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Application of low purity horseradish peroxidase enzyme to removal of oil from oily wastewater

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ABSTRACT

Application of low purity horseradish peroxidase (HRP) extracted from horseradish root for oil removal from oily wastewater was studied. Factors, such as oil concentration; contact time; HRP dose; H_2O_2 concentration; pH; temperature; Fe^{2+} ion concentration; and effect of emulsifier (Tween 80), were studied. Results indicate optimum HRP concentration for 20, 60, and 120 mg/L oil were 2 U/mL and pH 7.5. Removal efficacy of oil increased with an increase in H_2O_2 concentration at first, and reached maximum of 58.33% at H_2O_2 concentration of 4 mM, and then decreased. Temperature has significantly effect on oil removal. Presence of Fe^{2+} ion in mixture solution has no effect on enzymatic treatment. Results of this study were found to be independent of enzyme purity and therefore, it was possible to utilize crude enzyme preparation instead of purified one. Experimental data of initial reaction rates were fitted using analytical equation proposed by Michaelis–Menten.

Keywords: Low purity; Horseradish peroxidase (HRP); Oil; Removal

1. Introduction

Oil contaminated wastewater comes from a variety of sources such as crude oil production, oil refineries, the petrochemical industry, metal processing, compressor condensates, lubricant and cooling agents, car washing, and restaurants [1]. Oily wastewater contains toxic substances such as phenols, petroleum hydrocarbons, and poly aromatic hydrocarbons, which are inhibitory to plant and animal growth, and equally, mutagenic and carcinogenic to human beings. Similarly, oily wastewater has high oil content, a high chemical oxygen demand, and contains color [2]. Therefore, industries have been linked to environmental pollution resulting from oil spill, oily effluent discharge into bodies of water, and oily sludge discharge into the environment indiscriminately, untreated, or in conditions below standard discharge limits [3,4]. Methods to facilitate the removal of oil from wastewater include flotation [4], filtration [5], biological treatment (aerobic and anaerobic),

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coagulation [6] (chemical-coagulation and electro-coagulation) [7], adsorption [8], advanced oxidation processes [9], and land treatment [10]. Although these methods have proven effective in the treatment of oily water, they exhibit limitations such as restricted application conditions, high operation costs, corrosion, and recontamination. Furthermore, in the removal of dilute emulsified oil and dissolved oil from water, these methods have limited efficiency [9].

Recently, the enzymatic approach has attracted much interest in the removal of phenolic pollutants from aqueous solutions as an alternative strategy to conventional chemical and microbial treatments that pose some serious limitations [11–14]. Oxidoreductive enzymes such as peroxidases and laccase are participating in the degradation/removal of aromatic pollutants from various contaminated sites [15–20]. Peroxidases have been isolated from many species of plants, animals, and micro-organisms. These enzymes are able to act on a variety of aromatic compounds in the presence of hydrogen peroxide. The function of the latter is to oxidize enzyme into a catalytically active form, which is capable of reacting with the phenolic contaminant. The polymeric products have limited water solubility and tend to precipitate quite readily [13].

The enzymatic treatment process has several limitations including (1) the prohibitive cost of treatment, (2) the potential formation of residual products that remain in aqueous phase, and (3) enzyme turn-over [14]. On the other hand, enzymatic degradation has the advantages of convenient operation, mild reaction conditions, a high efficiency, a wide operation range, and the elimination of recontamination, so it can be a suitable solution to the above problems in the treatment of oily water [21].

As far as we know, there are limited reports about the application of HRP to the treatment of oily water. Only Li et al. reported research on the application of horseradish peroxidase and hydrogen peroxide to oil removal from oily water [22]. In this research, pure HRP with an activity level of 250 U/mL was applied. The effect factors of oil removal using HRP, such as pH, concentrations of HRP and H_2O_2, additives, Fe^{2+} ion, temperature, and reaction time were researched. Li et al. reported that, when the initial oil concentration was 120 mg/L, the remaining oil concentrations of the synthetic oily water and an actual oily wastewater reached 24.83 mg/L and 21.30 mg/L, respectively. In addition, they reported that the treatment of oily water using HRP and H_2O_2 is feasible [22].

