyes before and after degradation corroborate to the data obtained in the discoloration efficiency for similar dyes as described in the literature by Boucherit et al., [80].

3.6. Operating Stability of Methylene Blue and Navy Blue Dyes

Reuse is one of the main indicators to evaluate the application of immobilized enzymes in industries. The immobilized enzyme can be easily removed and evaluated for its efficiency [95]. The possibility of reuse lowers the cost in bioprocesses and consolidates the stability of the immobilized enzyme [96, 97]. Reuse of immobilized enzyme by physical adsorption on an organic carrier of coconut fiber, removing phenolic compounds in cationic (MB) and reactive (NB) dyes, was investigated. All discoloration efficiency in the two dyes were statistically significant ($p < 0.05$). Figure 7 shows the IRP performance used in the biodegradation of MB and NB dyes. The MB dye was reused ten times maintaining more than 50% of the initial efficiency, whereas for the NB dye there were only two reuse cycles. These results show that the higher discoloration efficiency obtained for MB can help improve the operational stability of bioprocesses. For dyes NB, immobilized by physical adsorption techniques, weak interactions are present that can lead to leaching of the enzyme adsorbed on the support, resulting in low operational stability [98]. Therefore, the IRP proved to be more vigorous in substituting the NB to MB dyes, as the immobilized system is easier to operate to cationic dye (basic and non-anionic) and can be reprocessed multiple times with the possibility of being used in industrial scale processes.

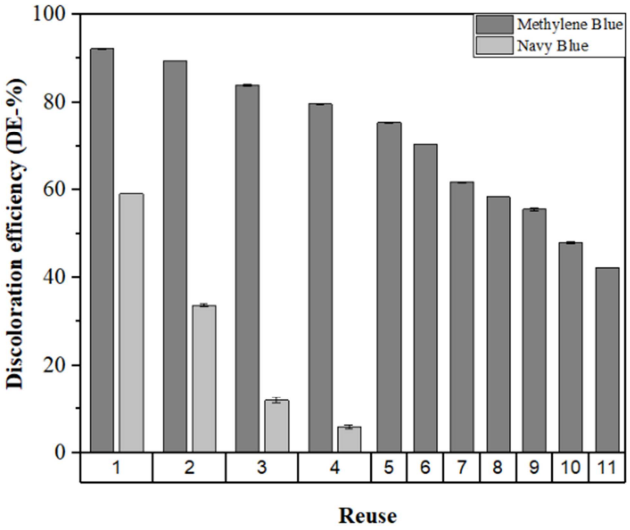


Figure 7. *Reuse cycles of immobilized radish peroxidase biocatalysts in the degradation of cationic dye methylene blue (A) and reactive dye navy blue (B).*

In the biocatalysts immobilized by physical adsorption, the operational stability was characterized by low reuse capacity with activity greater than 50% of the initial cycle. The low tolerance to the reuse cycles in the immobilized biocatalyst obtained by the physical adsorption technique can be attributed to the desorption of the enzyme due to weak bonds or the support [99]. This study also investigated the possibility of the influence of dye degradation by anionic charge of the reactive dye (NB). Other factors are that the operational stability data confirm the higher values of discoloration efficiency; the decrease in biocatalysts in subsequent cycles of reuse is likely due to denaturation of the enzyme carrier dye [100].

The efficiency reduction profile may be due to the smaller number of units (10^{-3} — U) of the biocatalyst used in the immobilization process. In some other cases, the loss of discoloration efficiency in the subsequent cycles can be attributed to the mass transfer limitation, the loss of catalytic activity that occurs during repetitive use. This fact too can be attributed to the enzymatic inactivation by the reaction and/or can be attributed to blocking enzymes inactivated by accumulation of polymerization products within immobilized peroxidase particles [101].

4. Conclusion

The crude extract obtained from the radish immobilized by the method of physical adsorption in support of coconut fiber presents potential for use in biodegradation processes of textile dyes. The highest immobilization efficiency (70%) was observed using 5.2 mg protein per gram support. For the biodegradation of methylene blue, the higher discoloration efficiency (87%) was obtained for IRP compared to FRP (DE = 26%). While for navy blue, practically the same discoloration efficiency (62%) was obtained for both biocatalysts. The results obtained in the present study revealed

the efficacy of radish peroxidase in the discoloration of cationic and reactive dyes, mainly the immobilized form, because a small amount (U) of crude extract compared to the free form is required, as well as the advantage of reuse. The performance in the biodegradation of methylene blue using FRP and IRP was highly dependent on the pH and amount of the biocatalyst, whereas for navy blue it was dependent on pH, molar ratio to H_2O_2, amount of biocatalyst, and time of contact. Therefore, radish peroxidase immobilized on coconut fiber can become an efficient and economical alternative in the development of an effective biotechnological process for the removal of textile effluents on a large scale.

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