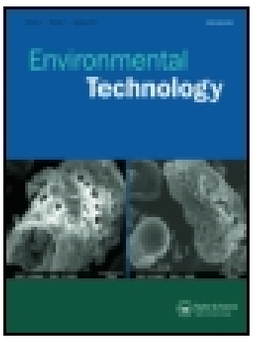

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Removal of acetaminophen in water by laccase immobilized in barium alginate

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ABSTRACT

This research has focused on the optimization of immobilized laccase condition and utilization in degradation of acetaminophen contaminated in aqueous solution. Laccase from *Lentinus polychrous* was immobilized in barium alginate. The effects of laccase immobilization such as sodium alginate concentration, barium chloride concentration and gelation time were studied. The optimal conditions for immobilization were sodium alginate 5% (w/v), barium chloride 5% (w/v) and gelation time of 60 min. Immobilized laccase was then used for acetaminophen removal. Acetaminophen was removed quickly in the first 50 min. The degradation rate and percentage of removal increased when the enzyme concentration increased. Immobilized laccase at 0.57 U/g-alginate showed the maximum removal at 94% in 240 min. The removal efficiency decreased with increasing initial acetaminophen concentration. The K_m value for immobilized laccase (98.86 μM) was lower than that of free laccase (203.56 μM), indicating that substrate affinity was probably enhanced by immobilization. The immobilized enzyme exhibited high activity and good acetaminophen removal at pH 7 and temperature of 35°C. The activation energies of free and immobilized laccase for degradation of acetaminophen were 8.08 and 17.70 kJ/mol, respectively. It was also found that laccase stability to pH and temperature increased after immobilization. Furthermore, immobilized laccase could be reused for five cycles. The capability of removal and enzyme activity were retained above 70%.

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KEYWORDS
Acetaminophen; laccase; immobilization; barium alginate; removal

1. Introduction

The presence of pharmaceuticals in aquatic environment has been considered as an emerging environmental problem since they are of organic toxicity, poor biodegradability and chemically persistent in the environment. A large number of pharmaceutical substances are produced and used yearly in human and veterinary medicine. Considerable amounts of these substances can reach the aquatic environment since many pharmaceuticals are not completely metabolized and ingested in the body of humans and animals. As a result, pharmaceutical residues are excreted with urine and feces into the sewage system [1,2]. Furthermore, the unused and expired pharmaceuticals are improperly disposed and they might be introduced into the sewage system. However, pharmaceutical substances are designed to have a complex structure and these substances often are not completely eliminated when using conventional wastewater treatments. As a result of incomplete removal, the pharmaceutical residues can be detected in final effluents and then they are released into lakes and rivers, leading to the contamination of surface water, ground water and drinking water [3,4]. Levels of these substances have been found at the range of nanogram to microgram per liter and some substances can reach a the range of milligram per liter [4]. Once the substances enter the environmental waters, they may have dramatic effects on aquatic life and human beings even at trace levels.

Acetaminophen (APAP), commonly named as paracetamol (N-acetyl-4-aminophenol), is one of the most popular and widely used medicines to relieve fever and pain due to tension headache, muscular aches, backache, toothache and general pain. It can be easily purchased in retail stores over-the-counter or as a prescribed medicine. APAP is considered to be safe for use at therapeutic dosage but acute overdose could cause fatal liver damage and death. When ingested in therapeutic dosage, about 58–68% of APAP is excreted from the body [5]. Due to the huge production and high consumption worldwide, APAP has become one of the most frequently detected pharmaceuticals in the aquatic environment. The quantities of APAP removed from contaminated waters by conventional treatment systems vary since these systems are not specially designed for pharmaceutical removal. The chance of APAP distribution to natural water thus increases and

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this can result in environmental impact on the ecosystem and aquatic organisms and human health effects. APAP has been detected in the aquatic environment with concentrations in the range of nanogram per liter to microgram per liter [6]. In surface water, APAP concentrations were found at levels up to 260 ng/l [7]. Up to 1.89 µg/l was detected in groundwater [8] and 30 µg/l in a sewage treatment plant [9]. Recently, the treatments of APAP by several physiochemical and electrochemical methods have been reported [3,10–12]. For example, the adsorption process removed APAP (100 ng/l–1 mg/l) with the percentage ranging from 45% to 85% [13,14]. About 53% removal of 5 mM APAP can be achieved by ozone oxidation [15]. However, these treatment processes have limitations on the disposal and the regeneration of absorbents, the formation of undesirable by-products, high treatment cost and high chemical consumption. As an alternative, the use of enzymes provides a benign option with advantages such as lower energy requirements, mild operational conditions and less undesirable pollutants after degradation.