Recently, free HRP was proved an effective alternative to treat oily wastewater. It can achieve quite high removal efficacy using HRP. With the addition of a reaction promoter, removal efficiency could reach as high as 79.3%. Nevertheless, the cost is still too high for industrial treatment [13].

None of the research shows the purity of enzyme effect on the removal of pollutants. The enzymatic treatment efficiency was found to be independent of enzyme purity, and therefore, it was possible to utilize a crude enzyme preparation instead of a purified one. This feature leads to a significant reduction in treatment costs, as the reduced amounts of enzyme make this method more economically competitive with the conventional treatment methods [12]. As far as we know, there is no report on the application of crude HRP to the treatment of oily water that has cost benefits toward pure HRP. This work aims at developing an enzymatic process to remove oil from oily waters with the application of low purity HRP. The present study focuses on the evaluation of parameters leading to the degradation of oil with concentrations in the range of 20–120 mg/L using crude enzyme preparation from horseradish roots. Reactions were conducted under contact time, oil concentration, HRP (0.5–4 U/mL), H_2O_2 (1–5 mM), effect of temperature (25–45˚C), Fe^{2+} ion, and using emulsifier (Tween 80 detergent) in the batch system. The experimental data of initial reaction rates were fitted using an analytical equation proposed by Michaelis–Menten [14].

2. Methods

Low purity HRP (Crude HRP) was extracted from horseradish roots purchased from local vegetable market as per the procedure given by Bhunia and co-workers [23]. The roots after cleaning with water were crushed in a wet grinder without addition of water and the extract was centrifuged (6,000 g, 6 min, 4˚C). The resulting supernatant was dialyzed using 12 kD membranes against 0.1 M phosphate buffer (pH 7.4) at 4˚C. The dialyzed enzyme extract was stored at 4˚C and used in treatment process, activity of low purity before any treatment process was assayed. One step of purification of low purity by ammonium precipitation in 0–35% and 35–90% saturation of ammonium sulfate to increase purification of HRP was carried out [14].

Oil used has 5.3% water; 1.12 density (g/ml); and kinematic viscosity 8 cSt properties. Aqueous solution of hydrogen peroxide (30% w/v, specific gravity 1.12) and 4-aminoantipyrine (AAP) were purchased from Sigma-Aldrich. Tween 80 Average MW1310 was purchased from Merck. All other chemical used were of analytical grade.

2.1. Peroxidase activity assay

HRP enzyme activity was measured using phenol, 4-aminoantipyrine, and hydrogen peroxide as substrates. The approach was to provide all components except enzyme near saturation concentration so that initial rate of reaction became directly proportional to amount of enzyme present. The assay mixture contained 2.5 ml 9.6 mM of AAP, 1 ml 100 mM of phenol, 1 ml 2 mM of hydrogen peroxide, 4.5–5.0 ml 100 mM of phosphate buffer pH 7.4 and 0.5–1 ml enzyme solution. The rate of reaction was measured by monitoring the rate of formation of the products which absorbed light at a peak wavelength of 510 nm upon addition of enzyme; thus, one unit of activity (U) used in this study is defined as the number of mM peroxide converted per min at pH 7.4 and 25˚C [12–14].

2.2. Experimental procedure

The overall experimental procedures included the preparation of synthetic oily water, reaction initiation, reaction interruption, and oil concentration. Batch reactions were carried out in glass vials of 100 ml capacity at pH 7.5 with different contact times (0–180 min); oil concentrations (20–120 mg/L); HRP amounts (0.5–4 U/mL); H_2O_2 amounts (1–5 mM); pH (5–9) amounts; temperature levels (25–45˚C); Fe^{2+} ion amounts (0–40 mM); and emulsifier levels (0–60 mg/L, Tween 80). The pH of the reaction mixtures was adjusted to a range of 5–9. The buffer was 0.2 M acetate in the pH range of 5–6 or 0.2 M phosphate in the pH range of 6–9. The Fe^{2+} ion concentrations in the reaction mixtures were adjusted to the range of 0–40 mM by adding a certain amount of ferrous sulfate. All solutions were prepared using deionized water. The reactions were started by the addition of H_2O_2 into the reaction mixtures. The reaction mixture was continuously agitated with a magnetic stirrer at speed of approximately 200 rpm during the reaction.

