# A Novel Xylan-Polyvinyl Alcohol Hydrogel Bead with Laccase Entrapment for Decolorization of Reactive Black 5

Wichanee Bankeeree,[a] Sehanat Prasongsuk,[a,*] Tsuyoshi Imai,[b] Pongtharin Lotrakul,[a] and Hunsa Punnapayak [a,*]

In an attempt to find a more efficient technique for biodegradation of the recalcitrant Reactive Black 5 (RB-5) dye, a composite xylan-polyvinyl alcohol (xylan-PVOH) hydrogel was used to immobilize laccase from the white-rot fungus *Trametes versicolor*. Xylan was prepared from the black liquor of pulp and paper effluent, and it was esterified with citric acid prior to cross-linking with polyvinyl alcohol (PVOH). The optimum composition for the immobilized laccase bead formation consisted of 4% (w/v) modified xylan, 10% (w/v) PVOH, and 15 $U.mL^{-1}$ crude laccase. The maximum decolorization of RB-5 (98.45 ± 1.96 %) was obtained within the first cycle (6 h) at 40 °C. In the eighth cycle, the reused beads were able to decolorize 55.35 ± 2.46 % of the RB-5. Moreover, the xylan-PVOH beads extended the optimum pH range of laccase activity from 6 to 10 and tolerated a temperature up to 10 °C higher than that of the free enzyme. These results suggest that the xylan-PVOH bead has great potential as the polymer matrix for enzyme immobilization, which has applications in wastewater treatment.

*Keywords: Reactive Black 5; Trametes versicolor; Alkaline tolerant; Immobilization; Hydrogel*

*Contact information: a: Plant Biomass Utilization Research Unit, Department of Botany, Faculty of Science, Chulalongkorn University, Bangkok, 10330, Thailand; b: Division of Environment Science and Engineering, Graduate School of Science and Engineering, Yamaguchi University, Yamaguchi, 755-8611, Japan; *Corresponding authors: phunsa@chula.ac.th; sehanat.p@chula.ac.th*

## INTRODUCTION

The effluents generated from textile plants are among the top sources of water pollution (Forootanfar *et al.* 2012). The major problem with these effluents is the persistence of synthetic dyes, which have carcinogenic potential (Solís *et al*. 2012). These dyes are recalcitrant due to the presence of azo-bonds ($R_1$–N=N–$R_2$) in the structure; hence, they are not totally degraded by conventional treatments including physical, chemical, and photochemical approaches (Singh *et al*. 2015). Due to these drawbacks, the use of biological or enzymatic techniques has gained more interest as an alternative approach because it is more efficient and environmentally friendly than traditional methods (Kandelbauer *et al*. 2004). Among various enzymes used for synthetic dye decolorization, multi-copper oxidases or laccases (EC 1.10.3.2) appear to be the most appropriate in treatment of azo dyes due to their non-requirement of toxic $H_2O_2$ for oxidation reaction (Baldrian 2006). These enzymes degrade azo dyes through a highly nonspecific free radical mechanism to form phenolic compounds, thereby avoiding the formation of toxic aromatic amines as the final products (Mirzadeh *et al.* 2014; Mohajershojaei *et al*. 2014). Decolorization of synthetic and textile dyes using free laccases produced by white rot fungi has been investigated in a number of studies, many of which exhibited efficiency between 80 and 90%, for example, the decolorization of Acid Orange 51 (87.87%) by crude laccase from *Trametes trogii* (Daâssi *et al*. 2013), Reactive Black 5 (87.07%) by crude laccase from *Trametes gibbosa* sp. (Adnan *et al*. 2014), and Malachite green (89.88%) by laccase produced from *Cerrena* sp. (Yang *et al*.

2015). One disadvantage for enzymatic decolorization, however, is the cost for the large-scale enzyme production and the instability of the free enzyme. The solution to this problem is to use immobilized enzymes that are more stable and therefore reusable. Enzyme immobilization, especially for decolorization of azo dyes, has been introduced using some polymeric carriers including polyamide 6,6 fibers (Silva *et al*. 2007), alginate/ gelatin blend with PEG (Ping *et al*. 2008), porous poly (GMA/EGDMA) (Arica *et al*. 2009), and silica (Wang *et al*. 2010). However, some of these polymers have low mechanical strength, durability, and/or high cost.

