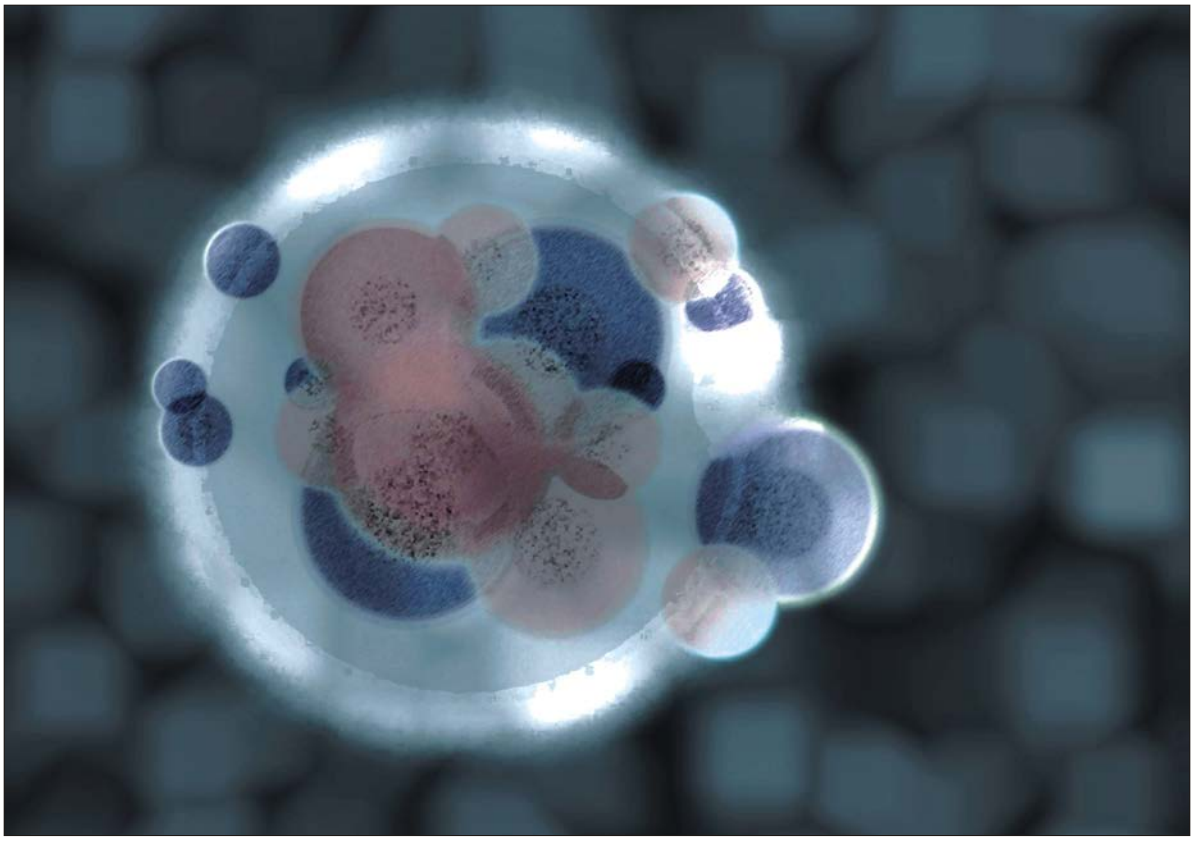

Journal of Biological Sciences

ISSN 1727-3048

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Journal of Biological Sciences 9 (6): 573-583, 2009
ISSN 1727-3048

Copper-Alginate Encapsulation of Crude laccase from *Lentinus polychrous* Lev. and their Effectiveness in Synthetic Dyes Decolorizations

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Abstract: Crude laccase from *Lentinus polychrous* Lev. was entrapped in alginate beads for application in decolorization of synthetic dyes. The percentages of alginate, types and concentrations of bivalent cations (Cu^{2+}, Zn^{2+}, Ca^{2+}) influenced on immobilized enzyme activity as well as immobilized yield. The sensitivity of the immobilized Cu-alginate enzyme towards pH change was lowest when compared to the immobilized enzymes of other bivalent cations including free enzyme. By using ABTS substrate, the optimum temperature of the Ca-, Cu- and Zn-alginate enzymes were 60, 55 and 55°C, respectively while the optimum temperature of the free enzyme was 50°C. The Cu- and Zn-alginate enzymes were well stabilized for a week in all tested pH values except for pH 8.0 (0.1 M Tris-HCl). The Cu- and Zn-alginate enzymes revealed better temperature stability than the Ca-alginate and the free enzymes. Decolorizations of four structurally different synthetic dyes by the immobilized Cu-alginate enzymes in a continuous pack-bed approach were reported. The Cu-alginate enzymes were being able to repeated use effectively more than two times in dye removal experiments.

Key words: Alginate bead, synthetic dyes, entrapment, laccase, *Lentinus* sp., ligninolytic enzyme

INTRODUCTION

Laccases (benzenediol:oxygen oxidoreductase, EC 1.10.3.2) are multi-copper oxidases which catalyze one electron transfer from its substrates coupling with reduce the molecular oxygen to water. These enzymes can be found in several sources including bacteria, fungi, insects and plants (Baldrian, 2006; Salis *et al*., 2009). The fungal laccase is secreted as one of ligninolytic enzymes which comprise of other two enzymes, lignin peroxidase (LiP; EC 1.11.1.14) and manganese peroxidase (MnP; EC1.11.1.13). From irregular structure of lignin render laccase can catalyst a wide range of substrates including ortho- and para-diphenols, aminophenols, polyphenols, polyamines, lignins and aryl diamines as well as some inorganic ions (Rodríguez Couto and Herrera, 2006). The enzyme has been received attention with high potential uses in industry such as the transformation of xenobiotics like synthetic dyes, chlorinated phenolics, polycyclic aromatic hydrocarbons, delignification in pulp and paper industry, organic synthesis and biosensors.

