PHENOL DEGRADATION BY CROSSLINKED ENZYMES AGGREGATES (CLEAS) FROM *BRASSICA RAPA* PEROXIDASE.

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ABSTRACT

Peroxidase from *Brassica rapa* (BRP) (EC 1.11.1.7) was immobilized as cross-linked enzyme aggregates (CLEAs) and used to treat waste water containing phenol.

Optimization of the reaction conditions of phenol degradation revealed an optimal contact time of 40 min at 90 $mg.L^{-1}$ initial phenol concentration and 2 mM of H_2O_2, leading to a maximum phenol removal yield of 90 % for 2.5 UI/mL of BRP-CLEAs.

The reusability of BRP-CLEAs was tested in recycled batches and it was demonstrated that more than 60% of their initial efficiency for phenol removal was conserved after five consecutive batches.

Keywords: Peroxidase, CLEAs, degradation, Optimization, phenol, reusability

1. Introduction

The increasing development of industrial processes generate a variety of molecules that may pollute air and waters due to negative impacts for ecosystems and humans (toxicity, carcinogenic and mutagenic properties)(Busca *et al.,* 2008). Among these pollutants, Phenol is considered as a priority pollutant. Due to its toxicity, it has been classified by numerous environmental institutions (ATSDR, 2007; NIOSH IDLH, 1997; U.S.EPA. 2003b).

The US National Institute of Occupational Safety and Health (NIOSH) fixed the concentration immediately dangerous to life or health at 960 mg/m^3 (NIOSH IDLH, 1997), while the US Environmental Protection Agency has established a provisional Reference Concentration for phenol of 0.006 milligrams per cubic meter (U.S.EPA, 2003b).phenol is released into the environment throw a variety of industrial activities such as: synthetic resins, petrochemical , petroleum refineries, , steel mills, coke oven plants, pharmaceuticals, paints, plywood industries and mine discharge (Moussavi and Mohseni,2008; Buscaa *et al.,*2008).Numerous remediation methods have been developed for the removal and decomposition of phenol, taking the example of biodegradation by microorganisms, advanced oxidation process, adsorption and finally enzymatic process.

Phenols enzymatic degradation by peroxidases in the presence of hydrogen peroxide is among the most studied subjects in recent years (Bansal and Kanwar, 2013; Narayan and Pushpa, 2012; Sukan and Sargin, 2013). The process presents many advantages such as simplicity under mild conditions, high turn-over, selectivity, less drawbacks when compared to chemical processes and lower cost. However, the commercialization of enzymatic processes is often hampered by a lack of availability, high price and/or limited stability under the operating conditions of the enzyme (Matijosyte *et al.,* 2010).Fortunately, many techniques, which showed impressive developments these last years, are available which may allow to improving enzyme features. Among the

available tools, immobilization has been revealed in the last years as a very powerful technique to improve enzyme properties (Mateo *et al.,* 2007; Hernandez and Fernandez-Lafuente, 2011).

CLEAs immobilization technique is among the recent biocatalyst advances which attracted increasing attention, due to its simplicity, broad applicability, high stability and high volume activity (Cao, 2005).The CLEAs methodology essentially combines two unit processes, purification and immobilization, into a single operation (Sheldon, 2011). So far, the CLEA technology has been applied to an increasingly wide selection of hydrolases, oxidoreductases, and lyases (Sheldon, 2011). However, no study has been reported on CLEAs of *Brassica rapa* peroxidase (BRP).

Thus the purpose of this study was to prepare CLEAs from *Brassica rapa* Peroxidase and use them as biocatalyst to treat waste water charged by phenol, the study covers initially the optimization of reaction conditions such as, reaction time, phenol and hydrogen peroxide concentration, BRP-CLEAs activity, and second their reusability in successive batches.