Xylan is the most abundant heteropolysaccharide in plant biomass that is commonly found as organic waste from the pulp and paper industry. Since xylan has been used in film and hydrogel preparation (Cao *et al*. 2014), the utilization of this polysaccharide as potential carriers for drug delivery and enzyme immobilization would be of interest because of their nontoxicity, biodegradability, and availability (Kumar *et al*. 2015). The preparation of hydrogels from xylan has been demonstrated by blending with other polymers such as chitosan (Karaaslan *et al*. 2010), 2-hydroxyethyl methacrylate (HEMA) (Silva *et al*. 2011), and N, N-methylene bisacrylamide (Peng *et al*. 2011) as a cross-linker. Among these synthetic polymers, cheap and non-toxic polyvinyl alcohol (PVOH) is of particular interest due to its excellent cross-linking and water soluble properties. Although attempts on xylan-PVOH hydrogel preparation have been reported (Tanodekaew *et al*. 2006; Venugopal *et al*. 2014), the bead formation capability and its application for laccase immobilization and dye decolorization have not been studied.

In order to optimize a multiple-step process, all factors involved in those steps must be considered, generally resulting in a large combination of experiments that is costly, time-consuming, and prone to errors. To minimize such large sets of studies, a statistical design must be used. The most widely used designs include Central Composite Design (CCD), Doehlert design, and Box-Behnken design (Bezerra *et al.* 2008). Among these three designs, Box–Behnken design requires the least experimental points, therefore it is more frequently applied to experiments with a large number of factors and variations (Ferreira *et al.* 2007). Based on results obtained from such experimental designs, Response Surface Methodology (RSM) is widely used to predict the optimal condition *via* regression analysis due to its exceptional accuracy (Clark and Williges 1973). The application of RSM in optimization of hydrogel bead formation and enzyme immobilization have been reported recently for cyclodextrin glycosyltransferase (CGTase) immobilized in alginate–gelatin mixed gel beads (Rakmai *et al.* 2015) and arsenate reductase immobilized in alginate-chitosan beads (Banerjee *et al.* 2016).

In this study, the decolorization of Reactive Black 5 by *Trametes versicolor* laccase immobilized in a novel xylan-PVOH bead was optimized to yield a nearly complete color removal. Box-Behnken design and RSM were employed to achieve the highest decolorizing efficiency. To reduce the cost of hydrogel substrates, xylan recovered from black liquor was used for the first time with great success. The beads also enhanced the enzyme stability and could be reused several times suggesting a high potential in practical wastewater treatment and other similar applications.

## EXPERIMENTAL

### Xylan Preparation from Black Liquor

Xylan was extracted from the black liquor of a commercial pulp and paper plant (Double A (1991) Public Co., Ltd, Thailand) using the method presented by Lisboa *et al*. (2005) with slight modifications. In brief, black liquor was treated with glacial acetic acid until pH was lowered to 3. The supernatant was harvested after centrifugation at 18,000 × *g* for 10 min and then mixed with two volumes of 95% (v/v) ethanol. After centrifugation (18,000 × *g* for 10 min), the xylan pellet was washed three times with 95% (v/v) ethanol and finally twice with acetone. The xylan pellet was then dried at 60 °C overnight and weighed. The modification of xylan was performed by separately dissolving xylan (3 g) and citric acid (10 g) in distilled water (10 mL) and then vigorously mixing the two solutions at 60 °C for 1 h. The mixture was dehydrated in a hot air oven at 100 °C for 1 h and then allowed to react at 120 °C for 5 h (Wang *et al*. 2013b). The obtained product was dissolved in distilled water for more than 1 h and precipitated with two volumes of 95% (v/v) ethanol. The precipitate was washed three times with 95% (v/v) ethanol and air dried overnight. The material weight was determined to calculate the yield. The chemical structures of the extracted and modified xylan were determined by Fourier transform infrared spectroscopy (FTIR) using a Vertex 70 spectrophotometer (Bruker, Ettlingen, Germany) within the wave number range of 500 to 4000 $cm^{-1}$.

### Bead Preparation

To prepare hydrogel beads, different amounts of PVOH (8 to 12% (w/v)) were completely dissolved in ultrapure water (80 mL) at 80 °C under continuous stirring and then cooled to 40 °C. The modified xylan (5 to 10% (w/v)) and sodium alginate (0 to 0.02% (w/v)) solutions were also prepared in ultrapure water (10 mL) and then added to the PVOH solution. After degassing to avoid air bubbles, the mixture was released in droplets through a needle (diameter 0.8 mm) *via* a peristaltic pump at a flow rate of 0.5 mL $min^{-1}$ into 1 L of developing solution consisting of 5% (w/v) boric acid and 1% (w/v) $CaCl_2$ at 4 °C. The resulting beads were stirred gently in this solution at 4 °C for 30 min and then washed five times with sterile distilled water to neutralize the pH and remove excess chemical reagents. The beads were stored in a 50 mM phosphate buffer (pH 6.0) at 4 °C prior to being used in the decolorization study.