Many dyes have been used in textile industry as well as for other industrial applications. Some of these dyes are released into wastewater. This dye wastewater is usually treated by physical or chemical treatment processes. However, these technologies are usually inefficient in the removal of color, costly and little adaptable to a wide range of dye wastewater. More environmental friendly processes have been introduced for dyes decolorization. Using enzyme for treatment of textile dye wastewater has been more interested (Enayatzamir *et al*., 2009). Since, laccase can use a broad range of substrates including synthetic dyes. The enzyme has been reported for several dyes decolorization (Michniewicz *et al*., 2008; Wong and Yu, 1999). The soluble laccase used in those applications have been known for some disadvantages such as the stability of enzyme, cannot reused lead to a high cost. In order to increase the potential use of laccase in wastewater treatment processes, their immobilizations are necessary for biochemical stability and reusability (Peralta-Zamora *et al*., 2003).

We earlier reported that the fungus, *L. polychrous* Lev. secreted some extracellular ligninolytic enzymes (Khammuang and Sarnthima, 2007), mainly laccase and manganese peroxidase which laccase was partially

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purified, characterized and production as well as studied synthetic dyes decolorization (Sarnthima *et al*., 2009). In this present work reported for the first time of alginate entrapment of the crude enzyme and applied in decolorization of synthetic dyes.

MATERIALS AND METHODS

Materials and enzymes: Alginic acid sodium salt from brown algae (Sodium alginate) was obtained from Fluka, Norway. Calcium chloride dihydrate ($CaCl_2 \cdot 2H_2O$), Zinc sulfateheptahydrate ($ZnSO_4 \cdot 7H_2O$) and Copper sulfate pentahydrate ($CuSO_4 \cdot 5H_2O$) were purchased from Merck, Germany. Synthetic dyes, Remazol Brilliant Blue R (RBBR) was a product of Sigma, USA. Indigo carmine was a product of Fluka, UK, Bromophenol blue was from Carlo erba, France and Methyl red was from Merck, Germany. All other chemicals were of analytical grade and used without further purification.

Crude laccase from *L. polychrous* Lev. was prepared according to previous report (Sarnthima *et al*., 2009). Crude enzymes (specific laccase activity 6.99 U mg^{-1}) were used in this study without further purification.

Enzyme immobilization on alginate: Ten milliliters of *L. polychrous* Lev. crude enzyme solution (total laccase activity 10 U) was added with sodium alginate solution (1-4%, w/v) and then the mixture was stirred thoroughly to ensure complete mixing for 20 min. As soon as the mixed solution was added drop-by-drop by means of a peristaltic pump equipped with a micropipette tip (yellow tip, P200) into 50 mL of $CaCl_2$ or $ZnSO_4$ or $CuSO_4$ solution (50-300 mM) (flow rate 3 mL min^{-1}), corresponding metal-alginate beads were formed. After 45 min of hardening in each solution, the beads (about 3-4 mm in diameter) were separated from the hardening solution by filtration through a strainer. They were washed on a strainer two times with distilled water in the same volume of hardening solution. The each filtered hardening solutions and the two washings were collected for immobilization percentage determination according to following equation:

$$\text{Immobilization (\%)} = \frac{A_{load} - A_{wash}}{A_{load}} \times 100$$

where, A_{load} is total loaded activity into the mixture of Na-alginate solution assayed using ABTS as substrate and A_{wash} is laccase activity detected in curing solution and two washing solutions assayed using the same substrate. All immobilized crude laccase alginate enzymes were kept in distilled water at 4°C. A larger batch preparation of selected immobilized alginate enzymes were performed using 50 mL crude enzyme-alginate mixture (total laccase activity 50 U) and used for following study; pH and temperature optima, pH and temperature stability and synthetic dyes decolorizations.

Enzyme activity assay: The laccase activity in solution was assayed based on the oxidation of ABTS (Sigma, USA), according to the modified method of Shin and Lee (2000), as previous described (Khammuang and Sarnthima, 2007). Laccase activity of immobilized enzymes was assayed in the same way as free enzyme in aqueous solution, except for 4 beads per tube were used instead of enzyme solution.

Protein determination: Soluble proteins in the crude extract were determined by the Bradford (1976) method using Bio-Rad Protein Assay Reagent (Bio-Rad) with bovine serum albumin as a protein standard.

Optima pH and temperature of immobilized alginate laccase: To figure out the optimum pH of immobilized Cu-, Zn- and Ca-alginate enzymes. The immobilized enzymes (4 beads/tube) were assayed for laccase activity using ABTS as substrate in 0.1 M sodium acetate buffers pH ranging from 3.5 to 5.5 at 32°C for 10 min in comparison to free soluble enzyme. For optima temperatures of each immobilized enzymes, the assays reactions were performed in 0.1 M sodium acetate buffer, pH 4.5 at various temperatures ranging from 30 to 70°C for 10 min with ABTS substrate.

pH and temperature stability of immobilized Cu-alginate laccase: pH stability of each immobilized enzymes were followed up its laccase activity periodically after kept the beads in different pH values ranging from 2 to 8 comparison with those kept in distilled water at 4°C (pH 2.0 of HCl-potassium chloride buffer, pH 3.5-5.5 of sodium acetate buffer, pH 7.0, 8.0 of Tris-HCl buffer). For temperature stability experiments, each bead types were incubated in 0.1 M sodium acetate buffer, pH 4.5 at different temperatures ranging from 30 to 60°C in comparison to those kept at 4°C. The laccase activity in each conditions was assayed every 30 min for 4 h at a standard assay condition.

Synthetic dyes decolorization by immobilized Cu-alginate laccase: Four groups of synthetic dyes, RBBR (anthraquinone), Methyl red (azo), Indigo carmine (indigoid) and Bromophenol blue (triarylmethane) were selected as dye models for study decolorization by immobilized Cu-alginate laccase from *L. polychrous* Lev.