The potential use of enzyme for the treatment of phenolic and non-phenolic compounds from water has been reported. In particular, oxidoreductive enzymes such as peroxidase, tyrosinase and laccase have shown their capabilities to oxidize these compounds including recalcitrant environmental pollutants such as pesticides, dyes, pharmaceutical and personal care products. According to literature reviews [16–18], as a group, laccase (benzenediol: oxygen oxidoreductase, EC 1.10.3.2) demonstrates strong potential use in various applications and does not need hydrogen peroxide for the oxidation unlike peroxidase. Laccase is a multicopper oxidase and is capable of catalyzing the oxidation of a wide range of phenolic compounds and aromatic amines through the reduction of molecular dioxygen to water [19]. The capability of many microorganisms to produce laccase has been reported from fungi [20], insects [21] and bacteria [22–24]. Recent studies indicated that most fungi, especially white-rot fungi, produce high yields of extracellular laccase [25]. Because of the extensive cultivation of *Lentinus polychrous* in Thailand and its ability to degrade many phenolic compounds [26,27], the white-rot fungus, *L. polychrous*, was chosen in this work. However, low stability, high cost associated with isolation and purification and poor reusability of the enzyme has limited its use. In order to enhance the utility of the enzyme in treatment processes, many efforts have been focused on the preparation of enzyme in immobilized form including a variety of support materials and methods of immobilization. Among the different immobilization methods, entrapment in alginate gel may be a good alternative since it presents the advantage of mild process, being simple, low cost, biocompatible matrix and causing little damage to the native structure of the enzyme. Alginate is a polysaccharide composed of guluronic and mannuronic acid and forms gel beads with divalent ions like barium, calcium, copper and strontium. Several studies have been published about the application of immobilized laccases using calcium alginate or copper alginate [28–30]. Although calcium has been widely used as the cross-linking ion, other divalent ions have higher affinity toward alginate and can form stronger gels compared to calcium. The affinity of alginate for divalent ions has been shown in the following order: $Pb^{2+} > Cu^{2+} > Cd^{2+} > Ba^{2+} > Sr^{2+} > Ca^{2+} > Co^{2+} > Ni^{2+} > Zn^{2+} > Mn^{2+}$ [30]. The degree of cross-linking depends on the ability of the ions to diffuse through the film and on their ionic size. Copper ions could then fill a larger space between the alginate molecules, providing a tighter arrangement than with calcium ions. However, the Cu^{2+} concentration can affect the strength of the gel bead and the pore size in the gel structure. The leakage of laccase from the cross-linking in the gel bead was promoted up to 45–55% at high concentrations of Cu^{2+} [29]. In 2001, Barranco-Florido et al. have reported that the use of Ba^{2+} as a gel-forming agent improves the mechanical resistance of the alginate matrix [31]. Therefore, to the best of our knowledge, the entrapment technique using barium alginate was then first applied to laccase to remove APAP from water in this work. Suitable conditions for laccase immobilization by this technique have not been well determined. Therefore, there is a need to thoroughly investigate the optimum condition before use. In addition, the capabilities of immobilized laccase to remove APAP at different operating conditions such as enzyme loading, APAP concentration, pH value, temperature and reusability were assessed.

2. Materials and methods

2.1. Materials

Trichloroacetic acid was purchased from Sigma-Aldrich Co. Ltd, Germany. Sodium alginate, Bovine serum albumin (BSA), Coomassie Brilliant Blue G250 and 2, 2′-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) were obtained from Fluka, UK. All other chemicals were of analytical grade and used without further purification.

2.2. Production of laccase

Laccase was produced by cultivation of the white-rot fungus *L. polychrous* Lev. (obtained from Rujira Farm,

Kalasin, Thailand) on sterile rice bran and rice husk as described in Sarnthima et al. [32]. After 14 days of cultivation, the solid culture was stirred with distilled water at a ratio of 1:3 (w/v) for 45 min and then filtered through a miracloth. The filtrate was clarified by centrifugation at 4000*g* for 10 min and the supernatant containing the laccase was used for the enzyme immobilization.

2.3. Activity assays

Laccase activity was assayed using ABTS as a substrate. The modified assay was previously described by Sarnthima and Khammuang (2007) [27]. Briefly, the assay mixture consisted of 0.1 M acetate buffer pH 4.5, 10 mM ABTS and the enzyme sample. The mixture was incubated at 32°C for 10 min and the reaction was stopped with 50% w/v trichloroacetic acid. The activity of the enzyme was detected at the absorbance of 420 nm. One unit of enzyme activity was defined as the amount of enzyme required to oxidize 1 µmol of substrate per minute.

Protein concentration was determined using the Bradford method [33] based on the color change of Coomassie Brilliant Blue G250 in response to binding with proteins. The protein content was measured at the absorbance of 595 nm. BSA was used as a standard to construct the calibration curve.