### Laccase Production

Laccase was produced from *T. versicolor* CBR-04 according to Galhaup and Haltrich (2001). After seven-day cultivation at room temperature (28±2 °C) on potato dextrose agar, mycelial discs (6 mm diameter) were transferred to a laccase production medium containing glucose (10 $g.L^{-1}$), $KH_2PO_4$ (1 $g.L^{-1}$), $MgSO_4$ (0.5 $g.L^{-1}$), $CaCl_2$ (0.14 $g.L^{-1}$), thiamine (0.0025 $g.L^{-1}$), and veratyl alcohol (0.4 mM), and incubated at the same temperature for another seven days under shaking condition (150 rpm). The culture supernatant was harvested by centrifugation (18,000 × *g*, 10 min) and concentrated ten-fold by ultrafiltration (10 kDa MW membrane cut-off, Amicon, Beverly, MA, USA). This crude laccase was used for activity assay and further immobilization study. Laccase activity was determined by the 2,2'-azinobis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) oxidation method according to Bourbonnais and Paice (1990). The assay mixture contained 0.5 mM ABTS, 50 mM sodium acetate (pH 6.0), and a suitable amount of either free or immobilized enzyme. Oxidation of ABTS was

monitored by determining the increase in $A_{420}$ ($\varepsilon_{420}$ = 3.6 × 104 $M^{-1}cm^{-1}$). One unit was defined as the amount of laccase that oxidized one μmol of ABTS substrate per min.

## Optimization of Laccase Immobilization

The optimal conditions for the laccase immobilization in the modified xylan-PVOH beads were determined by response surface methodology (RSM). The Box-Behnken experimental design with three factors at three levels including three replicates at the center point was used to fit the second order response surface (Box and Behnken 1960). The values and levels of the variables are presented in Table 1. The percentage of dye decolorization was taken as the response calculated from the decrease in absorbance ($\lambda_{max}$ 595 nm) of the reaction mixture containing the immobilized laccase (10 $g.L^{-1}$), 50 mM phosphate buffer (pH 6.0), and Reactive Black 5 (RB-5; 50 $mg.L^{-1}$) after incubation at 40 °C for 6 h as follows,

$$\text{Decolorization rate (\%)} = (A_{initial} - A_{final})/ A_{initial} \times 100 \quad (1)$$

where $A_{initial}$ is the initial absorbance and $A_{final}$ is the final absorbance at the given wavelength. The efficiency of immobilization, which refers to the amount of bound protein capacity in the prepared beads, was also analyzed. Protein in the enzyme solution and in the wash solutions was determined by Lowry's method (Lowry *et al*. 1951) with bovine serum albumin as a standard. The amount of bound enzyme was calculated as follows,

$$Q = [(C_i\text{-}C_f)V/m] \times 100 \quad (2)$$

where $Q$ is the percentage of immobilized enzyme in the modified xylan-PVOH beads, $C_i$ and $C_f$ are the initial and final enzyme concentrations in the solution ($mg.mL^{-1}$), respectively, $V$ is the volume of the solution (mL), and $m$ is the mass of the beads (mg).

Statistical analysis of the data was performed by the design software package Design-Expert (version 8.0.7.1, Stat-Ease, Inc., Minneapolis, USA) to evaluate the analysis of variance (ANOVA) and to determine the significance of each term in the equations of fit. The fitted polynomial equation was then expressed in the form of three-dimensional response surface plots to illustrate the main and interactive effects of the independent variables on the dependent variable. To verify the accuracy of the predicted model, the experiment was repeated using the optimized conditions. The prepared beads with the maximum efficiency of dye decolorization were selected for further study.

## Properties of Prepared Beads

*Morphological observation of the modified xylan-PVOH beads*

The inner pores and surface morphology of the optimized beads were analyzed by scanning electron microscopy (SEM) (JSM-6400; JEOL, Tokyo, Japan). The modified xylan-PVOH beads were frozen at -20 °C, dried under vacuum, and then sputter-coated with gold. The coated samples were then observed under the SEM operated at 15 KV.

*Effects of temperature, pH, and incubation period on RB-5 decolorization*

The optimal temperature and pH for RB-5 decolorization were determined by incubating the reaction mixture (10 mL) consisting of laccase at the same concentration (0.5 $U.mL^{-1}$ of free enzyme or 10 $g.L^{-1}$ of immobilized laccase) and RB-5 (50 $mg.mL^{-1}$) in the 50 mM reaction buffer, either sodium acetate buffer (pH 3.0 to 6.0) or phosphate buffer (pH 6.0 to 10.0), at various temperatures ranging from 30 to 60 °C. Blank controls contained the dye solution and the modified xylan-PVOH beads with and without heat-inactivated enzyme. The percent of dye decolorization was calculated as previously mentioned. All

experiments were done in triplicate, and the percent decolorization was reported. The UV-visible absorption spectrum of RB-5 treated with the immobilized laccase was also determined every 2 h for up to 10 h of incubation under the optimum conditions. Aliquots of solution were scanned from 200 to 900 nm on a UV-visible spectrophotometer (Shimadzu UV 1600, Japan); the untreated dye was the control.