The experiments were set in a continuous pumping into a pack-bed immobilized enzyme beads in a 10 mL-syringe (total laccase activity 1.8 U). Twenty milliliters of the representative dyes dissolved in distilled water at initial concentrations of 60, 16, 16 and 8 mg L^{-1} for RBBR, Methyl red, Indigo carmine and Bromophenol blue, respectively (with starting absorbance around 0.7 at its maximum absorption wavelength). The decolorization processes were performed at room temperature (~28±3°C) and periodically withdrawn every 10 min to measure the reduction of each dyes absorbance at its maximum absorption wavelength (λ_{max}: RBBR 591 nm, Methyl Red 524 nm, Indigo carmine 610 nm and Bromophenol Blue 592 nm). The decolorization ability of each dye was expressed in percentage calculated as following equation:

$$\text{Decolorization (\%)} = \frac{A_0 - A_t}{A_0} \times 100$$

where, A_0 is an absorbance at λ_{max} of each dye immediately measured after adding the enzyme solution and A_t is an absorbance at λ_{max} of each dye after each time intervals.

All reported data were averaged from triplicate experiments. All experiments were performed in the Protein and Enzyme Technology Research Unit, Faculty of Science, Mahasarakham University, during October 2008 to February 2009.

RESULTS AND DISCUSSION

In this study, crude laccase enzyme secreted by filamentous fungus, *L. polychrous* Lev. grown on rice husk-rice bran solid-state fermentation was investigated for alginate entrapment with three cation types, including Cu^{2+}, Zn^{2+} and Ca^{2+}. The alginate concentration and cation solution concentrations on enzyme entrapment were optimized. The effects of pH and temperature on catalytic reaction of the immobilized enzymes as well as pH and temperature stabilities were also studied. The synthetic dyes decolorization of model dye representatives were also reported with the most suitable immobilized ion-alginate enzyme in this study.

Enzyme immobilization on alginate: Effects of alginate concentration and cation type of hardening solutions were investigated by varying alginate from 1-4% (w/v) and fixed the salt concentration of Cu^{2+}, Zn^{2+} and Ca^{2+} - hardening solutions at a common value (0.2 M). Immobilization yield (%) and laccase activity of immobilized beads were considered altogether in order to find the best compromise. In Cu- and Ca-alginate enzymes, the immobilization percentages and activity of immobilized beads were in anti-parallel manners (the higher immobilization percentage, the lower activity of immobilized beads) as shown in Fig. 1a and c. Meanwhile, both values were in synchronizing in a case of Zn^{2+}-hardening solution (Fig. 1b). From the results in Fig. 1a-c, the suitable alginate concentrations for Cu-, Zn- and Ca-alginate enzyme entrapments were 2, 3 and 2.5% (w/v), respectively. For Cu- and Ca-alginate beads, those alginate concentrations were the values at the crossing points between immobilization percentages and activity of immobilized beads, whereas 3% (w/v) alginate was chosen for Zn-alginate enzyme entrapment instead of 4% of the crossing point. The Zn^{2+} salt in 4% (w/v) alginate beads yielded irregularly round shape (they were water-like drop with tail) and slight bigger than other percentages.

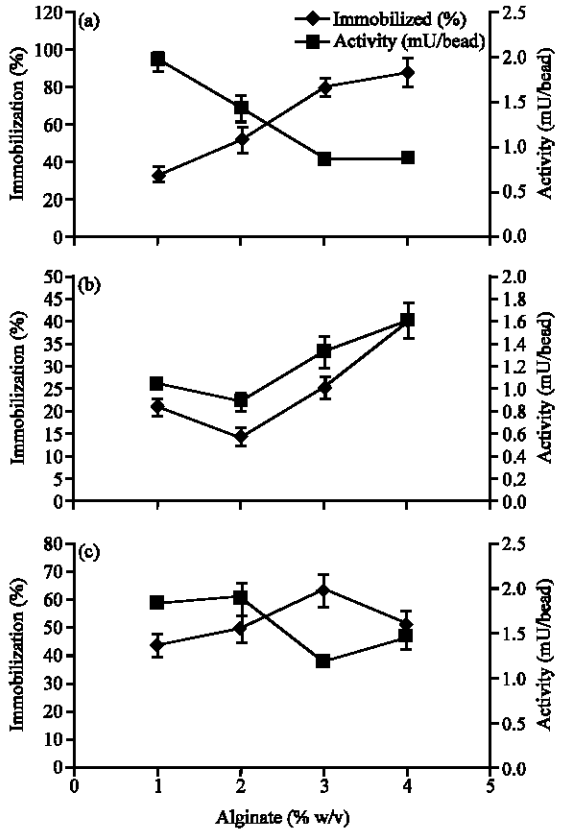

Fig. 1: Effect of alginate concentrations on the immobilizations and the laccase activity of immobilized enzymes. The immobilized (a) Cu-alginate enzyme immobilized (b) Zn-alginate enzyme and (c) immobilized Cu-alginate enzyme. The oxidation of ABTS (0.1 mM) was assayed in 0.1 M sodium acetate buffer (pH 4.5) at 32°C for 10 min

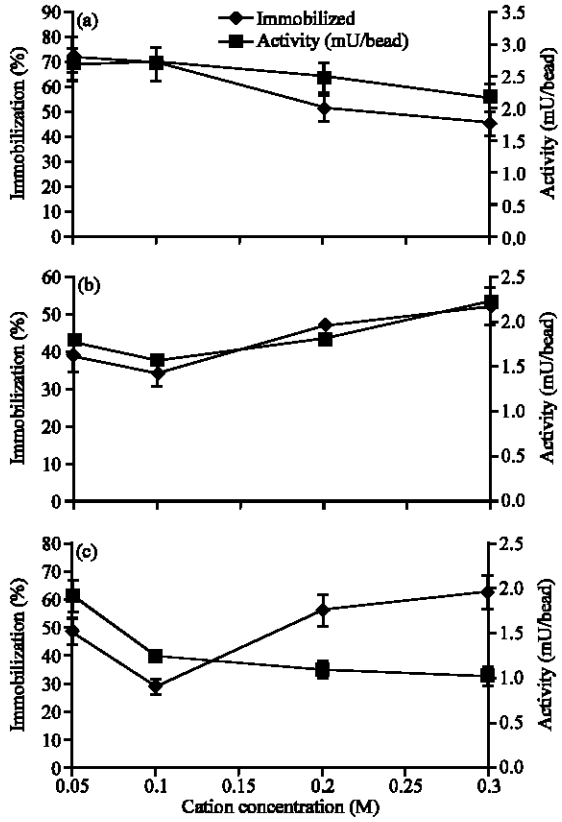

Fig. 2: Effect of curing solution concentrations on immobilizations of crude laccase from *Lentinus polychrous* Lev. The oxidation of ABTS (0.1 mM) was assayed in 0.1 M sodium acetate buffer (pH 4.5) at 32°C for 10 min. Immobilization percentages and activity of immobilized beads of (a) Cu-alginate (b) Zn-alginate and (c) Ca-alginate enzymes

Moreover, at this concentration of alginate, the mixture of the crude enzyme and alginate was difficult to thorough mix as it was very viscous making out off interest.