2.3. Analytical process

Oil concentrations in water are usually reported as a mass or volume unit in a given volume of water: either as milligrams per liter (mg/L) or microliters per liter (μl/L). Currently, five properties are used to measure oil in produced water. Four of these properties can be applied in the field and one in the laboratory. These properties are (1) direct weight measurement (US EPA Method 1664, etc.), (2) color, (3) IR absorption, and (4) ultraviolet (UV) fluorescence and particle counting methods. In this study, we applied direct weight measurement to the measurement of oil in effluent solution according to the standard methods for the examination of water and wastewater 20th edition (5520B). Liquid–liquid extraction with hexane, treatment with silica gel (for mineral oil and grease only), and gravimetric determination were used [22,23].

3. Results

In every group of experiment, removal efficacy of oil was inspected to evaluate the effect of oil treatment by crude HRP.

3.1. Effect of enzyme concentration and pH on oil removal

In order to determine the effect of enzyme concentration on oil removal from oily water, some sets of reactions were conducted under the conditions of different low purity HRP concentrations, in reaction mixture which have varied pH from 5 to 9 in nine levels at three concentrations of oily water (20, 60 and 120 mg/L) and 1–4 mM H_2O_2 for each oil concentrations, respectively. The results of the experiments were shown in Figs. 1–3.

Results show that at the beginning, there are direct relationship between removal efficacy and the amount of HRP to 2U/mL (Maximum removal efficiency was 58% in 20–120 mg/L oil at H_2O_2; 1 mM). But when the amount of HRP was reached to larger than 2 U/mL, an increment in the removal efficacy became negligible.

The effect of oil concentration in optimum condition is presented in Fig. 4. As for the reason of the efficacy increment becoming small when the amount of HRP was large at different oil concentrations. In all of the experiments, pH 7.5 was optimum to enzymatic treatment with crude HRP.

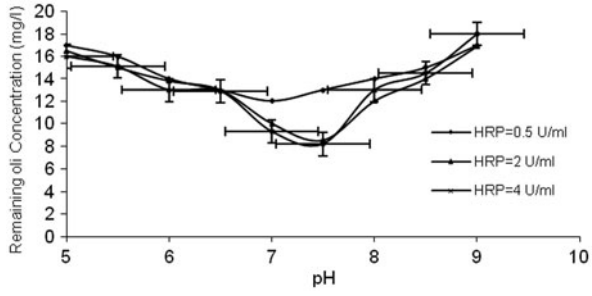

Fig. 1. Effect of enzyme concentration and pH on oil removal (20 mg/L oil; 180 min contact time; 1 mM H_2O_2; 25˚C).

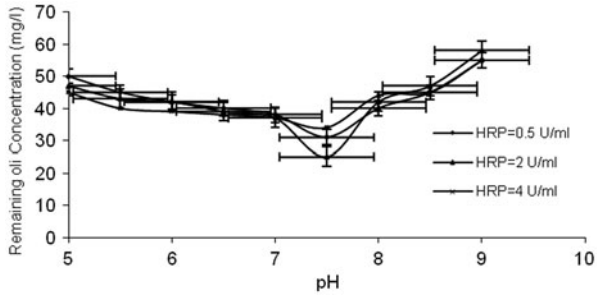

Fig. 2. Effect of enzyme concentration and pH on oil removal (60 mg/L oil; 180 min contact time; 2 mM H_2O_2; 25˚C).