*Storage stability and reusability*

Samples of the immobilized laccase were kept at either room temperature (28 ± 2 °C) or at 4 °C for 20 days. The decolorization efficiency of the immobilized enzyme was measured every day under the optimal conditions. The reusability of the modified xylan-PVOH beads immobilized with laccase was investigated under the optimal conditions for 6 h in each cycle. The used beads were recovered by filtration through filter paper (Whatman No. 1), washed three times with the same buffer at the end of each cycle, and then treated with the freshly prepared dye solution for the following cycle. The percentage of decolorization was determined as previously described for each cycle. The experiment was performed in triplicate.

### Data Analysis

Results are shown with one standard deviation derived from the three replicates. Statistical differences among the means of data were determined by one-way analysis of variance (ANOVA) and Duncan's multiple range test (DMRT) or Student's t-test (2 tailed) using the SPSS 17.0 software package (SPSS Inc., Chicago, USA). Differences at $P < 0.05$ were considered significant.

## RESULTS AND DISCUSSION

### Characteristics of the Extracted and Modified Xylan from Black Liquor

During chemical pulping of wood chips, hemicelluloses are partially depolymerized especially at the side chains resulting in the residues of dissolved xylan in the spent liquid, which is referred to black liquor (Jönsson *et al.* 2008). In this study, xylan was extracted from black liquor released during the kraft pulping process of *Eucalyptus grandis* from a commercial pulp and paper company in Thailand. The amount of dried xylan pellets obtained after the extraction process was 13.24 ± 0.22 $g.L^{-1}$ of black liquor, which indicated the high extraction efficiency because the xylan recovery was found to be at 98.06 ± 1.01 % (w/w) of the hemicellulose content in the black liquor. The structure of the extracted xylan was analyzed in the FTIR spectrum (Fig. 1A). The dominant absorption peaks appeared in the regions of 2800 to 3500 $cm^{-1}$ and 600 to 1800 $cm^{-1}$, which are typical of xylan (Gao *et al*. 2014; Chen *et al*. 2015). The peak at 1139 $cm^{-1}$ corresponded to the stretches of C-C in the main chain, while the peak at 1627 $cm^{-1}$ corresponded to carboxylate (C=O) in the salt or ester form of the xylan side chains (Deng *et al*. 2014). Typical characteristic bands of lignin at 1510, 1595, 1740, and 1770 $cm^{-1}$ (Popescu *et al*. 2007) were not observed, indicating a high purity of the extracted xylan. The broad band from the stretching of hydroxyl groups (-OH) appeared at 3435 $cm^{-1}$. The presence of hydroxyl groups on the backbone of xylan makes them suitable for chemical modification to enhance their cross-linking ability with other polymers and to form the beads (Edlund and Albertsson 2008; Kuzmenko *et al*. 2014). In this study, esterification between hydroxyl groups of the extracted xylan and carboxylic groups of citric acid was performed, resulting in the cross-linked xylan-citrate containing carboxylic acid groups (Wang *et al*. 2013b). The color of the xylan-citrate powder was slightly darker (data not shown) than the

unmodified xylan due to the changes in crystal structure and the color of citric acid at 120 °C (Salam *et al*. 2011). The success of this reaction was evidenced by the FTIR spectrum (Fig. 1B). The presence of a peak at 1730 $cm^{-1}$ in the modified xylan described the stretch of carboxylic acid (C=O) produced by the cross-linking reaction of xylan with citric acid (Grigoray *et al*. 2014). Moreover, the transmittance data from the stretching of hydroxyl groups was less than that of the unmodified xylan, suggesting that a number of hydroxyl groups was spent in the esterification with citric acid.

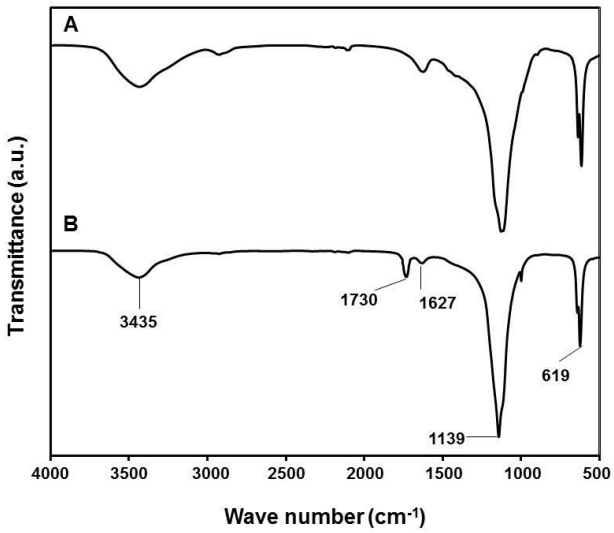


**Fig. 1.** FTIR spectra of the xylan isolated from black liquor of kraft pulping process of *Eucalyptus grandis* (A), and the modified xylan obtained from the esterification reaction with citric acid at 120 °C for 5 h (B)