After knowing the suitable alginate concentration for each salt ion, the concentrations of each hardening solutions were then optimized ranging from 50-300 mM. The results were as shown in Fig. 2a-c. The appropriate hardening solutions considering high immobilization percentages and activity of immobilized beads for enzyme encapsulation of Cu- and Ca-alginate were 0.05 M, whereas 0.3 M was the most suitable concentration for Zn-alginate enzyme entrapment.

Optima pH and temperature of immobilized ion-alginate enzymes: To investigate whether immobilized beads of each salt conditions showed similar or different

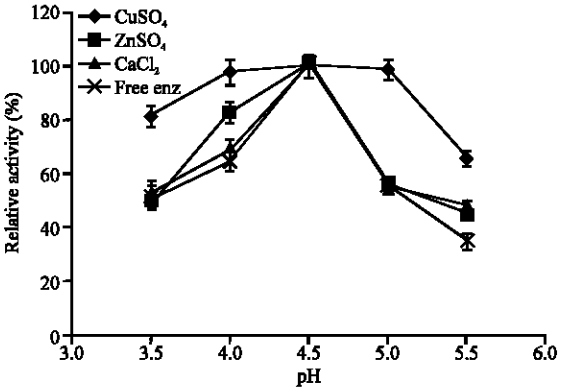

Fig. 3: Effect of pH on the laccase activity of soluble free and immobilized enzymes. The oxidation of ABTS (0.1 mM) was assayed in various pH values of 0.1 M sodium acetate buffer at 32°C for 10 min

characteristics comparison to free enzyme. Activity assays were done in different pH buffers ranging from 3.5 to 5.5 (0.1 M sodium acetate buffer) at 32°C for 10 min. The results found that the immobilized Ca- and Zn-alginate enzyme showed very similar pattern of pH effects on ABTS oxidation compared to that of the free enzyme in which pH 4.5 was the optimum. However, the immobilized Cu-alginate beads showed less pH sensitive on laccase activity. The latter immobilized enzymes revealed optimum laccase activity at pH 4.0-5.0 as shown in Fig. 3.

Study effect of temperature on laccase activity of the immobilized ion-alginate enzymes compared to the free enzyme also revealed the significant differences. The results were as shown in Fig. 4. All of the immobilized enzymes showed higher temperature optima than that of free enzyme. The Cu- and Zn-alginate enzymes had optimum temperature at 55°C, whereas the immobilized Ca-alginate enzyme had the highest temperature optimum at 60°C.

pH and temperature stability of immobilized Cu-alginate enzymes: The immobilized Cu-alginate enzymes were kept at different pH values ranging from 2.0 to 8.0 at 4°C and laccase activity were assayed periodically under a standard assay conditions. The results showed that the immobilized Cu-alginate enzymes were well stabilized when kept in tested buffer pH ranging from 3.5 to 5.5 for a week with interesting increased in laccase activity (Fig. 5a). These immobilized enzymes slightly decreased in activity (~15-20%) when kept in buffer pH 7.0 and 2.0

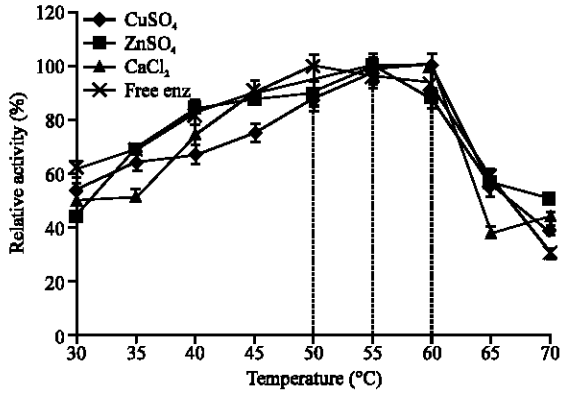

Fig. 4: Effect of temperature on the laccase activity of soluble free and immobilized enzymes. The oxidation of ABTS (0.1 mM) was assayed in various pH values of 0.1 M sodium acetate buffer at 32°C for 10 min

for 7 days. However, the immobilized beads were melting after 5 days soaking in (0.1 M) Tris-HCl buffer pH 8.0 as shown in Fig. 5a.

For the immobilized Zn-alginate beads, mostly well stabilized in all tested pH values and had higher laccase activity from the immobilized beads keeping in all pH of buffers, except for buffer pH 2.0 and 7.0. The results were shown in Fig. 5b. However, the effect of pH buffers showed strongly effect on laccase activity of immobilized Ca-alginate beads. The immobilized beads showed more than 50% activity lost at day 3 when kept in buffer solutions, pH ranging from 2.0 to 8.0. Whereas, beads kept in distilled water still remain the similar activity after 7 days (Fig. 5c).

Temperature stability experiments of each ion-alginate immobilized enzymes showed that all those three immobilized Cu-, Zn- and Ca-alginate enzymes very well stabilized when kept in 0.1 M sodium acetate buffer (pH 4.5) at 4°C. For Cu-alginate beads, when kept the immobilized enzymes at 30-40°C, the laccase activity lost about 15-30% after 4 h incubation. The remaining laccase activity of the immobilized enzymes, kept in the same buffer at 50°C and at the similar incubation times, was about 50%. However, when keeping temperature rose up to 60°C, the immobilized Cu-alginate enzymes lost its activity more than 70% within 30 min and nearly complete lost after 4 h (Fig. 6a).

In case of Zn-alginate immobilized enzymes, the results as shown in Fig. 6b, they more slightly stabled at 40°C than at 30°C (~15% activity lost at 30°C whereas a 100% remaining activity at 40°C after 4 h incubation). At 50°C, the Zn-alginate beads had remaining laccase activity higher than 50%. However, when kept at 60°C, its activity lost about 40% after 30 min and more than 90% lost after 4 h.