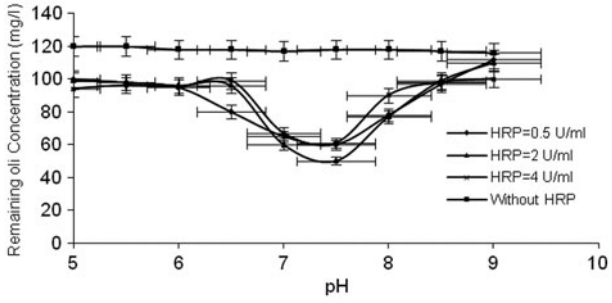

Fig. 3. Effect of enzyme concentration and pH on oil removal (120 mg/L oil; 180 min contact time; 4 mM H_2O_2; 25˚C).

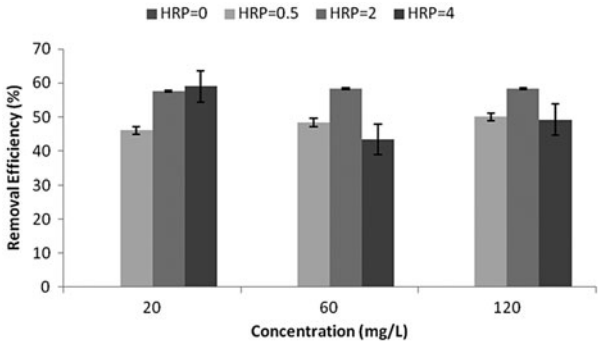

Fig. 4. Effect of oil concentration on oil removal (180 min contact time; 0–4 U/mL HRP; 4 mM H_2O_2; pH 7.4; 25˚C).

3.2. Effect of H_2O_2 concentration on oil removal

The H_2O_2 concentration was varied between 1 and 5 mM under HRP concentration of 2 U/mL. The results of experiments are illustrated in Fig. 5. According to the mechanism of HRP and H_2O_2 catalyzing, the results can be analyzed as the follows. When the H_2O_2 concentration was low, the amount of free radical products can be increased with an increase in the H_2O_2 concentration, so the coupling reaction of free radical forming polymers was promoted.

It is observed that the removal efficacy of oil increased with an increase in the H_2O_2 concentration at first, and reached maximum of 58.33% at the H_2O_2 concentration of 4 mM, and then decreased. Fig. 5 illustrated that excessive H_2O_2 inhibited the catalytic oxidation of the substrate by the crude HRP. The reason can be deduced as the follows. Excessive H_2O_2 could oxidize HRP, so the HRP and H_2O_2 which participated in the reaction were reduced. The byproduct of the oxidization of HRP by H_2O_2 might affect the microenvironment (after the reaction of the HRP and aromatic pollutants, HRP catalyzes the oxidation and polymerization of aqueous aromatic compounds in the presence of hydrogen peroxide, so this by product may adsorbed at the surface of the enzyme and cause inactivated this) around free and immobilized HRP and it is porous structure of carrier [11,14,16,24]. Too much polymerization products generated at the initial period because of over high H_2O_2 concentration inhibited the attacking of HRP to the substrate, so that the further HRP catalytic effect was inhibited [6,19]. In controls mixture: no H_2O_2, no oil removal was detected.

3.3. Effect of reaction temperature on oil removal efficiency

To examine the effect of temperature on oil removal, the polymerization and precipitation reactions were performed at optimal pH and temperatures from 25 to 45˚C under the same conditions. The results are presented in Fig. 6. The removal efficiencies decreased with an increase in the reaction temperature. It may be due to the lower solubility of the polymer at low temperature; that is, precipitation occurred without adsorption of enzyme on the polymers,

