Research Journal of Applied Sciences 13 (7): 425-430, 2018
ISSN: 1815-932X

Degradation of a Nonsteroidal Anti-Inflammatory Drug Using Horseradish Peroxidase Enzyme

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Abstract: Due to the persistent feature of some compounds commonly found in wastewaters, their treatment is very difficult through conventional processes. Therefore, alternatives among which arises the enzymatic treatment are in continuously sought. The objective of this research is to analyze the feasibility of degradation of a widely used pharmaceutical compound, diclofenac, using the enzyme Horseradish peroxidase as a catalyst of the reaction. Very promising results were obtained, since, there is no evidence of the use of this enzyme for the elimination of diclofenac compound. The influence of the initial concentration of enzyme and hydrogen peroxide in the removal efficiency is analyzed. A maximum value of removing close to 47% was reached when hydrogen peroxide and diclofenac were used in stoichiometric quantities. The operating conditions were also studied. It was observed that reaction can be performed in a pH range between 6-9 and temperatures from 20-50°C.

Key words: Diclofenac, enzymatic treatment, horseradish peroxidase, pharmaceutical compounds, removal efficiency, temperatures

INTRODUCTION

Pseudo-persistent contaminants in wastewater create a need to study different methods capable to degrade these contaminants in order to reduce its concentration in water sources and treatment plants, since, they could generate some problems therein. The growing attention to the pharmaceutical compounds as potential pollutants leads to the need to study different methods to be able to remove them from these sources. One of the possible methods is the enzymatic elimination which has proven to be very efficient in the removal of other recalcitrant contaminants such as phenol (Nicell *et al*., 1992; Aitken, 1993; Bodalo *et al*., 2006; Pramparo, 2008; Salazar, 2011) and phenol derivatives (Bayramoglu and Aryca, 2008).

In the last two decades, several processes have been implemented using different enzymes extracted from plants and microorganisms such as peroxidases from various sources including the horseradish peroxidase. Peroxidases are a type of enzymes that catalyze the oxidation of certain hydrogen donor's compounds such as phenols and aromatic amines by means of the Hydrogen peroxide (H_2O_2) as a precursor of polymerization (Duarte-Vazquez *et al*., 2003; Garcia *et al*., 2003; Bodalo *et al*., 2006).

Among the different pharmaceutical compounds that can be found, there are substances in everyday use as antibiotics, hormones, pain relievers and anti-inflammatories (Masuda *et al*. 2001; Verlicchi *et al*., 2012; Kummerer *et al*., 2000). Different researchers have reported the presence of these compounds in concentrations of μg/L in effluents from Wastewater Treatment Plants (WWTP) and the environment due to the persistence that exhibits these types of compounds (Kummerer *et al*., 1999; Cunningham *et al*., 2006). Because of the low efficiency of conventional processes used on the WWTP to treat this type of polluting compounds, usually physic-biologics, technologies using enzymes can be an interesting alternative. Horse Radish Peroxidase enzyme (HRP) catalyzes the oxidation and polymerization of aqueous aromatic compounds in the presence of hydrogen peroxide. The polymerized products have low solubility and are readily precipitated from solution thus demonstrating the potential for removing aromatic pollutants from wastewaters (Nicell *et al*., 1995). That is why this study proposes the use of this technique to analyze the possible degradation of pharmaceutical compounds using enzymes.

The objective of the present study is to analyze the feasibility of degradation of a non-steroidal anti-inflammatory such as diclofenac, using the enzyme Horse Radish Peroxidase (HRP) and the influence of the initial operating conditions.