For immobilized Ca-alginate enzymes, after 4 h incubation the activity remaining above 75, 70 and 40% when kept at 30, 40 and 50°C, respectively. While, more than 80% activity lost when kept the immobilized beads for 30 min at 60°C and completely lost after 3 h incubation (Fig. 6c).

Synthetic dyes decolorization by immobilized Cu-alginate enzymes: Since, the immobilized Cu-alginate enzyme showed the most promise characteristics among others, then such immobilized enzymes were selected for synthetic dyes removal experiments. To lower the effect of different pH on each dye decolorization, four structural different dye models were dissolved in distilled water. Initial concentrations of each dye adjusted according to the starting absorbance at its maximum wavelength around 0.7 were 60, 16, 16 and 8 mg L^{-1} for RBBR, Methyl red, Indigo carmine and Bromophenol blue, respectively. Detailed experiments were as described in Materials and Methods section. The results were shown in Fig. 7. For RBBR decolorization by the immobilized Cu-alginate enzymes, a rapid dye removal about 70% within 20 min and slightly increased in decolorization percentages until reached up to 90% at 60 min (Fig. 7a). The immobilized beads were able to reuse, but dye decolorization ability for cycle-2 and 3 were decreased to 60 and 30%, respectively (Fig. 7a).

For Methyl red decolorization by the immobilized Cu-alginate enzymes, this azo dye was rapidly decolorized to 70% within 20 min and slightly increased up to 90% after 150 min of treatment. The immobilized beads were well repeatable in the second and the third cycles with the highest decolorization percentages of 80 and 70%, respectively (Fig. 7b).

In case of Indigo carmine decolorization, only about 40% decolorization of this indigoid dye achieved within the first 10 min, after that decolorization percentages gradually increased until reached to 96% after 150 min. The reusability of the immobilized beads in later cycles during the similar treatment times found that high efficiency decolorization of Indigo carmine were observed 96 and 90% in cycle-2 and 3, respectively (Fig. 7c).

For Bromophenol blue decolorization, at the initial concentration such 8 mg L^{-1}, the immobilized Cu-alginate enzymes were be able to remove the dye about 40% within 10 min and then slightly increased up to 84% within 150 min (Fig. 7d). However, the decolorization ability in cycle-2 was decreased to only about 50% for the similar treatment time.

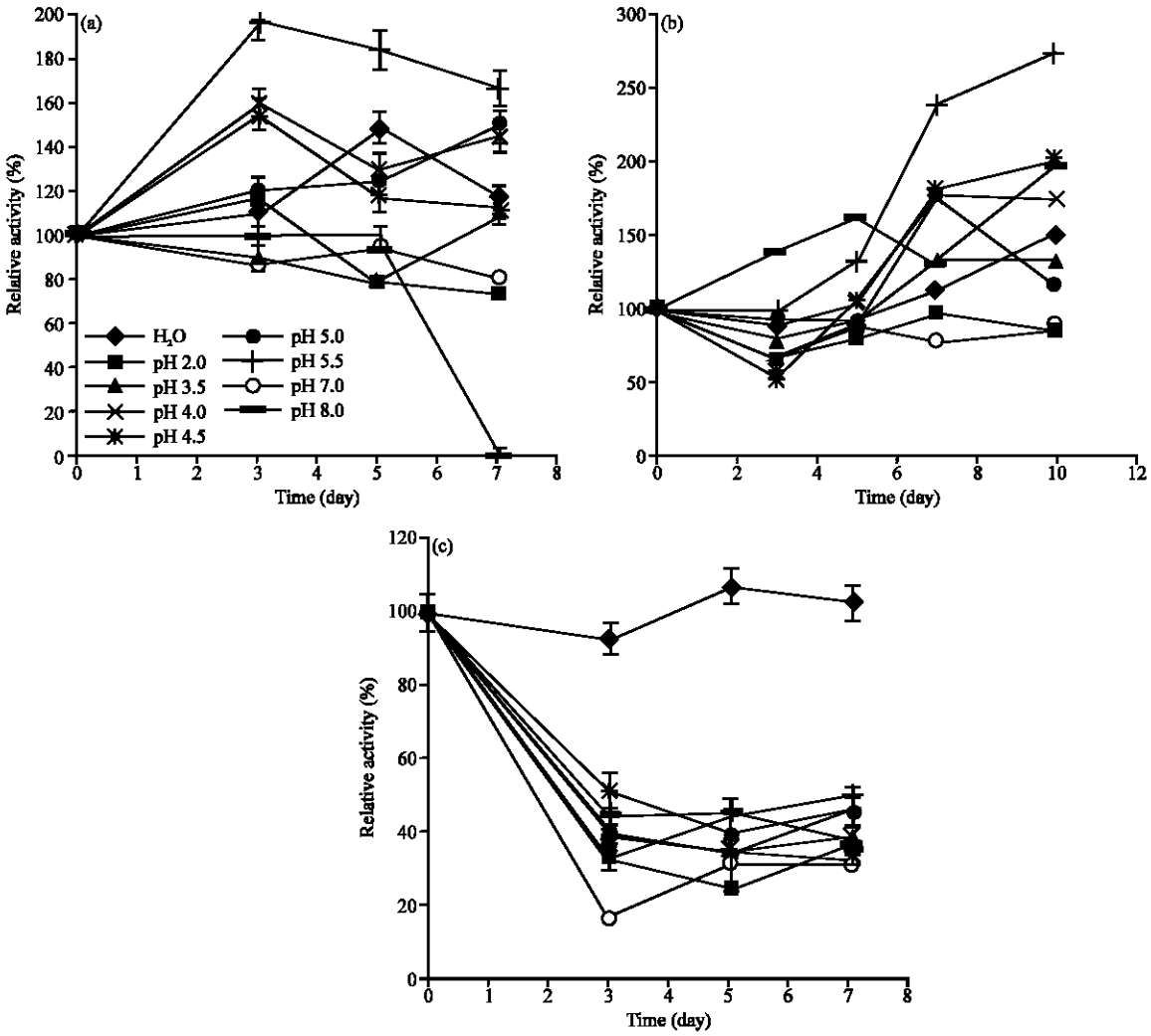

Fig. 5: Effect of pH of buffer on the laccase activity stability of (a) immobilized Cu-alginate enzyme, (b) immobilized Zn-alginate enzyme and (c) immobilized Ca-alginate enzyme. The oxidation of ABTS (0.1 mM) was assayed in 0.1 M sodium acetate buffer (pH 4.5) at 32°C for 10 min