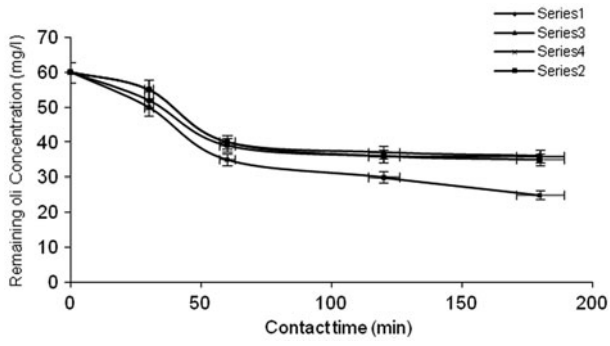

Fig. 5. Effect of H_2O_2 concentration on oil removal (60 mg/L oil; 0–180 min contact time; 0–5 mM H_2O_2; 2 U/mL HRP; pH 7.4; 25˚C).

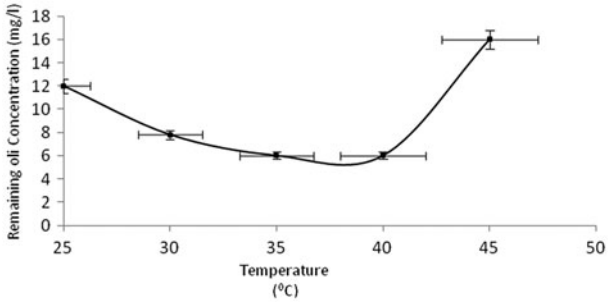

Fig. 6. Effect of reaction temperature on oil removal efficiency (20 mg/L oil concentration; 180 min reaction time; 2 U/mL HRP; 2 mM H_2O_2; pH 7.4)

resulting in extending catalyst lifetime at low temperature [12]. Another reason may be the lower concentration of free radicals, which reduce the enzyme inactivation. Also, at high temperature, solubility of oil was increased with the rising temperature to 40˚C. When the temperature was over than 40˚C, inactivation of HRP was occurred, so removal of oil was decreased. So the optimum temperature was 40˚C.

3.4. Effect of Fe^{2+} on oil removal efficiency

Normally there is Fe^{2+} ion in industrial wastewater because of the equipment corrosion; also HRP is a hem protein enzyme, so it is necessary to investigate its possible effects on enzymatic treatment of oily water. Tests were carried out at the Fe^{2+} ion concentration of 0–40 mM. The results of this study are shown in Fig. 7. Results show that Fe^{2+} ion has little effect on oil removal. It was reported that Fe^{2+} ion could interrupt the electron transport system of HRP and lead to the inhibition of substrate conversion [22]. According to our results, little change in oil removal with an increase in the Fe^{2+} ion concentration

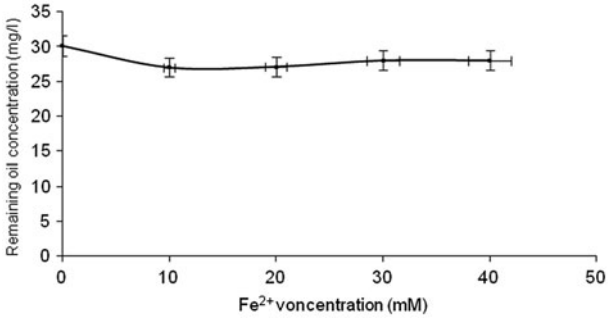

Fig. 7. Effect of Fe^{2+} on oil removal efficiency (60 mg/L oil; 180 min contact time; 4 mM H_2O_2; 2 U/mL HRP; pH 7.4; 25˚C)

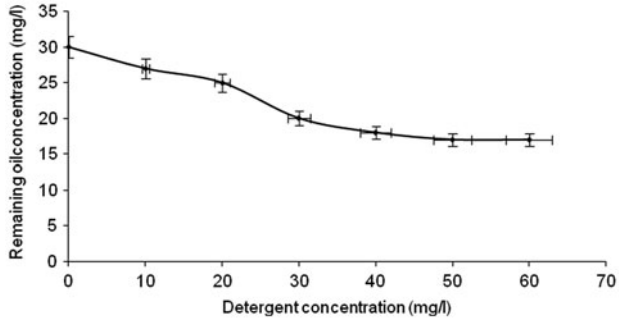

Fig. 8. Effect of detergent (Tween 80) on oil removal efficiency (60 mg/L oil; 180 min contact time; 4 mM H_2O_2; 2 U/mL HRP; pH 7.4; 25˚C)

was detected. So Fe^{2+} ion cannot significantly effects on HRP and H_2O_2 oxidation process.

