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Immobilization of peroxidase from fresh horseradish extract for anthraquinone dye decolorization

ABSTRACT

Synthetic dyes are used in all sectors of industry and represent a serious ecological problem. By the presence, anthraquinone dyes are right after the azo dyes. Unlike azo, anthraquinone dyes are considerably harder to degrade due to the presence of coupled aromatic rings in their structure. Horseradish peroxidase (HRP) proved to be an efficient and ecologically acceptable solution for synthetic dyes decolorization. For that reason, feasibility of peroxidase from fresh horseradish extract application in anthraquinone dye decolorization was the object of this research. Unpurified peroxidase (1.5 mg mL^{-1}), was immobilized onto fumed silica by adsorption. In order to optimize the decolorization of anthraquinone dye Acid Violet 109 (AV 109) 0.1 IU of obtained immobilized HRP was applied in a batch reactor. Under the optimal conditions (pH 4.0, dye concentration 30 mg L^{-1} , hydrogen peroxide concentration 0.1 mM) 55.1 % of initial dye concentration was decolorized, within 30 min. In addition, compared to free enzyme, immobilized HRP showed higher resistance towards inhibitory influence of the dye and hydrogen peroxide.

Keywords: horseradish peroxidase, immobilization, dye, wastewater.

1. INTRODUCTION

The rapid development of industry sector leads to sharp increase in water consumption. The resulting wastewater is characterized with intense staining, high suspended solids, chemical (COD) and biochemical (BOD) oxygen demand [1]. Thus, proper treatment of these waters is necessary to protect humans and the environment. One of the biggest problems in wastewater streams are dyes because they are highly visible even in small amounts (1 ppm). Colored water, if discharged in to the natural recipient, has negative effects on living organisms. Besides, human health problems such as liver, brain, central nervous system damage can be caused by toxic waters. Among all synthetic dyes used in industry, azo and anthraquinone are the most frequently applied ones. The use of classic physical-chemical methods, with adsorption being most commonly used, could not resolve the problem of colored wastewaters.

The main drawback of this method is that anthraquinone dyes have high water solubility and they poorly adsorb, with lower rate and lesser extent of decolorization as compared to azo dyes. Besides, anthraquinone dyes are extremely resistant to degradation due to fused aromatic rings present in their structure [2,3]. The use of oxidoreductive enzymes (E.C. 1.11.1.X) gain researchers attention because of the mild reaction conditions, low energy consumption and harmless by-products [4, 5]. Use of horseradish peroxidase (E.C. 1.11.1.7) appeared to be very efficient as well as eco-friendly method for decolorization of wastewaters [4,6-8]. Disadvantages of free enzyme application such as: instability, denaturation, impossibility of recovery and purification, contamination or proteolysis can be overcome by using their immobilized counterparts [9,10]. However, decolorization of wastewaters on an industrial scale requires stable, efficient and economically justified biocatalysts. These requirements can be met if the decolorization reaction is followed by the use of immobilized peroxidase. Fumed silica seems to be very prospective support for the enzyme immobilization because of the high adsorption affinity towards organic molecules like proteins and polymers. For that reason it was chosen in this work as a support for the adsorption of HRP. This paper is

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focused on the development of immobilized biocatalyst for the decolorization of anthraquinone dye from wastewaters. For the immobilization, peroxidase from fresh extract of horseradish roots (*Armoracia rusticana*) without purification was used. As a method of immobilization, adsorption was selected. In order to find optimal conditions for the decolorization of AV 109 dye, the experiments were performed in a batch reactor. The optimal conditions: pH, dye concentration and hydrogen peroxide concentration were evaluated. The obtained optimal parameters for decolorization with immobilized peroxidase were compared to the results obtained for the decolorization with free enzyme. Besides, operational stability of immobilized HRP was examined.

2. EXPERIMENTAL

2.1. Materials

Horseradish root was supplied from the local market. Pyrogallol and fumed silica were supplied from Sigma-Aldrich (USA). Hydrogen peroxide was purchased from E. Merck (Darmstadt). Model dye used in this paper, C. I. Acid Violet 109, was obtained from Lanaset (Lanaset Violet B). All other chemicals used in this work were of analytical grade.

2.2. Methods

Horseradish peroxidase extracts preparation

Horseradish extract was prepared as follows: horseradish roots, after cleaning with water, were crushed in a grinder without addition of water. The resulting extract was stored at 4°C and used for the immobilization and dye decolorization studies.