Enzyme immobilization on alginate: The immobilization results obtained in this work showed that the different types of cross-linking bivalent cations had a significant influence on the enzyme immobilization efficiency. The crude enzyme secreted by *L. polychrous* Lév. encapsulation in varying the alginate and metal ions concentrations showed differences in activity and immobilized yields. The results found that the increased alginate percentage showed the decreased laccase's activity (mU/bead) for both Cu- and Ca-alginate laccases. These results related to the consequences of losing the enzyme flexibility and limiting the substrate diffusion. Excepting, the Zn-alginate laccase's activity continually increased while increasing the alginate percentage. The several cations other than Ca-ions might reduce alginate porosity (Palmieri *et al*., 1994; Thu *et al*., 1996) that helps for enzyme flexibility. However, resulted the optimum alginate percentage was a range of 2-3% (w/v), for Cu-, Zn- and Ca-alginate that obtained the most efficient laccase activity and immobilized yields. The highest immobilized yield was obtained by using Cu-ions for gelifying agents that in agreement with the results of entrapped-fungal laccase (Palmieri *et al*., 1994). And also, it has been reported that Cu-alginate laccase from *Trametes villosa* was better support than Ca-alginate for laccase immobilization (Brandi *et al*., 2006).

The optimum salt concentration of Cu^{2+}, Zn^{2+} and Ca^{2+} was evaluated to obtain the maximal laccase activity and immobilized yields. The amount of Cu^{2+}, Zn^{2+} and Ca^{2+}

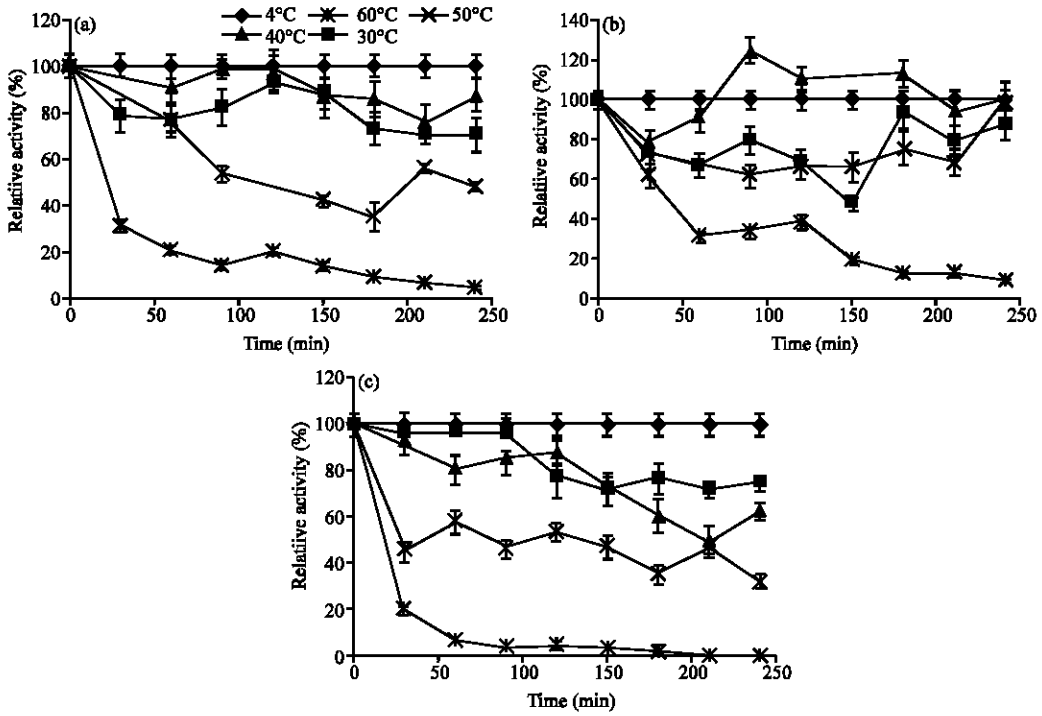

Fig. 6: Effect of temperature on the laccase activity stability of immobilized (a) Cu-alginate enzyme, (b) immobilized Zn-alginate enzyme and (c) immobilized Ca-alginate enzyme. The oxidation of ABTS (0.1 mM) was assayed in 0.1 M sodium acetate buffer (pH 4.5) at 32°C for 10 min

