pH AND TEMPERATURE INFLUENCE ON PHENOL REMOVAL FROM WATER BY HORSERADISH PEROXIDASE

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Abstract: Horseradish peroxidase represents one of the most exploited enzymes in the process of enzymatic phenol removal from aqueous solutions. It has a catalytic ability over a broad pH range, temperature, and contaminant concentration. In this study we have investigated the influence of pH and temperature on process of phenol removal by crude horseradish peroxidase from aqueous solution. Reaction was performed in the presence of low molecular polyethylene glycol (PEG 300) at different temperatures (4, 12, 17, 20, 25, 30, 35, 40 and 45 °C) and pH values (3, 4, 5, 6, 7, 8 and 9). Reaction was monitored by measuring of absorbance changes of the samples taken at certain time intervals from reaction mixture. Obtained results shown that phenol removal from aqueous solution increases by temperature increase up to 35 °C, after which this effect no longer exists. Also, phenol removal increases in the pH range of 3 - 7, while a further increase of pH value leads to the opposite effect. Based on this it can be concluded that phenol removal from aqueous solutions greatly depends on peroxidase activity, because this temperature and pH values represents the optimum values of peroxidase enzymatic activity.

KEYWORDS: PHENOL REMOVAL, HORSERADISH PEROXIDASE, pH, TEMPERATURE

1. Introduction
Phenols belongs to a group of compounds that important for the environment because they possess a toxic and carcinogenic properties [1–3]. Due to their widespread applications, they are present as pollutant in many industrial wastewater streams [2,4,5]. Although there are many conventional methods for removal of phenolic compounds from wastewaters that include chemical, physical and biological processes, they are not always applicable [1,6]. From these reasons methods that includes enzyme for wastewater treatment were developed and enzymes such us peroxidases, laccases and polyphenol oxides usually were used [2,7,8].

First application of horseradish peroxidase (HRP, EC 1.11.1.7) in the process of phenolic compounds removal from aqueous solutions, by Klübian and collaborators (1980) was proposed [9]. Advantages of HRP on the other enzymes, it is a catalytic ability in wide range of pH and temperature as well as and contaminant concentrations [7,10]. In the presence of hydrogen peroxide HRP could catalyze the oxidation of different aromatic compounds such as phenols, biphenols, benzenes, anilines and related heteroaromatic compounds to form phenoxy radicals [11,12]. Created phenoxy radical products spontaneously polymerize to water-insoluble polymers that can be separated from solution by filtration or sedimentation [12–15]. Unfortunately, as other conventional methods for removal of phenolic compounds from wastewater, enzymatic process also have limitations [10]. One of the biggest problems is excessive treatment cost appears because the high cost of the purified enzyme and the potential formation of residual products in the aqueous phase [10]. This problem can be reduced using of protective additives and potentially inexpensive enzymes sources [10,12,16].

In addition to the previously mentioned problems, inactivation of HRP during the process of phenol removal also was a major problem. As Klübian and Tu (1983) reported phenoxy radicals formed during the oxidation process, attack the catalytic active center of HRP which lead to the reduction and/or elimination of its catalytic ability [17]. The second hypothesis belongs to Nakamoto and Machida (1992) which assumed that inactivation of HRP was a consequence to a lack of contact between the substrate and enzyme, because HRP was captured in the newly formed polymer [18]. In some way, they managed to confirm their hypothesis because they showed that enzymes lifetime can be extended in the presence of highly hydrophilic additives with a higher affinity to the hydroxyl groups of the emerging polymer compared to HRP. Polyethylene glycol (PEG) and gelatin were particularly suitable because they could attack the polymer and thereby enable the enzyme to stay active in the solution. Results from investigations of Cooper and Nicell (1996) were demonstrated that crude extract from horseradish roots and high purity enzyme have similar removal potential in reaction of total phenols removal [12]. Also, almost all cases the crude extract achieved better total phenols removals than the pure HRP for the same enzyme concentrations except at the point of maximum removal [12]. The aim of this paper was to investigate the influence of temperature and pH on peroxidase activity, extracted from horseradish (low purified), as well as on the process of phenol removal in the presence of hydrogen peroxide and low molecular polyethylene glycol (PEG 300) from aqueous solutions.

2. Materials and Methods

2.1. Enzyme isolation
2 g of horseradish root (Armoracia rusticana syn. Cochlearia armoracia, found in the local market) is cleaned and chopped, and extracted during the period of 30 min with 20 ml 100 mM phosphate buffer (pH 7.0) at room temperature (20 °C). After extraction, the centrifugation is performed 15 min at 3000 rpm. The obtained supernatant is transferred to a test tube and the rest of the plant material is subjected to extraction again. Stock enzyme solutions are stored at 4°C and warmed to room temperature immediately prior to use.