Protein content determination

Immobilization efficiency was determined by measuring the amount of bound protein. The amount of bound protein was determined by subtracting the amount of protein in supernatant and filtrates from the offered protein mass, using modified method of Lowry [11].

Activity assay of free and immobilized HRP

Activity of free as well as immobilized HRP was determined using pyrogallol as standard substrate. Shortly, the assay solution consist of: 1 mL of pyrogallol (0.013 M), 10 µL hydrogen peroxide (3 % v/v) and 10 µL of enzyme solution; 3 mL of pyrogallol (0.013 M), 30 µL hydrogen peroxide (3 % v/v) and predetermined mass of immobilized biocatalyst, for free and immobilized HRP respectively. The catalytic activity was monitored by following the change in absorbance at 420 nm.

One unit of the activity was defined as the amount of peroxidase that will form 1.0 mg of purpurogallin from pyrogallol in 20 s at pH 7.0 and 20 °C [12].

Immobilization of horseradish peroxidase

100 mg of fumed silica was suspended in 1.2 mL of enzyme solution (1.5 mg mL⁻¹), prepared in the potassium phosphate buffer 0.1 M, pH 7.0. After 2 h shaking at room temperature immobilized HRP was removed from the supernatant and rinsed with immobilization buffer until no activity was detected in the filtrates. Immobilized HRP was stored at 4°C until use.

Decolorization of anthraquinone dye AV 109

Efficiency of obtained immobilized HRP was examined in the decolorization reaction of the selected anthraquinone dye, AV 109 often present in wastewater. In order to optimize the decolorization of AV 109 dye, using 0.1 IU of enzyme activity, pH (3.0, 4.0, 5.0, 7.0, 9.0), hydrogen peroxide (0.05-0.6 mM) and dye concentration (10-50 mg L⁻¹) were varied. The residual amount of dye was followed using UV-Vis spectrophotometer (UV Shimadzu 1700, Shimadzu Corporation, Kyoto, Japan) at maximum wavelength for tested dye (λ_{max} 590 nm AV 109). Decolorization was calculated using following mathematical expression [6]:

$$Decolorization(\%) = \left[\left(\frac{A_0 - A_t}{A_0} \right) \right] \times 100 \quad (1)$$

where A_0 is the initial absorbance of untreated dye solutions (control) and A_t is the absorbance of dye solutions after enzymatic treatment. Under the optimal conditions, immobilized HRP was subjected to reusability study in order to determine operational stability of obtained biocatalyst. Reuse of immobilized HRP was examined through consecutive cycles of AV 109 decolorization reaction. After first decolorization reaction, immobilized enzyme was separated from the reaction mixture, rinsed two times with immobilization buffer and applied in the next catalytic cycle.

3. RESULTS AND DISCUSSION

It was determined, after the immobilization, that 1 g of fumed silica adsorbe 3.4 mg of protein and the obtained preparation showed the activity of 22.1 IU g⁻¹. In order to make results comparable optimization experiments with free and immobilized HRP were done with the equal amount of the activity (0.1 IU). In the first experimental set optimal pH and hydrogen peroxide concentration were evaluated. Results are presented in Fig. 1.