2.4. Laccase immobilization

Laccase was immobilized within barium alginate beads. The required amount of sodium alginate was dissolved in deionized water under stirring condition. A quantity of laccase solution was added to the sodium alginate solution at ratio of 1:10 under gentle mixing. Beads were formed by dropping sodium alginate solution containing laccase through a syringe fitted with a needle into 100 ml $BaCl_2$ solution. After 60 min of hardening, the beads were removed from the $BaCl_2$ solution. After completing immobilization, the derivatives were washed twice with distilled water and drained. All immobilized beads were stored at 4°C until further use.

In order to achieve high immobilization yield, low enzyme leakage and to preserve enzyme activity, the different weights of sodium alginate (1–5%w/v), barium chloride (1–9%w/v) and time of gel formation (30, 60, 90, 120 and 150 min) were tested. Immobilization yield was defined as the percentage of the total immobilized enzyme activity to free enzyme.

The stability of immobilized laccase was studied by incubating the beads in the buffers of different pH (5.0, 7.0 and 9.0) for different time intervals. After incubation in buffer, the beads were washed with assay buffer and their activity was determined by ABTS.

2.5. APAP removal studies

Effect of parameters such as laccase concentration (0.03–0.57 U/g alginate), APAP concentration (1–100 mg/l), pH value of APAP solution and temperature were studied on APAP removal using laccase immobilized in barium alginate. The experiments were conducted in 500 ml conical flasks. The reaction mixture, containing 50 g of immobilized laccase and 300 ml of APAP solution, was incubated statically for 4 h at room temperature. Samples were withdrawn from the flask during incubation time and the residual APAP concentration analyzed. All the experiments were examined in triplicate. To determine possible removal of APAP due to adsorption onto the alginate beads, heat denatured laccase was immobilized and used in place under the same experimental condition.

APAP concentration was determined using the spectrophotometric method as modified by Nagendra [34]. The method is based on the reaction of APAP with 2 mM potassium ferricyanide and 0.1 M ferric chloride. The mixture was set for 10 min. Then 1 ml of 5 M hydrochloric acid was added. After 20 min, Prussian bluish green colored product was formed and measured at the absorbance of 715 nm. The absorbance values were transformed to APAP concentration using a calibration curve.

2.6. Reusability of immobilized laccase

The experiments were carried out in batch mode by repeatedly reusing a defined amount of immobilized laccase in removal of APAP. After each cycle lasted 60 min, the beads were separated and washed with deionized water. The reaction solution was then replaced with fresh APAP solution.

2.7. Scanning electron microscope

Scanning electron microscope (SEM) was used to study the morphology of immobilized laccase in barium alginate. The alginate beads were fixed with 2.5% glutaraldehyde and then dehydrated in ethanol. The dried samples were sputter-coated with gold and micrographs were observed using SEM (JEOL, JSM-5410LV, Tokyo Japan). All the images were taken at a magnification of 3500×.

2.8. Statistical analysis

Experiments were carried out in triplicate and the mean values were calculated using the Microsoft Excel Spreadsheet Program. The values in the results correspond to mean values with a standard deviation lower than 5%.

3. Results and discussion

3.1. Optimum conditions of laccase immobilization

Immobilization using alginate as a support is affected by many factors. To obtain the optimal condition for immobilization, three factors were taken into consideration; immobilization yield, enzyme leakage and stability. The experiments have been examined using different concentrations of sodium alginate, barium chloride and gelation time. The strength of alginate beads is highly dependent on cross-linking between alginate anions and Ba^{2+} cations to form beads. The results are presented in Table 1.

Firstly, to study the effect of alginate on the formation of immobilized laccase, the alginate concentration was varied from 1%w/v to 5%w/v, maintaining constant the $BaCl_2$ concentration (5%w/v) and gelation time (60 min). As presented in Table 1, the alginate concentration increased from 1%w/v to 3%w/v, and the immobilization yield increased (89.01–93.29%). Further extension of concentration up to 5%w/v resulted in only slight improvement. For consideration in terms of enzyme leakage, the lower alginate concentrations gave higher enzyme leakages which may have been due to the lower strength of the alginate matrix, making the beads more fragile, resulting in enzyme leakage from the beads [35]. Additional alginate concentration may affect the pore size of the beads and the degree of cross-linking of the gelling agent. The lower concentration of alginate results in the greater pore size of the beads leading to higher leakage [28].

The operational stability of the immobilized enzyme is one of the important considerations for economical use.