## Bead Formation

The hydrogel beads were prepared by copolymerization of the esterified xylan and polyvinyl alcohol (PVOH). The structure of the modified xylan-PVOH bead is presented in Fig. 2.

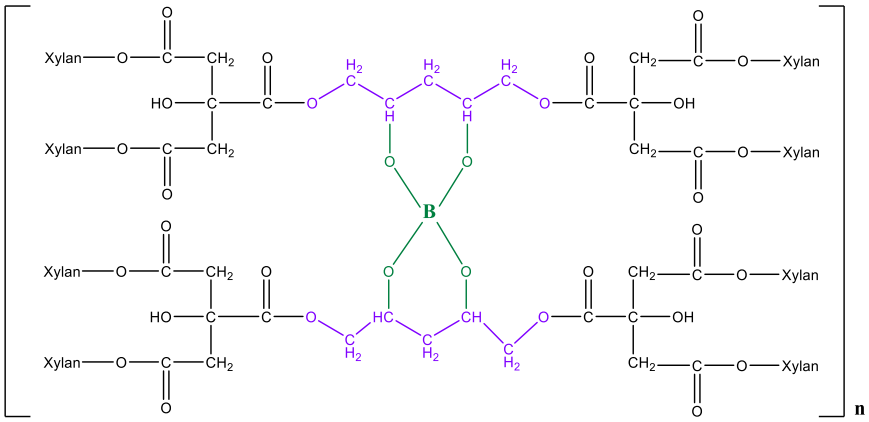


**Fig. 2.** Diagram of the modified xylan-PVOH bead structure. Modified xylan, PVOH, and boric acid are represented by the black, purple, and green sections, respectively.

The mixture of the modified xylan and PVOH was dropped into 5% (w/v) boric acid solution to allow crosslinking between the two polymers. Brownish spherical gel beads with an average size of 3 mm were formed. The high tendency of bead agglomeration was also found due to the slow rate of the crosslinking reaction. Bead agglomeration is not desirable for any further application; therefore, the addition of alginate into the mixture of modified xylan and PVOH was used to prevent agglomeration. The agglomeration of beads was entirely prevented when sodium alginate was added to the modified xylan and PVOH mixture at ratios of 0.02: 8: 4, 0.02: 10: 6, and 0.02: 12: 8; agglomeration persisted at lower concentrations of sodium alginate (data not shown). The amount of sodium alginate used here was lower than that previously reported for other PVOH bead formation (0.05 % (w/v) of sodium alginate) when the percentage of PVOH in the beads was kept in a range of 10 to 12.5 % (w/v) (Dave and Madamwar 2006).

## Optimization for Laccase Immobilization into the Modified Xylan-PVOH Beads

In the preliminary experiments, the effect of three independent parameters including concentration of modified xylan, PVOH, and crude laccase on bead formation and enzyme immobilization was investigated using a one factor at a time approach, to select operational ranges of variables where response in the term of dye decolorization was most favorable (data not shown).

Subsequently RSM using the Box-Behnken experimental design was applied within these ranges of parameters to obtain maximum response. The observed and predicted responses from 15 experimental trials containing different combinations of the three compounds at three different concentrations are shown in Table 1.