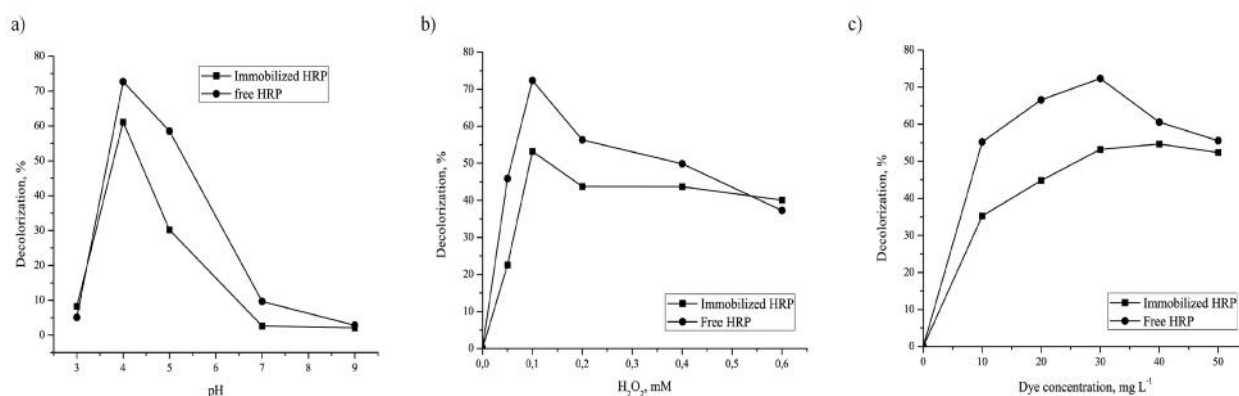


Figure 1 - Optimum a) pH (reaction volume 50 mL, enzyme 0.1 IU, dye concentration 30 mg L⁻¹, hydrogen peroxide concentration 0.1 mM); b) hydrogen peroxide concentration (reaction volume 50 mL, enzyme 0.1 IU, dye concentration 30 mg L⁻¹, pH 4.0); c) dye concentration (reaction volume 50 mL, enzyme 0.1 IU, dye concentration 30 mg L⁻¹, pH 4.0, hydrogen peroxide concentration 0.1 mM, contact time 40 min)

Fig. 1a clearly indicates that both, free and immobilized HRP have optimum at pH 4.0. This can be explained by the fact that adsorption is immobilization technique with the mild conditions and that native conformation of the enzyme often does not change during the immobilization [13]. The similar orientation resulted in the similar affinity of the enzyme molecule towards substrate. pH 4.0 seems to be the best, where 72.7% and 61.1% of AV 109 dye was decolorized using free and immobilized HRP, respectively. A smaller decolorization efficiency of immobilized HRP can be due to diffusion limitations and more difficult approach of the substrate to the active site. Namely, during the adsorption, enzyme molecules can be densely packaged, forming an obstacle to the incoming substrate molecule and thus lowering the decolorization power of immobilized HRP molecule [14]. Besides, for the immobilization unpurified HRP was used, where number of impurities could also adsorb on the support surface and hinder the bound HRP active center. Under the optimal pH, optimal concentration of hydrogen peroxide was evaluated. It is evident from Fig. 1b that immobilization of HRP resulted in reduced adverse impact of hydrogen peroxide on the peroxidase activity. By increasing the concentration of hydrogen peroxide up to 0.1 mM, increase in decolorization efficiency is evident. With further increase in hydrogen peroxide concentration decolorization of AV 109 dye falls off sharply using free peroxidase. On the other hand, using immobilized HRP a slight decrease in the decolorization at hydrogen peroxide concentration of 0.2 mM is evident, where from further increase in hydrogen peroxide concentration resulted in almost constant decolori-

zation of AV 109 dye. Nevertheless, after immobilization of HRP on fumed silica, hydrogen peroxide inhibitory effect is reduced. After pH and hydrogen peroxide, optimum dye concentration was examined (Fig. 1c). Apparently, immobilization had protective effect on the HRP because inhibitory influence of dye was less pronounced. In both cases, optimum dye concentration was 30 mgL⁻¹, where 53.2 % and 72.3% of AV 109 dye was decolorized using immobilized and free peroxidase, respectively. Increasing the dye concentration exerted a strong inhibitory effect on free HRP as opposed to immobilized, which has proven to be considerably less sensitive to the inhibition by AV 109 dye.

Industrial application of enzymes is based on the possibility to use immobilized biocatalyst more times in the desired reaction. Under the optimal conditions, operational stability of immobilized peroxidase was examined (Fig. 2a). Through three consecutive cycles higher decolorization efficiency of immobilized than free HRP was evident. Namely, after three cycles immobilized HRP decolorized 27.0 mg L⁻¹ of AV 109 dye, meanwhile free HRP 21.0 mgL⁻¹. It is evident that after third cycle peroxidase lost its catalytic activity probably because of active center covering by reaction products [15]. In addition, immobilized HRP obtained in this study was more effective than dye-decolorizing peroxidase which has maintained its activity during only two cycles of application [16]. Fig. 2b donates the UV-Vis spectra of the initial dye solution as well as the spectra of dye solutions after decolorization with free and immobilized HRP (the first reaction cycle). Although a higher decolorization was observed with free HRP, the

immobilized HRP can be applied in three consecutive cycles which clearly indicates the advantage of application of immobilized HRP in comparison to the free HRP. If we take into account that peroxidase used in this study was

from fresh horseradish roots extract, the obtained results indicate a high potential of unpurified HRP application in the decolorization reactions, significantly increasing the economic feasibility of the overall process.