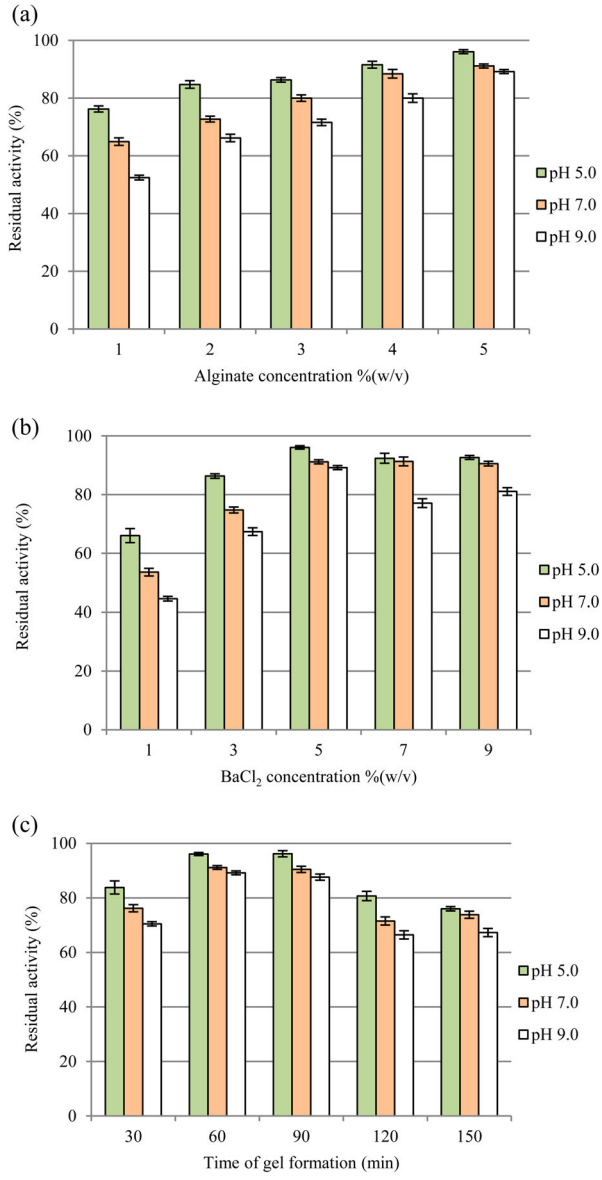

Figure 1. pH stability of immobilized laccase. (a) Effect of alginate concentration; (b) Effect of $BaCl_2$ concentration; (c) Effect of gelation time.

The stability of immobilized laccase with different alginate concentrations was also tested by incubating the

Table 1. Immobilization yield and enzyme leakage under different immobilized conditions.

Test 1	$BaCl_2$ concentration 5%(w/v)				
Sodium alginate %(w/v)	1	2	3	4	5
Immobilization yield %	89.0 ± 0.8	89.6 ± 0.5	93.3 ± 0.3	93.5 ± 0.1	93.6 ± 0.1
Enzyme leakage after 24 h (%)	38.5 ± 1.2	31.6 ± 3.1	25.3 ± 1.5	18.9 ± 2.3	16.7 ± 2.7
Test 2	Sodium alginate concentration 5%(w/v)				
$BaCl_2$%(w/v)	1	3	5	7	9
Immobilization yield %	90.3 ± 0.5	93.1 ± 0.7	93.6 ± 0.1	94.7 ± 0.2	95.2 ± 0.2
Enzyme leakage after 24 h (%)	55.6 ± 1.2	40.0 ± 1.8	16.7 ± 2.7	27.62 ± 1.9	29.5 ± 2.2
Test 3	Sodium alginate and $BaCl_2$ concentrations 5%(w/v)				
Gelation time (min)	30	60	90	120	150
Immobilization yield %	94.4 ± 0.2	93.6 ± 0.2	94.2 ± 0.2	93.9 ± 0.5	91.6 ± 0.4
Enzyme leakage after 24 h (%)	29.5 ± 1.3	16.7 ± 2.7	30.5 ± 1.7	32.5 ± 2.1	31.6 ± 2.1

beads in buffers of various pHs (5.0, 7.0 and 9.0) at 25°C for 240 min (Figure 1(a)). Then the residual activity was determined. Immobilized laccase with 5%w/v alginate maintained the highest residual activity. The residual activities of immobilized laccase at pH 5.0, 7.0 and 9.0 were 96%, 91% and 89%, respectively. Consequently, the alginate concentration of 5% w/v was selected for the next step because of high immobilization yield, low enzyme leakage and remaining good activity in the buffers of various pH. However, the optimum concentration of alginate may differ with respect to the enzyme; for example, Rehman et al. [35] mentioned that alginate concentration had a significant effect on the formation of a strong cross-linked gel and entrapment of the enzyme. The maximum immobilization yield of pectinase was observed with 3%w/v of alginate concentration, whereas the concentration beyond this point decreased the immobilization yield. Ghattas et al. [36] studied factors affecting lipase immobilization with calcium alginate for monoolein production. The optimum sodium alginate concentration was only 2% w/v. They conclude that an increase in sodium alginate retarded penetration of calcium to the interior of the beads, resulting in decreased cross-linking and porosity.