2. Methods

2.1. BRP-CLEAs Preparation

Cross-linked aggregates of *Brassica rapa* peroxidase (BRP) were prepared by following a standard protocol (Sheldon, 2011) (Tandjaoui *et al.,*2015).Three volumes of ice-cold Acetone were slowly added to 12 ml of crude peroxidase solutions with a specific dilution of 3/4 (V/V) with phosphate buffer. Mixture was kept under constant agitation on 300 rpm orbital shaker for 45 minutes at 4 °C. The physically aggregated proteins obtained were subject to chemical cross-linking using a given amount of glutaraldehyde (25%,v/v) which was added drop-wise to achieve final concentrations of 2 %. Aggregated enzymes (CLEAs) were finally separated using centrifugation then thoroughly washed with distilled water and stored in phosphate buffer (50 mM) pH 7 at 4°C until use.

2.2. Activity measurements

The activity of BRP-CLEAs in aqueous solution was determined spectrophotometrically at 470 nm by the standard guaiacol method (Egley *et al.,* 1983).The assay mixture consisted of 4.05µL of 98% guaiacol, 2 mg of CLEAs, 3.9 ml of phosphate buffer (50 mM, pH 7). The reaction was started by the addition of 5µl of H_2O_2 (0.8M), ending up with a volume of 4mL. The change of absorbance with time was monitored using a SHIMADZU UV–VIS spectrophotometer, model UV mini-1240. One unit (1.0 U) of peroxidase activity was defined as the amount of enzyme protein that catalyses the oxidation of 1.0 µmol of hydrogen peroxide per min at 25°C and pH 7(molar extinction coefficient, 4279 M^{-1} cm^{-1}).

2.3. Experimental protocol for phenol degradation:

Batch experiments were conducted to remove phenol by BRP-CLEAs. Reactions were carried out in 20ml volumetric vials equipped with a magnetic stirrer and closed with PTFE-coated silicone rubber septa (PerkinElmer, France) sealed with aluminium caps. Mixtures consisted of 5 ml of phosphate buffer solution (pH=7, 10mM) as reaction medium containing a specific concentration of phenol. BRP-CLEAs (5mg) were added to each vial. The reaction was initiated by adding hydrogen peroxide (5 µL) using a micro-syringe of 25 µL throw the rubber septa. The vials were maintained at room temperature (25±3) °C under magnetic stirring of 400 rpm. At the end of the reaction, mixtures were centrifuged at 5000 rpm and the supernatant was assessed for residual phenol concentration.

Aiming to maximise phenol degradation, the kinetics of phenol degradation was followed under different conditions as follows: initial concentrations of phenol (20 to 160 mg/L), hydrogen peroxide from 0.5 to 20 mM and BRP-CLEAs amount from 2.5 to 20 mg which corresponds to an activity from (0.4 to 3.5 UI/ml).

The efficiency of phenol removal from aqueous phase was evaluated by the calculation of two parameters: the conversion using the equation below (Eq. 1). The initial rate (V_o) is determined by extrapolating the slope of the time course of the substrate concentration to time zero.

$$\text{Conversion (\%)} = \left(1 - \frac{[\text{phenol}]_r}{[\text{phenol}]_0}\right) \times 100 \quad (1)$$

Where $[phenol]_0$ and $[phenol]_r$ are the initial and residual concentrations of phenol in the reaction medium respectively

2.4. Analytical methods

Residual phenol concentration was assessed using a colorimetric assay in which the phenolic compounds within a sample reacts with 4-AAP (4-Aminoantypirrine) in the presence of potassium ferricyanide reagent under alkaline conditions (Bayramoglu and Arıca, 2008; Wei *et al.,* 2013) as follows: A sample of 2.4 ml from the treated solution was withdrawn using a syringe and diluted with phosphate buffer (pH7) (phenol concentration up to 0.2 mM). The mixture was added to 0.3 ml of potassium ferricyanide (83.4 mM) and 0.3 ml of 4-aminoantipyrine (20.8 mM). After few minutes, the mixture absorbance was measured at 510 nm using an extinction coefficient $\varepsilon_{2} = 9.8\times10^{-3}$ L.mg^{-1}.