**Table 1.** Box-Behnken Design Matrix with Experimental and Predicted Values of RB-5 Decolorization

| Trial | Variable Factor | | | Dye Decolorization (%)[b] | Predicted Values (%) | Bound Protein Capacity (%) |
|---|---|---|---|---|---|---|
| | MX[a] (% (w/v)) | PVOH (% (w/v)) | Laccase (U/mL) | | | |
| 1 | 6 (+1) | 12 (+1) | 15 (0) | 96.56 ± 2.03 | 81.35 ± 4.19 | 89.46 ± 2.84 |
| 2 | 4 (0) | 8 (-1) | 10 (-1) | 83.55 ± 1.97 | 87.52 ± 4.19 | 62.48 ± 1.76 |
| 3 | 4 (0) | 10 (0) | 15 (0) | 98.45 ± 1.96 | 98.49 ± 4.19 | 74.99 ± 2.33 |
| 4 | 4 (0) | 8 (-1) | 20 (+1) | 79.56 ± 1.34 | 81.53 ± 4.19 | 70.84 ± 1.83 |
| 5 | 2 (-1) | 12 (+1) | 15 (0) | 70.56 ± 2.84 | 73.70 ± 4.19 | 75.95 ± 2.47 |
| 6 | 2 (-1) | 10 (0) | 10 (-1) | 75.83 ± 1.48 | 74.66 ± 4.19 | 68.95 ± 2.49 |
| 7 | 6 (+1) | 10 (0) | 10 (-1) | 84.61 ± 1.53 | 83.79 ± 4.19 | 82.93 ± 1.74 |
| 8 | 6 (+1) | 10 (0) | 20 (+1) | 78.51 ± 1.94 | 79.68 ± 4.19 | 87.84 ± 1.97 |
| 9 | 4 (0) | 12 (+1) | 20 (+1) | 90.64 ± 1.66 | 86.68 ± 4.19 | 76.83 ± 2.03 |
| 10 | 2 (-1) | 10 (0) | 20 (+1) | 78.44 ± 2.03 | 79.26 ± 4.19 | 72.75 ± 1.44 |
| 11 | 6 (+1) | 8 (-1) | 15 (0) | 82.71 ± 1.78 | 79.57 ± 4.19 | 72.94 ± 1.53 |
| 12 | 2 (-1) | 8 (-1) | 15 (0) | 80.46 ± 1.40 | 77.64 ± 4.19 | 60.87 ± 1.56 |
| 13 | 4 (0) | 12 (+1) | 10 (-1) | 82.16 ± 1.22 | 80.19 ± 4.19 | 72.88 ± 2.74 |
| 14 | 4 (0) | 10 (0) | 15 (0) | 99.98 ± 1.96 | 98.49 ± 4.19 | 70.35 ± 2.33 |
| 15 | 4 (0) | 10 (0) | 15 (0) | 98.03 ± 1.96 | 98.49 ± 4.19 | 72.94 ± 2.33 |

[a]MX = modified xylan ; [b] Mean ± one standard deviation derived from three replicates

The mean of RB-5 decolorization was taken as the response and analyzed in a statistical manner using regression. The maximum decolorization of RB-5 was found to be 99.98 % in trial 14. However, when results from three identical trials (3, 14, and 15) were averaged (98.01 %), it was very close to the predicted value of 98.82%. The second-order regression equation provided the levels of dye decolorization as a function of variables. The equation was presented in terms of coded factors and is shown as follows,

$$Y = 25.91A+33.07B+5.72C+0.36AB-0.22AC+0.31BC-3.13A^2-1.97B^2-0.26C^2-162.81 \quad (3)$$

where $Y$ is the response value or dye decolorization (%). $A$, $B$, and $C$ are the xylan (% (w/v)), PVOH (% (w/v)), and laccase ($U.mL^{-1}$) concentrations, respectively. The model for enzyme immobilization in this study was significant, based on the $F$-value (6.14) and the model terms value of Prob > $F$ (0.0299) (Table 2).

The coefficient of determination ($R^2$) was calculated to be 0.98. Also, there was good correspondence between the predicted and experimental values. The model showed insignificance in the lack of fit ($P$ = 0.1528). This result indicated that the second-order model equation was adequate for the prediction of enzymatic decolorization across the specified range of variables employed. Three-dimensional response plots and their corresponding contour plots were drawn on the basis of the model equation to investigate the interaction among the variables and to determine the optimum concentration of each compound for maximum decolorization.

**Table 2.** Analysis of Variance (ANOVA) of the Box-Behnken Experimental Model Developed for RB-5 Decolorization

| Source | Coefficient factor | Sum of Squares | Df | Mean square | *F*-value | P-value (Prob > *F*) |
|---|---|---|---|---|---|---|
| Model | 98.49 | 970.85 | 9 | 107.87 | 6.14 | 0.0299* |
| *A*-Modified xylan | 2.39 | 45.60 | 1 | 45.60 | 2.60 | 0.1681 |
| *B*-PVOH | -0.55 | 2.38 | 1 | 2.38 | 0.14 | 0.7281 |
| *C*-Laccase | 0.13 | 0.12 | 1 | 0.12 | 7.115E-003 | 0.9361 |
| *AB* | 1.44 | 8.27 | 1 | 8.27 | 0.47 | 0.5233 |
| *AC* | -2.18 | 18.97 | 1 | 18.97 | 1.08 | 0.3464 |
| *BC* | 3.12 | 38.88 | 1 | 38.88 | 2.21 | 0.1970 |
| $A^2$ | -12.52 | 578.96 | 1 | 578.96 | 32.95 | 0.0022* |
| $B^2$ | -7.89 | 229.98 | 1 | 229.98 | 13.09 | 0.0153* |
| $C^2$ | -6.62 | 161.67 | 1 | 161.67 | 9.20 | 0.0290* |
| Residual | | 87.85 | 22 | 17.57 | | |
| Lack of Fit | | 78.65 | 20 | 26.22 | 5.70 | 0.1528 |
| Pure error | | 9.19 | 2 | 4.60 | | |
| Cor Total | | 1058.70 | 42 | | | |