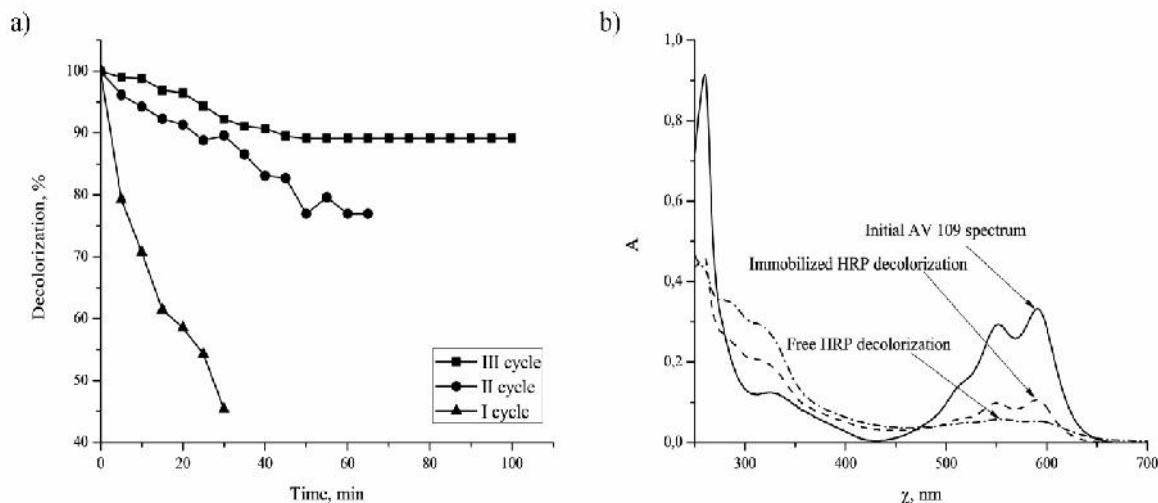


Figure 2 - a) reusability (reaction volume 50 mL, enzyme 0.1 IU, dye concentration 30 mg L⁻¹, pH 4.0, hydrogen peroxide concentration 0.1 mM); b) Initial, after decolorization with free and immobilized HRP AV 109 spectrum

4. CONCLUSION

In this paper a significant effort was made for the optimization of enzyme catalyzed anthraquinone dye decolorization from wastewater. The obtained results represent a good base for the industrial scale biocatalyst development. Further research should be directed towards optimizing the immobilization procedure. Additionally, increasing the stability of the resulting biocatalyst should also be the subject of further studies. The development of a stable biocatalyst leads to the ultimate objective, which is application in the appropriate bioreactor configuration with high efficiency.

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IZVOD

IMOBILIZACIJA PEROKSIDAZE IZ SVEŽEG EKSTRAKTA RENA I PRIMENA U DEKOLORIZACIJI ANTRAHINONSKIH BOJA

Sinteti ke boje se primenjuju u gotovo svim granama u industriji i predstavljaju ozbiljan ekološki problem. Po zastupljenosti, antrahinonske boje se nalaze na drugom mestu, odmah posle azo boja. Za razliku od azo boja, antrahinonske boje se zbog prisustva kondenzovanih aromati nih prstenova teže razgra uju. Peroksidaza iz rena se pokazala kao efikasno i ekološki prihvatljivo rešenje za dekolozaciju sinteti kih boja. Iz tog razloga je predmet ovog rada mogu nost primene nepre iš ene peroksidaze iz svežeg ekstrakta rena u dekolozaciji antrahinonskih boja. Nepre iš ena peroksidaza (1.5 mg mL^{-1}) imobilisana je adsorpcijom na silikatne estice. 0.1 IU dobijene imobilisane peroksidaze je upotrebljena u cilju optimizacije dekolozacije antrahinonske boje AV 109 u šaržnom reaktoru. Pod optimizovanim uslovima (pH 4.0, koncentracija boje 30 mg L^{-1} , koncentracija vodonik-peroksida 0.1 mM, vreme kontakta 40 minuta), 55.1 % ispitivane boje je uklonjeno. Pored toga, imobilizacijom je zna ajno umanj en inhibitorni uticaj boje i peroksida na peroksidazu.

Klju ne re i: peroksidaza iz rena, imobilizacija, boje, otpadna voda.

Nau ni rad

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