3. Results

3.1. Effect of reaction time and phenol concentration:

The kinetics of any enzymatic reaction is affected by substrate concentration. Figures 1.A; shows the evolution of phenol concentration with time. At constant peroxide (1mM) and enzyme concentration (0.82UI/ml) , the effect of initial phenol concentration (C_0) on the initial rate V_0 and conversion yield (%) , is reported in Figure.1.B It is clear that V_0 as well as the removal yield depend on initial substrate concentration (C_0).

At fixed hydrogen peroxide concentration and activity, the reaction proceeds fast and only 40 minutes were sufficient to rich the equilibrium. Longer time did not affect significantly, phenol conversion. On other hand, the curve of initial phenol concentration versus initial reaction rate V_0 shows that the rate increases rapidly and linearly at low phenol concentrations (0-100) mg.L^{-1}, but it gradually levels towards a limiting value at high substrate concentrations. Whereas conversion decrease gradually form its maximal values (69%) for high phenol concentrations.

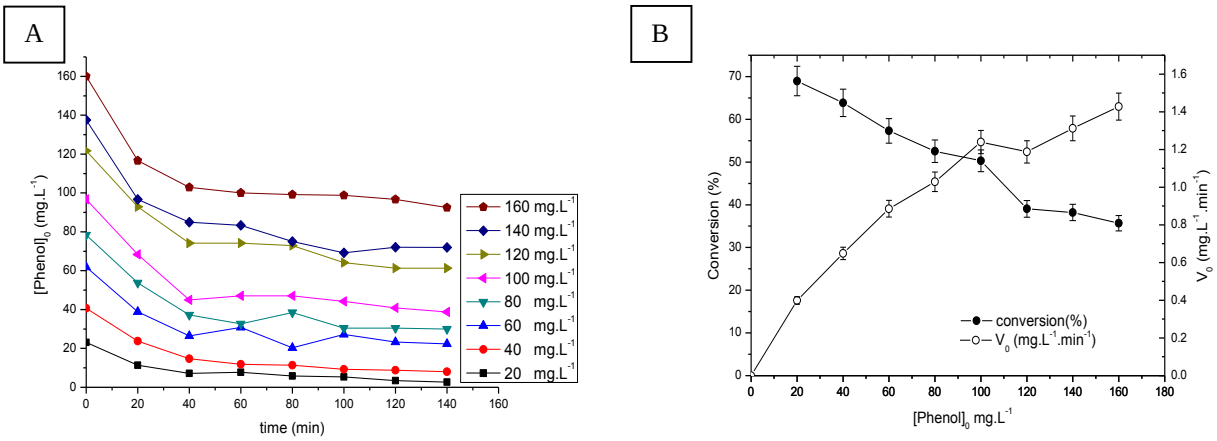

Figure.1: Effect of phenol concentration on the kinetics of enzymatic degradation by BRP-CLEAs, Reaction mixture: 5 mL of phosphate buffer (pH 7,10mM), 5µL of H_2O_2 (1M), 5mg CLEAs (0.82UI.mL^{-1}) at room temperature.

3.2. Effect of hydrogen peroxide concentration:

Figure 2 illustrates the effect of hydrogen peroxide concentration on the reduction of phenol concentration after 40 minutes of reaction time and for initial phenol concentration of 90 mg.L^{-1}, Hydrogen peroxide concentration in the reaction medium was varied by injecting four times volumes of 5µL every 10 minutes from freshly prepared solutions of peroxide.

For the investigated range of hydrogen peroxide concentration (0.5-20 mM), the rate and the percentage of phenol conversion increased with peroxide concentration to reach an optimum

between the concentrations of 2 to 3mM (Fig.2). Maximal conversion was about 60 to 65 % with an initial rate of 1.41 $mg.L^{-1}\,min^{-1}$.

At peroxide concentrations higher than the optimum values detected, the rate and the yield of phenol conversion decreased dramatically. This decrease was most likely due to a deactivating effect of the hydrogen peroxide on peroxidase which irreversibly oxidize the enzyme ferriheme group essential for peroxidase activity.