$R^2$ = 0.980; Adj $R^2$= 0.987; Coefficient of variance = 4.99%; Significant at $P$<0.05

The effects of the three variables are shown in Fig. 3. The canonical analysis revealed a maximum enzymatic decolorization of 98.49% under the optimal conditions of 4% (w/v) xylan, 10% (w/v) PVOH, and 15 $U.mL^{-1}$ laccase. The model predicted that the decolorization of RB-5 could reach up to 98.49 % when performed in the mixture containing these three components at the optimized concentrations. The validity of the predicted results by the

regression model was confirmed by a repeated experiment under the optimal concentrations. The results obtained from three replications showed that the actual RB-5 decolorization (98.45 ± 2.87 %) was close to the predicted value (98.49 ± 4.19 %). This data implies that the empirical models derived from the RSM can be used to adequately describe the relationship between the factors and response.

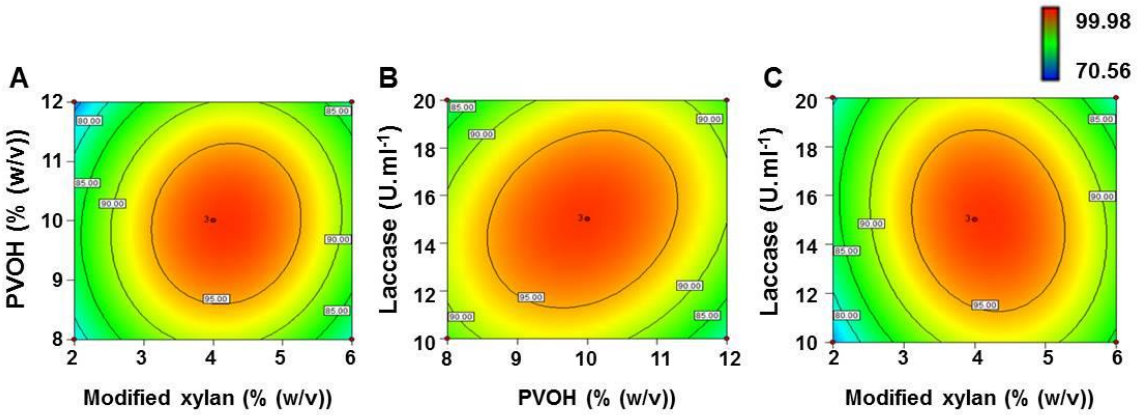


**Fig. 3.** Contour plots showing interactions between different concentrations of PVOH/xylan (A), laccase/PVOH (B), and laccase/xylan (C) on the response of RB-5 decolorization

The effects of all three variables on the bound enzyme capacity were also analyzed. The amount of bound protein reached a peak value of 89.46 ± 2.33 % in trial 1 (6 % (w/v) xylan, 12 % (w/v) PVOH, and 15 $U.mL^{-1}$ of laccase enzyme), while the dye decolorization (96.56 ± 1.96 %) in this trial was lower than that of trial 14 (99.98 ± 1.96 %) (Table 1). These results were possibly due to the porosity of the prepared beads. A high concentration of PVOH and the modified xylan might lead to the high density of physical cross-linking sites, resulting in the formation of small pores on and inside the beads, which probably limits the diffusivity of the substrate (Zhan *et al*. 2013). In contrast, the low concentration of the modified xylan and PVOH may cause a reduction in mechanical strength and the increment of the pore size resulting in enzyme leakage (El-Tanash *et al*. 2011). The equilibrium point between the amount of bound enzyme and dye decolorization was found at the same laccase concentration of 15 $U.mL^{-1}$. Efficiency of dye decolorization of the immobilized laccase varied widely depending on carriers, such as an alginate/gelatin blend with PEG (82 %) (Wang *et al*. 2008), calcium alginate beads (78.2 %) (Daâssi *et al*. 2014), and porous silica beads (76 %) (Mirzadeh *et al*. 2014). The aim of this study was to obtain the decolorization efficiency higher than those previously reported at the same or lower cost. The highest RB-5 decolorization efficiency ever reported was 95% by *Trametes villosa* laccase immobilized in γ–amino-propyltriethoxy silane modified $Al_2O_3$ (Sathishkumar *et al*. 2014). Based on the optimization results, the highest decolorization efficiency of *T. versicolor* CBR-04 immobilized in the xylan-PVOH beads was slightly higher at 98.45 %, while the immobilization cost was calculatedly much lower due to the low value of PVOH and xylan recovered from black liquor compared to the γ–amino-propyltriethoxy silane modified $Al_2O_3$ (Sathishkumar *et al*. 2014).