MATERIALS AND METHODS

Diclofenac was used in commercial presentation type ampoule of 3 mL containing 75 mg of sodium diclofenac per ampoule. Several dilutions of the compound were

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prepared in water and were measured for absorbance using a spectrophotometer UV, Model Hach DR 5000. Using a scanning range of wavelength between 190 and 900 nm, it was determined that wavelength for maximum absorption of diclofenac is 275 nm. This value was used to observe the behavior of the compound in the degradation process. The enzyme Horse Radish Peroxidase (HRP) was obtained from Sigma Aldrich (P8250-50KU) with an activity of 181 U/mg according to the method of the pyrogallol. A stock solution was prepared in distilled water. The aliquots were stored at -4°C. The stock solution of Hydrogen peroxide (H_2O_2) was prepared by dissolving the required amount of 30% hydrogen peroxide supplied by Bioquigen to obtain a final concentration of 100 mM.

General procedure: Different concentrations of H_2O_2, HRP and diclofenac were analyzed. The different solutions of diclofenac were prepared by adding distilled water until obtaining the desired concentration. Both diclofenac and the solution of HRP were maintained within the reactor before adding the appropriate amount of hydrogen peroxide. Reaction time began when H_2O_2 was introduced into the reactor. In all experiments several liquid samples were taken 3 mL to different reaction times to perform the analytical procedure.

The pH variation experiments were conducted using a pHmeter brand Ezdo, Model PL-700AL. The acidic pH was obtained by adding sulfuric acid (H_2SO_4) and the basic pH with sodium hydroxide (NaOH) directly to the diclofenac solution. The initial pH of the solution was verified during the reaction and it was established by the respective measurements.

Furthermore, for the study of the initial temperature influence, the temperature changes were established using a shaker brand Wisd, Model MSH-20D which allowed the temperature to be constant. In both cases, once having obtained the desired pH or temperature conditions, the reaction began immediately after adding the Hydrogen peroxide (H_2O_2) to the reactor.

RESULTS AND DISCUSSION

Analysis of the optimal diclofenac concentration: Three different dilutions of diclofenac were prepared in 1-3 L, to obtain a final concentration of 75, 37.5 and 25 mg/L, respectively. A decrease in the absorbance value was obtained when the diclofenac concentration was diminished. This is related to the Beer-Lambert's Law in which absorbance is related with the concentration of the product, although, it is proportional only to a certain extent. That is why from the obtained results, it was decided to work with a diclofenac concentration of 25 mg/L to have the absorbance value lower than 1.0 and ensure proportionality between concentration and absorbance.

Fig. 1: Observation of the colour produced in different reactions depending on the initial condition

Assay for the enzymatic elimination of diclofenac: Various experiments were conducted to analyze if the enzyme Horseradish peroxidase is able to degrade or remove the selected compound. In the first instance, the influence of the hydrogen peroxide concentration was studied. The degradation test consisted of the measurement of the absorbance change of the solution at the time. To do this, 100 mL of diclofenac with a concentration of 25 mg/L was placed in a batch reactor, continuously agitated with magnetic stirring at room temperature. The necessary amount of enzyme and hydrogen peroxide were added to the solution. Once hydrogen peroxide is added to the sample, reaction time starts and the value of absorbance at 275 nm it is regularly measured. The reaction is approximately evaluated over a period of time between 2-3 h.

Analysis of the initial H_2O_2 concentration influence: Experiments to analyze the influence of the initial concentration of H_2O_2 were carried out in batch conditions. The concentration of HRP was maintained at $4*10^{-4}$ mM in all cases. In every experiment, the solution changed color to yellow or orange, depending on the concentrations used, after the addition of H_2O_2. Darker solutions were obtained when more H_2O_2 was added to the sample, however when excess of H_2O_2 was added, the solution had a final color a little clearer as shown in Fig. 1.