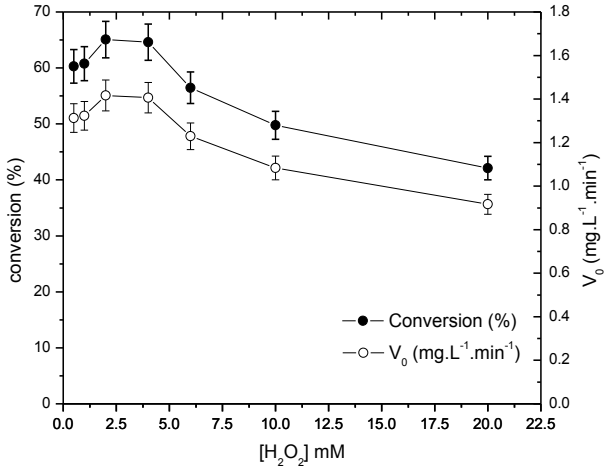

Figure.2: Effect of hydrogen peroxide concentration on phenol degradation using BRP-CLEAs. Reaction mixture: 5 ml of phosphate buffer (pH=7, 10mM), 90$mg.L^{-1}$ of phenol, 5×4µL of H_2O_2, 5mg CLEAs (0.82UI/ml) at room temperature

3.3. Effect of BRP-CLEAs activity

The fourth parameter that could enhance the reaction of phenol degradation is the BRP-CLEAs concentration or activity in the reaction medium. All above experiments were carried out with a constant mass of enzyme 5 mg (≈0.86 UI/mL). To examine the impact of the BRP-CLEAs concentration, the molar ratio of phenol to hydrogen peroxide was kept constant at its optimal value (2), and the enzyme activity was varied from 0.4 to 3.5 UI/ml by weighing the correspondent amount of BRP-CLEAs

Results obtained are represented in figure 4. Data elucidate that enzyme concentration has a significant effect on phenol degradation.

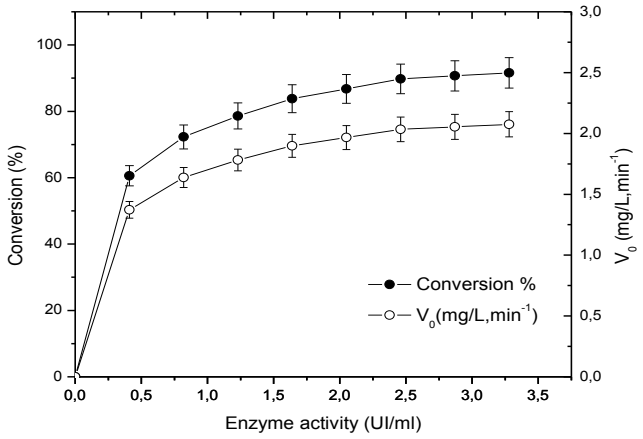

Figure.3: Effect of BRP-CLEAs on phenol degradation .Reaction mixture: 5 ml of phosphate buffer (pH=7, 10mM), 90$mg.L^{-1}$ of phenol, 5×4µL of H_2O_2 (2mM) at room temperature.

The reaction rate increased rapidly when concentration was raised from 0.4 to 2.0 UI/ml. This result was predictable since the availability of enzymatic sites induce an attraction with substrate molecules and subsequently an increase in the number of molecules converted per time. Maximum performance of BRP-CLEAs was achieved at an optimal concentration of 2.5 UI/ml which correspond to 90% of phenol degradation.

3.4. BRP-CLEAs reusability

Reusability is one of the most researched characters in immobilized enzymes, as it is one of the most important criteria in the selection of immobilization technique. A fixed amount of BRP-CLEAs were introduced in successive batches and phenol degradation experiments were conducted under optimal conditions. At the end of the reaction, CLEAs were separated by centrifugation and washed by distillated water to be introduced in the next batch. The efficiency of BRP-CLEAs was evaluated on term of residual removal capacity which was calculated by the following equation:

$$\text{Residual Removal capacity (\%)} = \frac{\text{conversion of batch (x)}}{\text{conversion of batch (1)}} \times 100$$