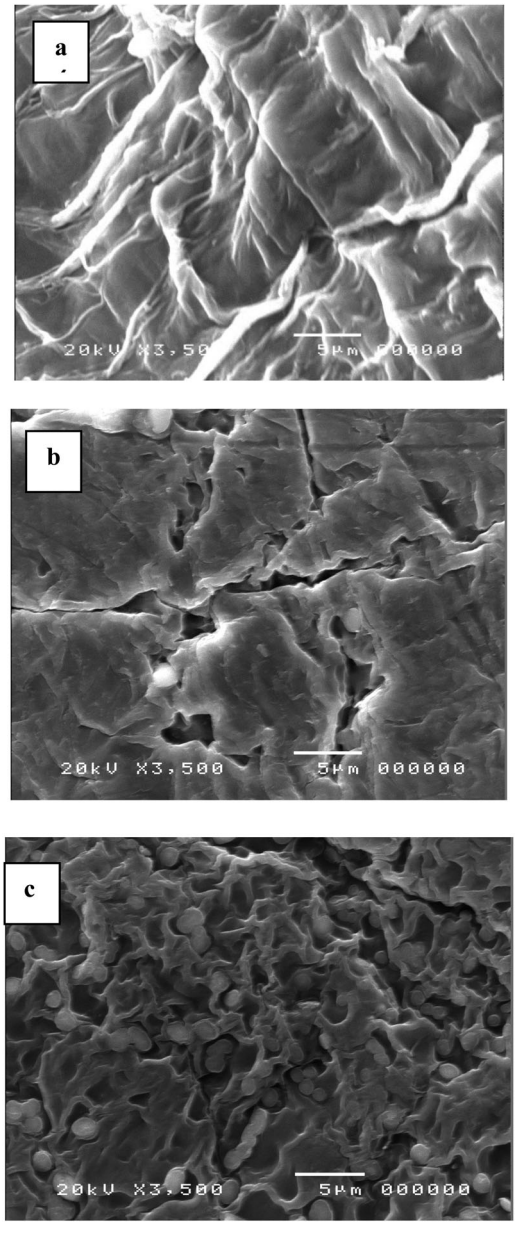

Figure 2. SEM images of the immobilized bead surface before use (a), a bead after one cycle use (b) and a bead after five cycles of use (c)

In case of changing $BaCl_2$ concentrations, the sodium alginate was kept constant at 5%w/v, the immobilization yield was increased with increasing of $BaCl_2$ concentration (Table 1). Laccase may easily leak out from the beads at a low $BaCl_2$ concentration because of unstable cross-linked gel formation and low dispersion of Ba^{2+} cations to form beads. Then an increase in $BaCl_2$ concentration from 1% to 5% decreased the enzyme leakage. The minimum enzyme leakage was observed at 5%w/v. Furthermore, immobilized laccase with 5%w/v $BaCl_2$ also maintained the highest residual activity (Figure 1 (b)). Above the concentration of 5%w/v, the residual activity tended to decrease. Then $BaCl_2$ probably damaged or had a little effect on the enzyme activity. These results indicate that 5%w/v $BaCl_2$ provided the best conditions for beads' hardening to maintain the stability, although the percent immobilization yield was maximal at a $BaCl_2$ concentration of 9%w/v. However, some previous studies mentioned that the diffusion of high molecular weight substances out of the beads into the bulk solution was little affected by increases in divalent ion concentration but it was greatly limited by increases in alginate concentration [28,37].

To determine the proper time of exposure of the beads to the hardening solution, the concentrations of both alginate and $BaCl_2$ were fixed at 5%w/v. As presented in Table 1, there was no significant difference in the immobilization yield. The minimum enzyme leakage was achieved in 60 min of gelation time. Above 60 min, the leakage increased. The residual activity of immobilized laccase in the buffers of various pHs obviously decreased as the gelation time increased from 90 to 150 min (Figure 1(c)). This could be because longer gelation time provides more time for cross-linking in the matrix and this might cause some damage to the hybrid matrix. Bhujbal et al. [38] studied the effect of gelation time on the stability by incubating the beads from 5 to 20 min and found that gelation time of more than 15 min decreased the strength of the beads. Similar previous studies have suggested that increasing the gelation time caused beads to become weak [38,39].

From these results, 5% sodium alginate, 5% barium chloride and a gelation time of 60 min were chosen as the optimum conditions for the formation of immobilized laccase with suitable enzyme yield, minimum enzyme leakage and good stability. These conditions were then used throughout the rest of the experiments.

3.2. Scanning electron microscopy

The alginate beads' structures were studied using SEM and the images are shown in Figure 2. Figure 2(a) shows the surface of immobilized laccase on barium alginate at the optimum sodium alginate of 5%w/v, barium chloride of 5%w/v and gelation time of 60 min. The characteristic of surface structure was tightly compact. The outer surface of the immobilized bead after use in APAP removal in the fifth cycle (Figure 2(c)) was apparently more porous and rougher than in the first cycle (Figure 2(b)). The pores were evident on the surface to provide the leakage of laccase out of the beads.