This fact is due to the amount of products formed in the reaction increased with an increase in the amount of H_2O_2 and moreover, the inactivation of the enzyme causes lower levels of conversion and therefore, fewer

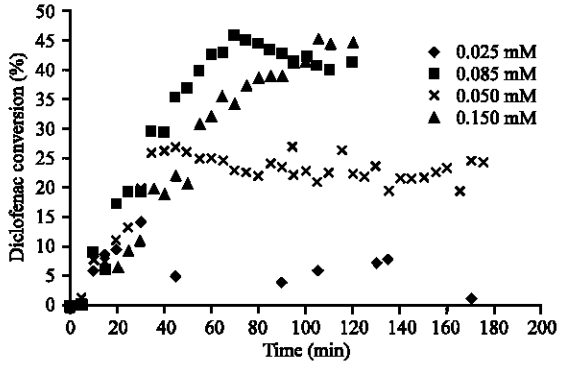

Fig. 2: Influence of the initial H_2O_2 concentration. (Diclofenac) 0: 0.084 mM, (HRP) 0: $4*10^{-4}$ mM, pH 6.8 and T = 20°C

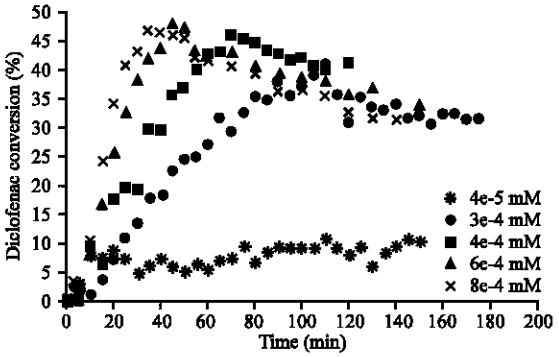

Fig. 3: Influence of the initial HRP concentration. (Diclofenac) 0: 0.084 mM, (H_2O_2) 0: 0.085 mM, pH 6.8 and T = 20°C

colors in the solution. This was evidenced with the occurrence of different peaks in the scanning of absorbance during the reaction. The results obtained from the removal of diclofenac for 4 different H_2O_2 initial concentrations are shown in Fig. 2.

As it can be observed in the figure when a low concentration of H_2O_2 was applied, there was almost no reaction. As to the quantity of hydrogen peroxide was increasing, a greater removal of diclofenac was obtained, until it reached a maximum value of elimination or conversion. From this maximum value when more H_2O_2 was added, higher performance in reaction was not obtained or on the contrary, the amount of removed diclofenac decreased. This is due to the enzyme inhibition. For other compounds, it was determined that the optimum concentration of H_2O_2 to be used is an equimolar ratio with the compound to treat, for example, phenol (Nicell *et al*., 1992; Vasudevan and Li, 1996; Pramparo, 2008). In this case, since, the concentration of diclofenac is 0.084 mM it would be assumed that behavior should be similar. This is confirmed in Fig. 2 where it can be observed that the maximum removal is obtained using an equivalent quantity of 0.085 mM of H_2O_2.

It was obtained around 47% of conversion of diclofenac when using an equimolar ratio of H_2O_2. With an increase in the concentration of peroxide, the conversion of diclofenac decreased as well as the reaction rate. More experiments are needed to determine a possible inactivation of the enzyme to increased levels of H_2O_2.

Analysis of the initial HRP concentration influence: Several experiments were carried out to analyze the influence of the initial concentration of enzyme in the reaction. They used 6 different concentrations of HRP, varying between $4*10^{-5}$-$8*10^{-4}$ mM. Figure 3 as the initial concentration of HRP was increased, it increases the amount of removed diclofenac. When concentrations are used at $<3*10^{-4}$ mM a significant reaction is not evidenced, even after 180 min of reaction. However, with HRP concentrations $>3*10^{-4}$ mM, good percentages of elimination are obtained, reaching a maximum value of removal close to 47% using a concentration of HRP $8*10^{-4}$ mM.

Analysis of the diclofenac initial concentration: The influence of the initial diclofenac concentration was studied for two conditions, a H_2O_2 initial concentration of 0.085 mM and stoichiometric ratio to diclofenac concentration for initial diclofenac concentrations in the range of 0.042-0.084 mM. The enzyme concentration was kept at $6*10^{-4}$ mM. The experimental results of the influence of the initial diclofenac concentration for an initial concentration of 0.085 mM of hydrogen peroxide can be observed in Fig. 4.