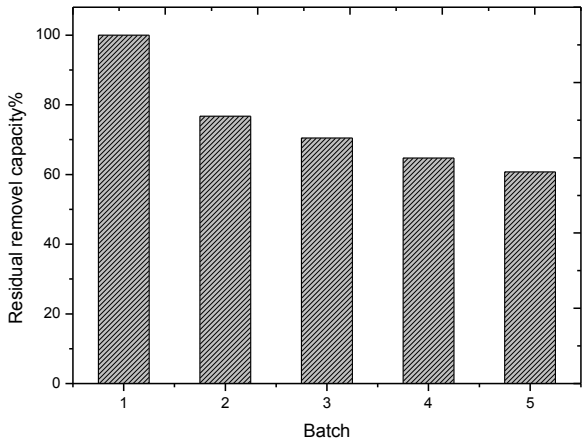

Figure.4: Reusability of BRP-CLEAs for phenol degradation in batch processes ([phenol] = 90mg.L^{-1}, [H_2O_2] =2mM, BRP-CLEAs activity= 2.5UI/mL).

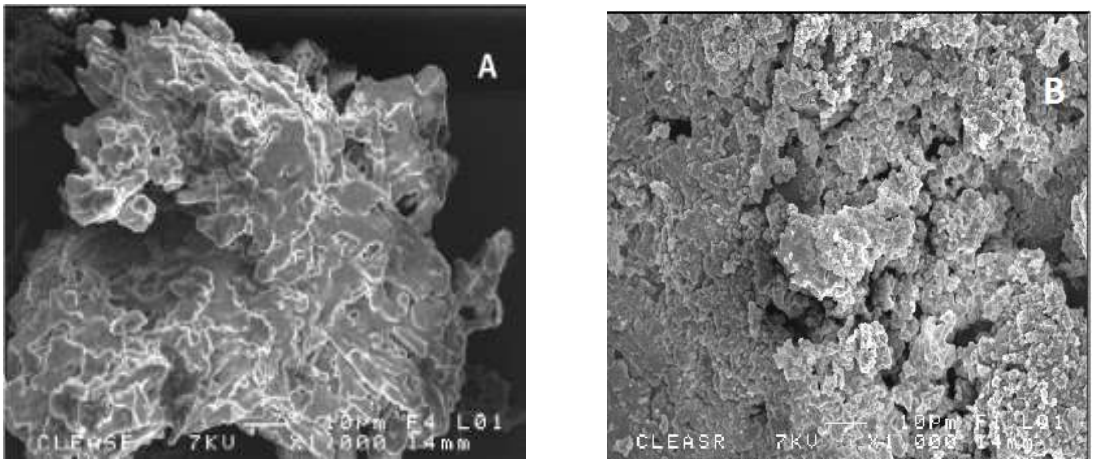

Figure.5: Scanning electron microscopy of BRP-CLEAs before (A) and after (B) with a magnification of ×1000 .

BRP-CLEAs showed that the capacity for phenol degradation was conserved during five consecutive batches even after washing operation and reuse. Nevertheless, the degradation yield decreased significantly after the first batch, and then it decreased less dramatically between the

second and the fifth batch. In all cases, the residual removal capacity was higher than 60.7% which demonstrate that BRP-CLEAs could be reused and keep an acceptable level of their catalytic efficiency. The gradual loss in removal capacity could be explained by the inhibition of BRP-CLEAs due to the accumulation of by-products on their active site or to the partial loss of peroxidase from CLEAs solids during the washing operation.

After reuse, BRP-CLEAs were analysed by electron microscopy in order to compare the shapes of particles before and after treatment. As shown in figure 5, the main change was detected in CLEAs surface which lost its smoothness and became less homogeneous.

4. Conclusions

Use of CLEAs from BRASSICA RAPA peroxidase seems to be a promising new technique for the removal of phenol from wastewater. It was demonstrated that the process is significantly affected by reaction time, substrates and enzyme concentrations which were optimized to obtain a maximal degree of phenol elimination (90%). The effectiveness of BRP-CLEAs in reusability was evaluated as well and the fact that their removal capacity was significant after the fifth batch, demonstrate that this form of immobilisation of peroxidase could be a promising technique for wastewater treatment.

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