3.3. Effect of initial APAP concentration on the removal and kinetic studies

The influence of initial APAP concentration on the removal by laccase immobilized in barium alginate was investigated. The reaction system was controlled at pH 7.0 and 25°C. APAP concentration was varied from 1 to 100 mg/l. As shown in Figure 3, the APAP removal continuously increased during the initial 120 min, followed by a gradually slower removal during the remaining period. It was also found that APAP removal at the same reaction time was lower when higher concentration of APAP was used. The highest removal (94%) was clearly obtained at 1 mg/l of APAP. Since the binding between substrate and the enzyme was limited, higher concentration of substrate then needed more reaction time to reach the same level of removal. Thus APAP concentration at 1 mg/l was applied for subsequent experiments in this study.

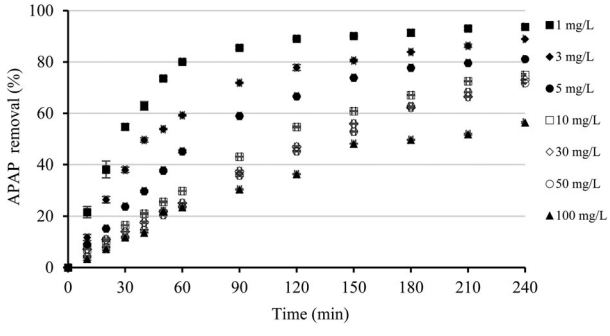

Figure 3. Effect of APAP concentration on the removal.

In general, when the substrate concentration is gradually increased and the enzyme concentration is kept constant, the reaction rate will increase until it reaches the maximum. After reaching the equilibrium state, any further addition of the substrate will not affect the reaction rate. This indicates that the concentration of substrate has a significant influence on the enzyme. The effects of APAP concentration on the initial reaction rate of both free and immobilized laccase are shown in Table 2. The results showed that the initial rates of reaction of both forms increased when higher APAP concentration was employed. At the same APAP concentration, the immobilized laccase had lower initial rate in comparison to free laccase. The data from Table 2 are also used to determine the kinetic parameters for both free and immobilized laccase. Maximum reaction rate (V_{max}) and Michaelis–Menten constant (K_m) are evaluated from the Lineweaver–Burk plot of 1/V versus 1/S. V_{max} is defined as the highest reaction rate when all the enzyme is completely saturated with substrate and K_m value indicates a measure of the dissociation of enzyme–substrate complex. K_m values of free and immobilized laccase as calculated from Table 3 are 203.56 and 98.86 µM, respectively; and corresponding V_{max} values are 15.75 and 1.38 µmol/min. It was observed that there was a decrease in K_m value after immobilization. Comparison of K_m value for a specific enzyme in both free and immobilized states can result in an increase or decrease. The apparent K_m decreases probably because the charges on the support and the substrate are opposite which leads to affinity of the substrate to the polymer matrix than by increase of the enzyme–substrate affinity. The other reason might be because the freedom of immobilized enzyme is less than that of free enzyme, the probability of immobilized enzyme active sites contacting substrate is less than that for the free enzyme, leading to a decreased affinity between immobilized enzyme and substrate [40]. In contrast, an increase in K_m after immobilization implies that immobilized enzyme has an apparent lower affinity for substrate

Table 2. Initial reaction rates of free and immobilized laccase as a function of APAP concentration.

APAP concentration (µM)	Initial reaction rate (µM min^{-1})		1/Initial reaction rate (µM^{-1} min)	
	Free laccase	Immobilized laccase	Free laccase	Immobilized laccase
7	0.5	0.09	2.00	11.30
20	1.32	0.20	0.76	5.10
33	1.89	0.26	0.53	3.78
66	4.17	0.33	0.24	3.03
198	9.11	0.83	0.11	1.21
331	12.04	1.32	0.08	0.76
662	16.96	2.62	0.06	0.38

Table 3. Kinetic constants for the oxidation of acetaminophen by free and immobilized laccase.

Kinetic parameter	Free laccase	Immobilized laccase
V_{max} (μM min^{-1})	15.75	1.38
K_m (μM)	203.56	98.86

which might be caused by the following reasons: (i) steric hindrance found by the substrate molecules in reaching the active site on the support, (ii) loss of enzyme flexibility for substrate binding and (iii) diffusion resistance to solute transport near the particles of the support [41–43]. The immobilization procedure also influences the V_{max} value. The decrease of maximum reaction rate after immobilization is probably due to the substrate diffusion limitation effect caused by support.