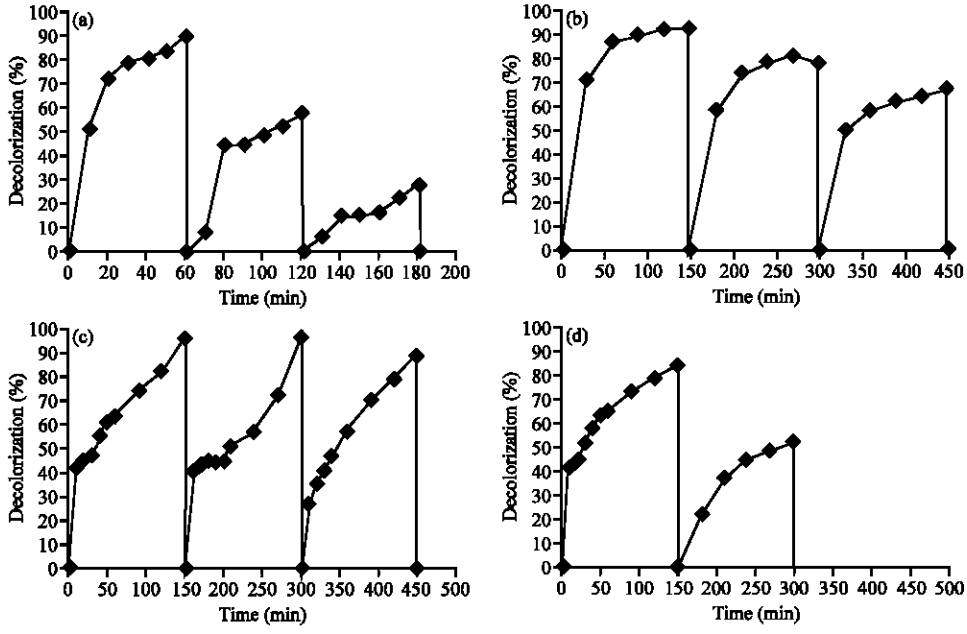

Fig. 7: Decolorization by immobilized Cu-alginate enzyme of synthetic dyes, (a) RBBR, (b) Methyl red, (c) Indigo carmine and (d) Bromophenol blue. Initial concentrations of RBBR, Methyl red, Indigo carmine and Bromophenol blue in each round were 60, 16, 16 and 8 mg L^{-1}, respectively. The treatments were performed using 10 mL pack-bed immobilized Cu-alginate enzyme per column (total laccase activity 1.8 U) and at room temperature (~28±3°C)

showed certain affect in alginate laccase's activity and immobilized yield. The similar activity/immobilization profile could be found in Cu- and Zn-alginate laccase, the higher immobilization yield, the higher activity. The different profile could be observed in 0.2 and 0.3 M $CaCl_2$, which found that the immobilized yield was increased while the activity of bead (mU/bead) was decreased. Laccase activity was affected by those three metal ions tested; especially Ca^{2+} which caused activity lost more than 70% of its initial activity when only 10 mM Ca^{2+} presence in the assay reaction (data not shown). Therefore, at a high concentration of this cation, lower activity of immobilized bead observed might be due to enzyme's activity inactivation by Ca^{2+}, apart from substrate get through barrier. The high concentration of $CaCl_2$ showed effect in alginate-laccase's activity which effect was similar to the alginate-dipeptidylpeptidase's activity (Mittal *et al*., 2005).

Optima pH and temperature of immobilized ion-alginate enzymes: The optimum pH of free and immobilized laccase was 4.5. These results were slight different from the previous report that the pH in carrier was lower than the bulk pH, causing the observed pH shift. This problem could be dissolved by using the solution with a high ionic strength (Tischer and Kasche, 1999). In addition, the alginate is able to absorb H^+ within beads that resulted in decreasing the H^+ outside beads and shifting the optimum pH (Lu *et al*., 2007). The Cu-alginate enzymes revealed the highest activity than Zn- and Ca-alginate enzymes. The Cu-alginate enzymes, activity was also observed in broadly pH ranges from 3.5 to 5.5.

The optimum temperature for free laccase activity from *L. polychrous* Lév. was 50°C while the immobilized enzymes showed laccase activity optimum at higher than 50°C. The results found that the relative activity of Cu-, Zn- and Ca-alginate laccases was gradually increased from 30°C to their optimum points. The boarder temperature ranges were also reported for laccase (Lu *et al*., 2007; Dodor *et al*., 2004). The immobilized-alginate and free laccases revealed the closely relative activity's profile for all Cu^{2+}, Zn^{2+} and Ca^{2+}. Our results agreed with the previous report that optimum activity of immobilized laccase was at a higher temperature than free laccase (Cho *et al*., 2008).

pH and temperature stability of immobilized ion-alginate enzymes: The stability of pH and temperature was investigated in ion-alginate enzymes. The previous report, enhancing the storage stability of laccase could be obtained by using the alginate or alginate/carbon beads (Khani *et al*., 2006). The pH stability of free and immobilized enzymes was determined in a range of pH 2-8 and incubated in distilled water as a control. The activity/pH profile of the Zn-alginate laccase was slight decreased in the starting date until the fourth day. Its activity was clearly observed to increase dramatically to the maximal point at 10 days. The relative activity of immobilized Cu-alginate enzymes showed over 100% for all pH ranges except pH 2 and 7. Interestingly, distilled water and studied buffer had a certain pH level but they contributed the Zn-alginate laccase activity at the different levels. These phenomenons might be occurred with the different types of ions in the solution that not improved the Zn-alginate activity. However the Ca-alginate laccase showed the contrast activity profile to the Zn-alginate laccase at the start date of experiment. Its activity was rapidly decreased from 1st to 3rd date, after that slightly increased in all pH ranges. Especially, the distilled water could maintain the relative activity to a nearby initial activity for 7 days. For Cu-alginate laccase, activity/pH profile increased in a range of pH 3.5-5.5 whereas, its activity decreased from the initial in pH 2, 7 and 8. These pH ranges are harsh condition for Cu-alginate laccase's capability. Consequently, the Cu-alginate laccase's beads start melted in the buffer pH 8.0 and completely dissolved after 5 days. The melting beads might be caused by the electrostatic interaction between H^+/OH^- and the matrix in alkaline solution. In addition, the Ca-alginate beads were found to be unstable in buffer solutions containing phosphate and citrate ions (Gaserød *et al*., 1999; Taqieddin and Amiji, 2004). At mean while, the distilled water could keep the relative activity's laccase over the initial activity and also, this activity could be obtained in all Cu-, Zn- and Ca-alginate laccases. The distilled water was reported to be negligible effect in montmorillonite-laccase activity of *Trametes villosa* (Ahn *et al*., 2002). Concluding, pH 5.5, showed the highest stability and activity for Cu-, Zn and Ca-alginate laccase even if the relative activity of Ca-alginate laccase was lower than the initial activity. And also, pH could affect the ion-alginate laccase. Especially, pH showed strongly effect in Ca-alginate laccase which its activity lost over 50% within 3 days in all pH ranges.

The previous report found that immobilized laccase showed a higher thermal stability than free enzyme at the same temperature (Reyes *et al*., 1999; Cho *et al*., 2008). The immobilized laccase could maintain the activity at broadly temperature ranges (30-50°C) even gradually decreased activity at higher temperature. The immobilized-laccase activity was extremely lost up to 70% at high temperature (60°C). The Ca-alginate laccase's activity completely lost within 3 h. The highest thermal stability was observed at 40°C for all Cu- and Zn-alginate enzymes.