3.5. Effect of detergent on oil removal efficiency

The positive effect of additives such as surfactants, polyethylene glycol, and gelatin on the oxidation of phenol catalyzed by peroxidases has been demonstrated in a number of earlier investigations [25]. Tween 80 (Sorbitan oleate ester or ethylene $oxide_{20}$) was used as detergent at 0–60 mg/L at optimum conditions (HRP 2 U/mL; H_2O_2 4 mM; 60 mg/L oil; 40˚C at 180 min contact time). The effects of surfactant on extent of oil removal in aqueous batch tests are shown in Fig. 8. Experimental results reported in this study indicate potential of nonionic surfactant (Tween 80) to positively influence HRP-mediated oil oxidation process. These observations indicate that using Tween 80 surfactant in oil removal could potentially increase enzyme performance and reduce treatment costs. It should be mentioned that surfactant binding to enzyme may activate enzyme, resulting in enhanced

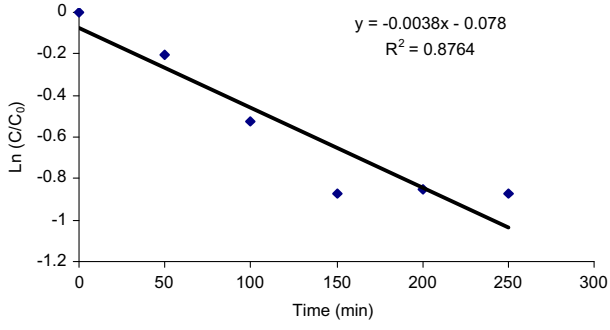

Fig. 9. Determining reaction order by plotting (first-order reaction).

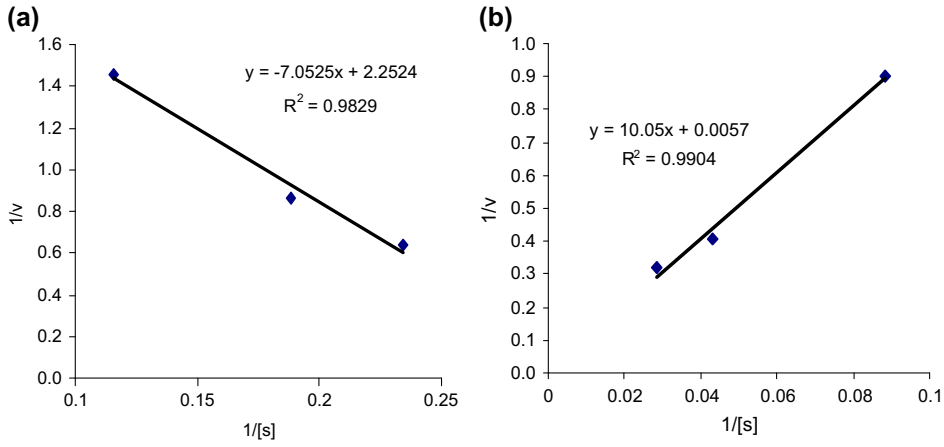

Fig. 10. (a) Michaelis–Menten model and (b) Michaelis–Menten model with inhibition by substrate.