3.4. Effect of laccase concentration on APAP removal

The effect of the concentration of enzyme used during the immobilization step was studied. The optimization of enzyme concentration was considered to achieve the maximum APAP removal and initial reaction rate relative to the economical use of the enzyme. Five different enzyme concentrations immobilized in barium alginate were used to remove initial APAP concentration of 1 mg/l at pH 7.0 and 25°C. The reaction was conducted for 240 min.

The relationship between enzyme concentration immobilized in alginate and APAP removal is presented in Figure 4. The results showed that the enzyme concentration had a significant influence on the APAP removal. The percentage of APAP removal gradually increased with increasing in enzyme concentration from 0.03 to 0.57 U/g-alginate. More than 90% of APAP removal was observed at 0.57 U/g-alginate enzyme concentrations and this concentration was applied for the next experiments. Moreover, the effect of enzyme concentration on the initial rate of enzymatic APAP degradation is also shown in Figure 4. Plotting of the initial rate versus concentration indicated that rate of degradation seemed to be linearly proportional to the enzyme concentration when using enzyme up to 0.29 U/g-alginate and after this concentration the initial rate slowly increased. The progress curve shows a hyperbolic form. The initial rate tends to almost reach a maximum rate when higher enzyme concentration is used.

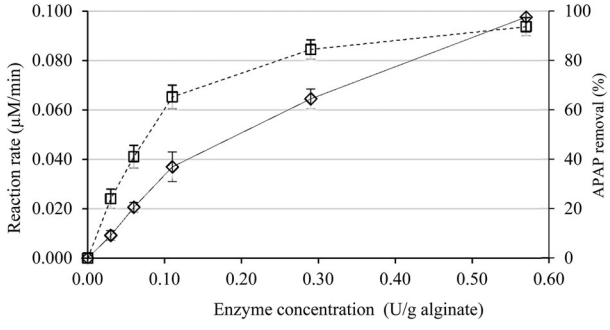

Figure 4. Effect of the concentration of enzyme used during the immobilization step on initial reaction rate and APAP removal.

3.5. Effect of pH on APAP removal

The role of pH in the range of 4.0–8.0 on APAP removal by immobilized laccase was determined at 25°C for 240 min. The immobilized laccase and APAP concentrations were kept constant at 0.57 U/g-alginate and 1 mg/l, respectively. The optimum pH value was observed at pH 6.0 and 7.0 with maximum removal of both pH values at about 94%. The removal efficiency decreased rapidly to less than 5% at pH 8, then less dramatically between pH 4 and 5 with removal of about 48% and 69%, respectively.

Relative activities of both free and immobilized laccase as a function of pH are also compared in Figure 5(a). It can be seen that the relative activity of immobilized enzyme was higher over most of the pH values as compared to the free enzyme. A hundred percent of relative

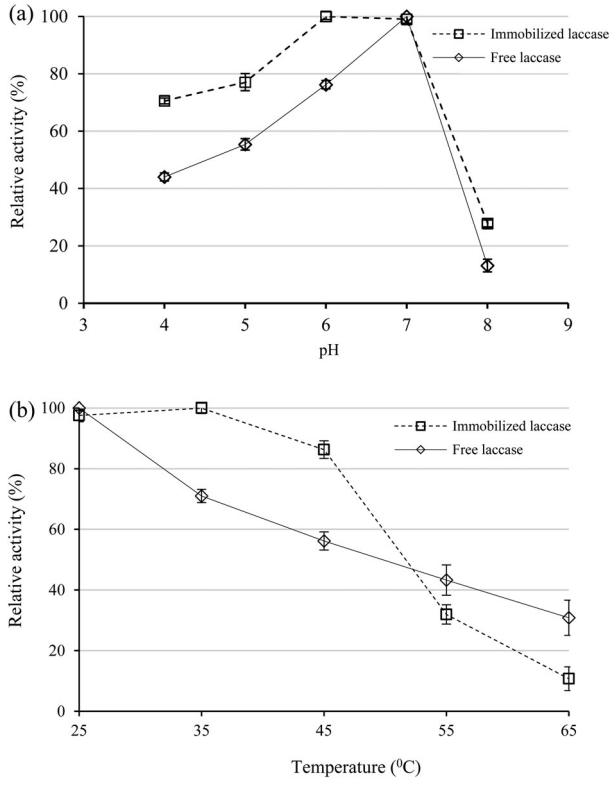

Figure 5. Activity profile as a function of (a) pH and (b) temperature for free and immobilized laccase.

activity for the free laccase was obtained at an optimum pH value of 7 while for the immobilized laccase it was from pH 6 to 7. Above pH 7.0 both forms of the enzyme drastically lost the activity. These results indicated that the immobilized form was able to tolerate a broader range of pH values and was less sensitive to pH changes than the free enzyme which was likely to be an advantage in the application of immobilized enzyme. This may be because of the protection effect provided by the binding with the carrier. Strong interaction between the enzyme and carrier could prevent conformational changes in enzymes [44].