2.2. Experimental protocol
Phenol oxidation reactions were carried out in glass flasks at different temperatures (4, 12, 17, 20, 25, 30, 35, 40 and 45 °C) and pH values (3, 4, 5, 6, 7, 8 and 9) using 30 ml reaction volumes. Reaction mixtures contained 2.0 mM phenol, 300 mg/ml PEG and 1.2 U/ml sample of HRP. After 60 minutes, aliquots are pipetted into the tubes and HRP activity and phenol content were examined.

2.3. HRP activity assay
Peroxidase activity is determined by method of Soysal and Styölemez (2005), with slight modifications [19]. 2.1 ml of 100 mM acetate buffer (pH 7.0) is measured, followed by addition of 0.2 ml sample solution, 0.2 ml of 0.125% solution of o-dianisidine in methanol; the mixture is vigorously vortexed, and reaction is started by addition of 0.5 ml 8.8 mM H₂O₂. Absorbance change is recorded as a function of time at 460 nm, and activity of HRP is calculated by using following equation:

\[ A[U/ml] = \text{tg}_a \cdot \frac{R}{\varepsilon} \]  

where: \( \text{tg}_a \) - slope of the plot, 
\( R \) - the total ratio volume of the reaction mixture and the enzyme volume, 
\( \varepsilon \) - molar extinction coefficient (\( \varepsilon_{460} = 11.3 \text{mM}^{-1} \text{cm}^{-1} \)).
The blank contained all reagents except the hydrogen peroxide, which is replaced by aqua destillata.

One unit of peroxidase activity is defined as the amount of enzyme that transformed 1 µmol of α-dianisidine per minute.

2.4. Phenolic compound assays

Phenol content is determined as described by Nicell et al., (1995), with slight modifications [13]. Aliquots (2.5 ml) from batch reactions are pipetted into the tubes at different time intervals (20, 40, 60, 80, 100 and 120 min) and reactions are stopped by adding 2.5 ml 96% ethanol solution. After, samples are treated with alum and pH is adjusted to 6.3 to optimize precipitation, using a stock solution of 0.1 M NaOH and 0.1 M HCl. Samples are centrifuged 10 min at 10000 rpm. Residual phenol concentration are determined by direct spectrophotometric measurement of the absorbance at 269 nm on UV-VIS.

3. Results and Discussion

In previous research of Savic and collaborators (2014) [20], phenol removal from aqueous solutions by raw peroxidase (extracted from horseradish) in the presence of hydrogen peroxide and PEG 300 as well as the influence of PEG 300, phenol and hydrogen peroxide on HRP activity and phenol removal was investigated. Obtained results were demonstrated that the presence of PEG 300 shows a stabilizing effect on HRP activity. The highest phenol removal was achieved when the concentrations of PEG 300, phenol and hydrogen peroxide were 300 mg/L, 2.0 mM and 2.5 mM, respectively, and that was the reason to select the same concentrations of the reaction participants for further research.

This study represent a continuation of previous research and in this study the influence of temperature and pH on peroxidase activity, as well as and their influence on the process of phenol removal in the presence of hydrogen peroxide and low molecular polyethylene glycol (PEG 300) from aqueous solutions were investigated.

Results from investigations of temperature influence on HRP activity on Fig. 1 were presented. Based on obtained results it could be concluded that there are three regions of HRP activity changes: first region from 4 to 20 °C with a intensive increasing of HRP activity, second from 20 to 35 °C with small changes of HRP activity, and third from 35 to 45 °C with decreasing of HRP activity. These results are in agreement result reported by Veitch (2004) where the highest activity was at 35 °C [21].

Obtained results from investigations of pH influence on HRP activity were presented on Fig. 2. Results indicate that increasing of HRP activity from 3 to 6 was progressive, between 6 and 7 changes were small, while at higher pH values HRP activity decreases. Also, if we take into account previously reported results of Veitch (2004) at different range of pH values, it can be concluded that results from our study are in agreement [21].

Influences of temperature and pH on phenol removal by HRP on figures 3 and 4 were presented, respectively. By looking of Figure 3 it can be noticed that the amount of phenol removed have two different parts. In first, with increasing of temperature (from 4 to 35°C) removed phenol amount also increases, even up to 66% from starting concentration, and second, where increasing of temperature leads to a reduction in the removed amount of phenol. If we take into consideration results from investigations of temperature influence on HRP activity, it could be concluded that changes of HRP activity with increasing of temperature were similar as achieved changes in the removed amount of phenol. A similar conclusion can be made by looking figures 2 and 4. Changes of HRP activity induced by changes of pH values follow changes in the removed amount of phenol. Based on these results, it can be concluded that phenol removal in great measure depends of HRP activity.
4. Conclusion
Based on obtained results it could be concluded that phenol removal from aqueous solution increases by temperature increase up to 35 °C, after which this effect no longer exists. Also, phenol removal increases in the pH range of 3 – 7, while a further increase of pH value leads to the opposite effect. Based on this it can be concluded that phenol removal from aqueous solutions greatly depends of peroxidase activity, because this temperature and pH values represents the optimum values of peroxidase enzymatic activity.

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5. Literature