Figure 4 shows an increase in the diclofenac initial concentration caused a little higher reaction rate; however, the final diclofenac conversion is similar in all cases. This fact is due that the H_2O_2 initial concentration was the same in all experiments, 0.085 mM. This concentration is the optimal value for an initial diclofenac concentration of 0.084 mM (stoichiometric ratio). If the initial concentration of diclofenac is lower, an excess of H_2O_2 is available in the reaction solution, provoking an enzyme inhibition. That is why no longer extension of the reaction is obtained. The maximum diclofenac conversion is given by the hydrogen peroxide concentration.

In the case that stoichiometric diclofenac and H_2O_2 initial concentrations were used, the results were different as it can be observed in Fig. 5.

When more diclofenac was added in the reactor, higher final conversions were obtained. In this case, the

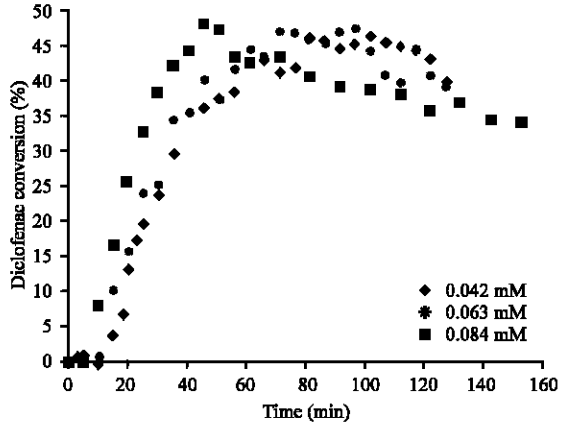

Fig. 4: Influence of the initial diclofenac concentration. (H_2O_2) 0: 0.085 mM, (HRP) 0: $6*10^{-4}$ mM, T = 20°C and pH = 6.8

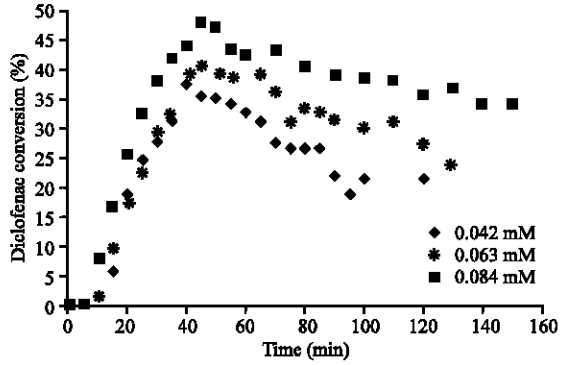

Fig. 5: Influence of the initial diclofenac concentration. (H_2O_2) 0: stoichiometric ratio, (HRP) 0: $6*10^{-4}$ mM, T = 20°C and pH = 6.8

enzyme is not inhibited by the excess of H_2O_2 concentration and the maximum diclofenac conversion is obtained for higher values of diclofenac.

This fact is provoked by the ratio of the enzyme concentration to the diclofenac concentration. When the diclofenac concentration is increased, it is necessary to add a higher amount of enzyme to the reaction mixture in order to obtain the same diclofenac conversion. In this case, enzyme was enough to react with a high concentration of diclofenac and as the H_2O_2 initial concentration was the optimal value in all experiments as higher diclofenac in solution higher removal of the compound.

The higher removal percentage is found in a 100 mL sample (0.084 mM) in a shorter time of reaction. For the other both cases in which a lower concentration of the compound is used in the sample with a higher dilution value (75 and 50 mL), some removal rates are reached but not the maximum percentage. However, the initial reaction rate is quite similar in all cases. When it comes to compare the different diclofenac concentrations between the stoichiometric amounts of hydrogen peroxide is shown that the behaviour of the reaction is similar, the maximum removal rates happens approximately in the same range of time of the reaction one.