3.6. Effect of temperature on APAP removal

Temperature is a crucial reaction parameter for any catalytic reaction which increases the chance of molecular collision–interaction. To determine optimal reaction temperature, the effect of the temperature on the reaction of immobilized enzyme catalyzed APAP removal was investigated at constant enzyme concentration (0.57 U/g-alginate), APAP concentration (1 mg/l) and pH (7.0). The highest percentage of APAP removal for free laccase was obtained at 25°C and above this point, the enzyme gradually lost the activity while the percentage of removal for immobilized laccase increased with temperature increasing in the range between 25°C and 35°C (Figure 5(b)). The temperature in this range did not have a great difference on the final APAP removal efficiency. In case the temperature was further increased (45–65°C), the removal decreased. The results were in a good relation with retaining enzyme activity (data not shown). The maximum retaining enzyme activity was at 35°C. Above 65°C the immobilized laccase retained a few percentages of activity. This phenomenon is expected since the active sites of any enzyme are temperature sensitive and beyond a specific temperature, the active sites are denatured.

The effect of temperature on enzyme kinetics is often described with the Arrhenius equation. Then the Arrhenius plot for the removal of APAP catalyzed by free and immobilized laccase was established within the temperature range of 25–55°C as data shown in Table 4. Increase in the rates of APAP degradation was observed when the temperature increased. Natural logarithm of the reaction rate constant is graphed against 1/T (plotting not shown). The correlation of the data provided a straight line. Activation energy (Ea) was evaluated from a slope equal to –Ea/R. The calculated Ea values for free and immobilized laccase on the change of APAP concentration were 8.08 and 17.70 kJ/mol, respectively. Lower Ea value for free laccase indicates the lesser energy requirement and maximum velocity for the corresponding biotransformation as compared to immobilized laccase. It is often found that the activation energy of the immobilized form is higher than that of the free enzyme. The reason for this could be in reduction of conformational flexibility, resulting in higher activation energy for the molecule to reorganize and attain the proper conformation for binding to the substrate [45].

3.7. Repeated use of immobilized laccase

Since most of the enzymes are expensive, the ability to be used repeatedly without significant fall in activity is one of the most important properties of immobilized enzymes. In this study, the reusability of immobilized laccase was tested in batch reactions and the same conditions were used for all batches. The activity observed for each repetition was compared with the initial activity, assuming that it was 100%. The catalytic activity of the immobilized laccase was lasting under repeated use. The immobilized laccase was able to remove APAP more than 70% even after five cycles. After the 2nd, 3rd, 4th and 5th cycles, the percentages APAP removal were 83%, 78%, 75% and 74%, respectively. There was no drastic decrease in removal which could be due to the optimum conditions for the formation of immobilized laccase within barium alginate. However, the gradual decrease in removal in the subsequent cycles might be related to enzyme inactivation, conformational changes by repeated uses, the blocking of some pores of beads by the accumulation of substrate or reaction products or the leakage of enzyme from beads during washing at the end of each cycle [36]. Several studies have reported about the reuse of immobilized laccase. Daâssi et al. [28] found that laccase from *Coriolopsis gallica* immobilized in calcium alginate beads retained more than 70% of its activity after seven cycles of Remazol Brilliant Blue R (RBBR) decolorization and less

Table 4. Reaction constants of free and immobilized laccase as a function of temperature.

	Reaction constant (k)			−Ln (reaction constant (k))	
Temperature (K)	Free laccase	Immobilized laccase	$1/T \times 10^3$	Free laccase	Immobilized laccase
298	0.072	0.024	3.354	2.627	3.716
308	0.083	0.028	3.245	2.492	3.578
318	0.091	0.040	3.143	2.392	3.230
328	0.097	0.045	3.047	2.330	3.107

than 30% of its activity for Bismark Brown R [28]. A laccase from *Pleurotus ostreatus* immobilized in copper alginate could decolorize RBBR 20 times with high activity [46] and a laccase immobilized in alginate/gelatin beads retained 50% of its initial activity after 10 cycles of Reactive Red B-3BF decolorization [47].

4. Conclusions

Laccase produced from *L. polychrous* was immobilized within barium alginate beads. Optimum condition of immobilization was obtained at 5% (w/v) sodium alginate, 5% (w/v) barium chloride and gelation time of 60 min. The immobilized laccase was applied for the removal of APAP in water. The effect of the various reaction parameters was determined. The results showed that the immobilized laccase had a good potential to remove APAP. Maximum enzymatic removal of APAP was at neutral pH and the optimum temperature was at 35°C. Further study on the degradation products of APAP transformation by immobilized laccase is required. Considering the APAP transformation and toxicity studies after the APAP transformation are necessary to evaluate before discharge to the environment.

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