Table 1
Apparent Michaelis–Menten parameters for the reaction of HRP with oil and H_2O_2

Oil (mg/L)	V_{max} (mM/min)	K_m (mM)	Cumulative error
20	0.45 ± 0.01	0.06 ± 0.001	0.016
60	1.02 ± 0.02	0.13 ± 0.002	0.024
120	2.04 ± 0.1	0.28 ± 0.01	0.073

Table 2
Apparent Michaelis–Menten with inhibition parameters for the reaction of HRP with oil and H_2O_2

Oil (mg/L)	V_{max} (mM/min)	K_m (mM)	K' (1/mM)	Cumulative error
20	1.05 ± 0.01	0.32 ± 0.002	0.24 ± 0.01	0.001
60	1.99 ± 0.02	0.65 ± 0.003	0.55 ± 0.01	0.001
120	2.97 ± 0.03	1.27 ± 0.02	0.65 ± 0.03	0.007

substrate conversion. Activation of enzymes by non-ionic surfactants has been widely documented [5,26]. These results show that from 0 to 30 mg/L Tween 80 increasing 25%in oil removal was detected. Increasing the normalized surfactant concentration to more than 30 mg/L resulted in only a small incremental increase in oil removal.

3.6. Kinetic study

Determining of the reaction order was carried out by curve fitting. Plot of $\ln C/C_0$ vs. reaction time shows that these enzymatic reactions occur in the first-order reaction by $R^2 = 0.876$. Determining reaction order by plotting, Michaelis–Menten model and Michaelis–Menten model with inhibition by substrate are shown in Figs. 9 and 10 respectively.

These models proposed by Michaelis–Menten that is usually used in enzymatic treatments. Eq. (1) is defined by Cornish-Bowden [14]:

$$V_i = \frac{V_{max}[H_2O_2]}{K_m[H_2O_2]} \quad (1)$$

where the variables V_i and $[H_2O_2]$ are, respectively, the apparent rate of the oil consumption and the H_2O_2 initial concentration. The apparent maximum reaction rate, V_{max}, and the apparent Michaelis constant K_m, were estimated by the least-squares approximation with the solver of MS Excel. Table 1 presents the values obtained for the kinetic parameters.

An alternative kinetic model based on Michaelis–Menten with inhibition was proposed.

The equation of the apparent reaction rate for the 120 mg/L of oil is at Eq. (2):

$$V_i = \frac{2.97[H_2O_2]}{1.36 + [H_2O_2] + 0.65[H_2O_2]} \quad (2)$$

Apparent Michaelis–Menten with inhibition parameters for the reaction of HRP with oil and H_2O_2 are presented in Table 2.

4. Discussion and conclusions

The results of this study have demonstrated the applicability of using crude horseradish peroxidase (HRP) enzyme for treating oily water. In summary, the experimental results reported in this study indicate optimum HRP concentration for 20, 60, and 120 mg/L oil were 2 U/mL and pH 7.5, so the oil concentration was not effect on HRP enzyme needed. Also, mixture with H_2O_2 and free HRP has not significantly effect on oil removal, these results in earlier of our publication were approved for dye detoxification. Removal efficacy of oil increased with an increase in the H_2O_2 concentration at first, and reached maximum of 58.33% at the H_2O_2 concentration of 4 mM, and then decreased. Also HRP alone has no effect on oil removal. Temperature has significant effect on oil removal but increasing over than 40˚C, inactivation of HRP was occurred, so removal of oil was decreased. So higher temperature to enzymatic treatment of oil was 40˚C. Presence of Fe^{2+} ion in mixture solution has no effect on enzymatic treatment. This study indicates the potential of the nonionic surfactant (Tween 80) to positively influence the HRP-mediated oil oxidation process. Results of this study were found to be independent of the enzyme purity and therefore, it was possible to utilize a crude enzyme preparation instead of a purified one. This feature leads to a significant reduction in treatment costs. These reduced amounts of enzyme can make this method more economically competitive with the conventional treatment methods. Enzymatic reactions occur in the first-order reaction by $R^2 = 0.876$. The enzymatic reaction in the batch reactor follows the Michaelis–Menten equation with inhibition when the kinetic data are obtained as a function of the initial H_2O_2 concentration. But, different kinetic parameters were found for different initial oil concentrations. The treatment of oily water using crude HRP and H_2O_2 is feasible.

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