Analysis of the initial operating conditions influence: The optimisation of the process variables is necessary to avoid the enzyme inactivation (Nicell *et al*., 1992). This optimisation has the objective on extending the catalytic lifetime to improve the economical feasibility of the process because the lifetime is one of the most important characteristics.

Some researchers have presented works about the optimisation of the process variables. Nicell *et al*. (1992) studied the HRP catalysed polymerisation and precipitation of aromatic compounds from wastewater. In this study, the removal of phenol, 3-chlorophenol and 4-methylphenol was clearly function of the value of the pH. The HRP established some activity between pH's 4 and 10 but only a high efficiency between 6 and 9. In the same way the optimum temperature was found to be below 35°C. Other authors demonstrated that 100% of phenol removal is achievable in a large range of pH (5-9) and temperature (0-60°C) (Pramparo, 2008).

Finally in a comparative study between HRP and soybean peroxidase, the maximal phenol elimination was reached at neutral pH and moreover, both enzymes are also able to work at basic medium, better and at slightly acid medium (Bodalo *et al*., 2006). No significant differences were found in the behaviour of the reaction with both enzymes in the range of temperature studied (25-40°C).

Influence of the pH: Diclofenac removal efficiency as a function of pH was studied in the range of 4-10. The initial diclofenac concentration was 0.084 mM (25 $mg \times L^{-1}$) and the initial H_2O_2 concentration of 0.085 mM. The concentration of HRP was kept to $6*10^{-4}$ mM. The temperature was always 20°C. The colour of the reaction solution turned instantly after the addition of the peroxide but its intensity depended of the initial pH checked. The results of the diclofenac removal as a function of the pH value can be seen in Fig. 6.

The pH 4 value shows a low removal rate. After 75 min of reaction, a raise in the absorbance was noticeable. This fact can be related to enzyme denaturation which makes the enzyme lose its own properties and stability. Even though a higher removal is identified with a 5.5 pH value, it does not reach the maximum removal rate.

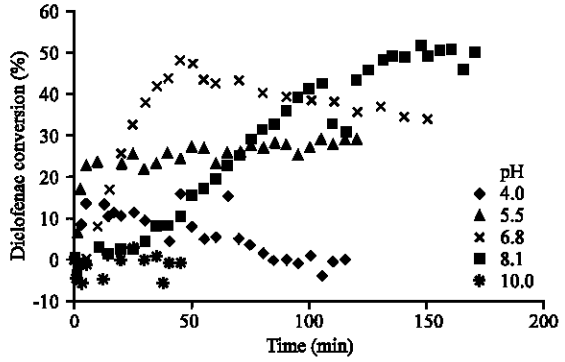

Fig. 6: Influence of the initial pH value. (Diclofenac) 0: 0.084 mM, (H_2O_2) 0: 0.085 mM, (HRP) 0: $6*10^{-4}$ mM and T = 20°C

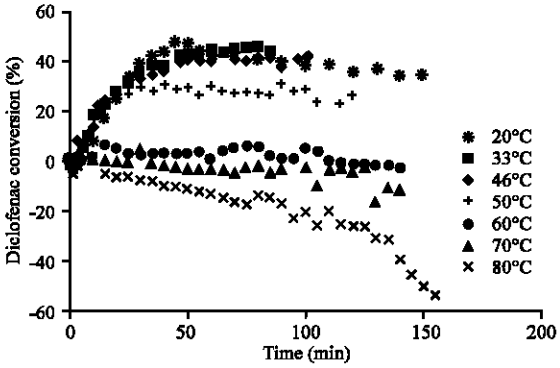

Fig. 7: Influence of the initial temperature on the reaction. (Diclofenac) 0: 0.084 mM, (H_2O_2) 0: 0.085 mM, (HRP) 0: $6*10^{-4}$ mM and pH = 6.8

In addition to this as the pH increases, the reaction achieves a higher removal rate but with a significant time variation. For a 6.8 pH value is observed a 47% removal rate after 45 min and for an 8.1 pH value is reached a 51% removal percentage after 140 min of reaction. This fact shows that at pH values higher than the optimal value, the activity of the enzyme begins to change and decrease. A lack of enzymatic activity is observed at very high pH levels. For instance, a 10 pH value does not present any removal of the compound.