Synthetic dyes decolorization by immobilized Cu-alginate enzymes: Due to superior characteristics of Cu-alginate laccase over other two immobilized enzymes, it was further selected for dye decolorization experiments. Four structural different dyes were used for testing the Cu-alginate enzyme which obtained the vantage characteristic in bead decolorization. The copper ions contained in laccase enzyme which essential for its activity. The Cu-alginate enzyme showed the highest removal efficiency in both RBBR and Methyl red than other dyes. The decolorization of Indigo carmine and Methyl red showed steady efficiency for 3 cycles. Both RBBR and bromophenol blue dyes revealed low removal efficiency after the first cycle and lost efficiency up to 50% after the second cycle. However, high removal efficiency was acquired in decolorized RBBR for 1st cycle and using only 60 min in each cycle. Present results showed ~90% decolorization of BRBB, Methyl red, Indigo carmine and Bromophenol blue in preparing 2% alginate and 0.05M $CuSO_4$. However, its efficiency quite a bit different in terms of time period, amount of dye in colorization process as well as enzyme loaded capacity in immobilized beads. Cu-alginate crude laccase from *Pleurotus ostreatus* (3% alginate type B, 0.15 M $CuSO_4$) as reported by Palmieri *et al.* (2005), a maximum of 70% RBBR decolorization achieved even after 20 cycles of stepwise dye additions in batch operations (100 U g^{-1} beads). Teerapatsakul *et al.* (2008) showed that the immobilized laccase from *Ganoderma* sp. KU-Alk4 (3.6% alginate type A, 0.15 M $CuSO_4$ as cross-linking agent) to be able to decolorize 100% Indigo carmine completed over 6 cycles, one day for each cycle (100 U mL^{-1} activity loaded). In the present study, the Cu-alginate crude laccases were prepared with a final activity of 1 U mL^{-1} which had activity only about 0.18 U mL^{-1} beads.

The reusability of immobilized crude enzymes in decolorization process in our study was considered in 3 cycles for four dyes, excluding Bromophenol blue (2 cycles). Results showed that decolorization of Bromophenol blue had lost efficiency in the 2nd cycle (less than a half of the 1st cycle) and not continued in the 3rd cycle.

The reusability of Cu-alginate enzymes was examined and found that RBBR showed the highest decolorized efficiency with short time, whereas Indigo carmine and Methyl red took longer time period for decolorization. The Cu-alginate enzymes were proper in reusability in decolorization process of Indigo carmine and Methyl red because of immobilized laccase efficiency in each cycle. Lower initial concentration of RBBR might give the better results for each cycle. And also, the storage stability of enzyme was depended on the chemical structure of the dye (Zille *et al.*, 2003). By the reusability of Cu-alginate enzymes, it could be guided to improve the continuous-wastewater treatment when effluent contained RBBR, Indigo carmine or Methyl red dyes.

In a previous report, complete decolorization of Indigo carmine was obtained after more than 30 h of treatment by an alginate-immobilized laccase from *T. hirsuta* (Rodríguez Couto and Sanromán (2005). Camarero *et al.* (2005) also reported a 100% indigo carmine decolorization in less than 1 h when mediators were used with laccase from *T. villosa*. In our study, no mediators were required in the treatment, which is an advantage since they are sometimes toxic, expensive and not environmentally friendly. Decolorization of the same dyes by the free crude enzyme from *L. polychrous* Lév. showed comparable in Indigo carmine decolorization (86% within 1 h), whereas decolorization of RBBR, Methyl red and Bromophenol blue showed significant improvement in efficiency (Sarnthima *et al.*, 2009). In that work, the dye removal experiments were performed at its pH optimum. The present work, we performed in the vicinity of water to lower the effect of other ion species in the system. However, the pH of dye solution in water were neutral (RBBR, pH 7.1) to basic pH values (Indigo, pH 7.3; Bromophenol blue, pH 8.4), except for acidic pH of Methyl red (pH 5.0). RBBR decolorization activity by this work was higher efficiency than that result obtained by the crude enzyme extracted from the spent mushroom compost (Khammuang and Sarnthima, 2007) and by crude enzyme from *Pleurotus sajor-caju* (Sarnthima and Khammuang, 2008) and partial purified laccase from *Ganoderma* sp.MK05 (Khammuang and Rakakrudee, 2009). High efficiency in Methyl red decolorization (100% within 1 h at dye concentration 100 mg L^{-1}) was obtained by *Galactomyces geotrichum* MTCC 1360 in de-ionized water at 30 °C and the degradation was a direct involvement of its numerous enzymes (Jadhav *et al.*, 2008). Due to differences in the core structure of synthetic dyes, the ability and stability of the enzymes to catalyze the oxidation of each dye is related to the structure of the dye. The immobilized-Cu-alginate enzymes showed much more promise over the others. Even though, the intermediates and metabolites produced during enzymatic treatments of synthetic dyes has been reported environmental friendly in several works. The end-products catalyzed by these fungal enzymes are needed to check to ensure the safety of the decolorized dye wastewater.

CONCLUSION

According to the results obtained in the present work, it can be concluded that the most optimized conditions for alginate encapsulation of the crude enzyme

from *L. polychrous* Lév. were 2% (w/v) alginate and 0.05 M $CuSO_4$ as hardening agent. The immobilized Cu-alginate enzymes showed broader pH optima values (4.0-5.0) and higher temperature optimum (55°C) as well as more stable to the pH and temperature changes. The immobilized crude enzymes were very effective in decolorization of synthetic dye models and be able to reuse more than two cycles in a packed-bed system. The scale up should be made to apply enzymatic decolorization approaches to real industrial dye-containing wastewater. Furthermore, the intermediates and metabolites produced during enzymatic treatments are underway characterizations in details to ensure the safety of the decolorized dye wastewater in our laboratory.

ACKNOWLEDGMENTS

This study was financed by the special budget from Mahasarakham University (Fiscal year 2008). We thank the Rujira Mushroom Farm for providing mushroom samples. The authors also thank PERCH-CIC for research encouragement and student's supports, Department of Chemistry, Faculty of Science, Mahasarakham University for instruments supports.

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