Therefore, the diclofenac removal efficiency increased as the initial pH value is augmented until the optimum value of pH is reached (6.8) where 47% of diclofenac conversion was attained. After this value, the activity of the enzyme decreased and it was null for pH 10. The obtained results agree with those published by Nicell *et al.* (1992), Bodalo *et al.* (2006) and Pramparo (2008) for phenol compound. The enzymatic treatment of aromatic compounds from wastewater is clearly a function of the pH value. The HRP establishes some activity between pH's 4 and 10 but only a high efficiency between 6 and 9. This statement is very interesting because the conditions of industrial effluents may vary over a wide range.

Influence of the temperature: In order to study the influence of the temperature, the reaction solution containing 0.084 mM of diclofenac (25 mg×L^{-1}) and $6*10^{-4}$ mM of HRP solution was introduced into the reactor and heated until the desired temperature, between 20 and 80°C. Diclofenac polymerizing reactions were started with the addition of the exact quantity of hydrogen peroxide into the reactor to have an initial concentration of 0.085 mM.

The influence of the temperature on the reaction can be observed in Fig. 7. In this case, for 0.084 mM of diclofenac and 0.085 mM of H_2O_2, the reaction was carried out at 20, 33, 46, 50, 60, 70 and 80°C. In some experiments, the solution turned dark brown after the addition of H_2O_2 signifying the polymer formation, depending on the initial value of the temperature.

As seen in Fig. 7, the maximum removal rate is reached at a 20°C (47%) after 45 min from the start of the reaction. It was found that for higher values than 60°C no significant removal percentages are presented as a clear inhibition of the enzyme is identified. When the temperature is 33°C, a 45% of removal is reached after 80 minutes. This value shows that there is not an optimal removal activity increasing or decreasing the temperature. These results match those found by Nicell *et al.* (1992) and Pramparo (2008) which state that the enzyme activity is inactivated at temperatures above 45°C. The results show that there is no need to be in control of the temperature during the reaction as beyond 10°C there is no significant removal activity which means that the HRP enzyme does not have major changes in its catalytic activity at room temperature.

It is possible to obtain a very good percentage of pollutant removal using temperatures of reaction between 20 and 50°C. This statement is very interesting because the conditions of industrial effluents and the processes in WWTP may vary over a wide range of temperatures.

CONCLUSION

In the study of the enzymatic removal of diclofenac using HRP, high degrees of removal were obtained in most studied conditions. It was determined that the optimum concentration of work for diclofenac is 25 mg/L. The optimal initial concentration found to H_2O_2, for greater conversion of diclofenac, must be equal to 1 ratio between the molar concentration of diclofenac and that of H_2O_2. An excess of hydrogen peroxide in the reaction mixture may inhibit the catalytic action of the enzyme through its conversion to an inactive form, resulting in lower levels of conversion of diclofenac.

The diclofenac elimination is affected by the initial pH of the solution. Different pH values between 4 and 10 where analyzed obtaining that for values into the 5-9 range achieve the maximum removal efficiencies. Instability of the enzyme is presented for higher pH values which prevents its catalytic activity. The reaction time needed in most of the analyzed cases was between 50 and 100 min. This speed could be increased by using a higher initial concentration of the enzyme but a cost analysis would be needed to verify its efficiency.

The influence of the temperature on the reaction was studied and it was observed that at normal room temperature (20-25°C) it is possible to obtain the maximum diclofenac conversion. In addition to this, the reaction time does not present optimal results when it comes to an alteration of the temperature of the sample. Good percentages of diclofenac conversion were obtained using temperatures of reaction between 20 and 50°C.

The results obtained in this research are very promising due to the lack of evidence of the use of this technology for treating diclofenac which is often found in wastewaters and difficult to remove because of its high persistence.

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