## Morphology of the Modified Xylan-PVOH Beads

The surface and intrastructure of the modified xylan-PVOH beads are depicted in Fig. 4. The gel beads were spherical with a smooth surface (Fig. 4A), and a large number of small pores were clearly visible (Fig. 4B). Such pores might serve as entrances for substrate diffusion. Cross-sectional images of the modified xylan-PVOH beads revealed the fibrous network with an average pore size of 0.15 ± 0.02 μm (Fig. 4C), while the average pore size of the 12 % (w/v) PVOH beads containing 0.05 % (w/v) sodium alginate was 1.43 ± 0.36 μm (Fig. 4D). This modified xylan-PVOH bead could be categorized as a macroporous material, but the PVOH bead could be classified as a gigaporous material that can provide more efficient cell or compound entrapment (microporous materials (less than 0.002 μm), mesoporous materials (0.002 to 0.050 μm), macroporous materials (0.05 to 0.20 μm), and gigaporous materials (more than 0.2 μm)) (Li *et al.* 2010). Pore size plays an important role in enzyme immobilization. Although several studies of enzyme immobilization have mainly focused on the micro- or mesoporous materials such as silica SBA-15 (Yang *et al*. 2013), chitosan with epichlorohydrin (Bayramoglu *et al*. 2012), and cellulose acetate (Güleç 2013), there are usually constraints of mass transfer and diffusion of the enzyme (Li *et al.* 2010; Jesionowski *et al*. 2014). With a small pore size carrier, the enzyme can primarily be immobilized on the surface, and it is not well protected by the carrier, leading to the remarkable decrease in activity recovery. However, the diffusion of enzyme into the deep internal regions of macroporous carriers could result in more protection of this modified xylan/PVOH bead with macroporous properties, making it an appropriate carrier for enzyme immobilization.

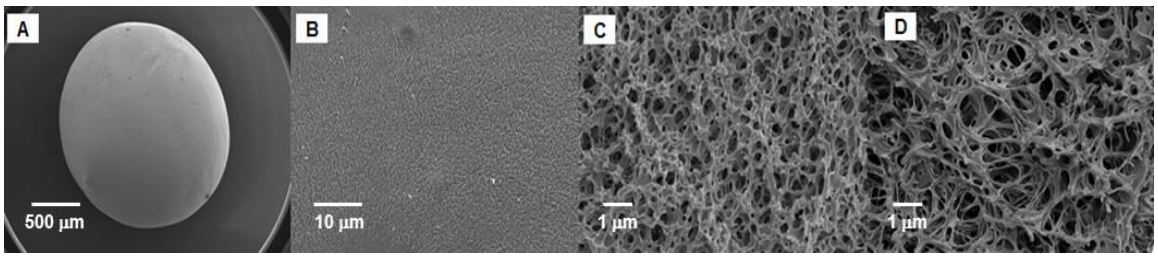


**Fig. 4.** Scanning electron microscope images of the modified xylan-PVOH beads. (A) morphology of the beads; (B) external surface at 2,000x magnification; (C) internal cross-section of the beads at 10,000x magnification; and (D) internal cross-section of PVOH beads at 5,500x magnification

## Effects of Temperature, pH, and Incubation Time on RB-5 Decolorization

The efficiencies of the free and immobilized laccases in RB-5 decolorization were compared across a range of temperatures and pH values (Fig. 5). The optimal temperature and pH for decolorization of RB-5 were identical (50 °C, pH = 6) for both free (93.22 ± 4.16 %) and immobilized laccase (98.98 ± 1.77 %); however, the immobilized enzyme was active over a broader pH range, especially between pH 8 to 10 (Fig. 5B). At 60 °C, more than 70 % of the RB5 was decolorized by the immobilized laccase at pH 5 to 10, and the efficiency did not significantly differ in this pH range.

The decolorization efficiencies were similar between the immobilized enzyme and the free enzyme only when the free enzyme was incubated at a pH of 5 to 7 (Fig. 5A). When performed at different temperatures, the dye decolorization efficiency of the free enzyme significantly decreased at all pH values as the incubation temperature increased from 50 °C to 60 °C. A higher decolorization efficiency was observed in the immobilized laccase at all temperatures compared with the free enzyme. This result might be due to the entrapment effect of the beads that protected the enzyme against heat and alkaline denaturation (Dey *et al*. 2003).

The stability of the laccase immobilized in the modified xylan-PVOH beads at a high temperature and alkaline environment makes it suitable for a wider range of applications than the free enzyme.

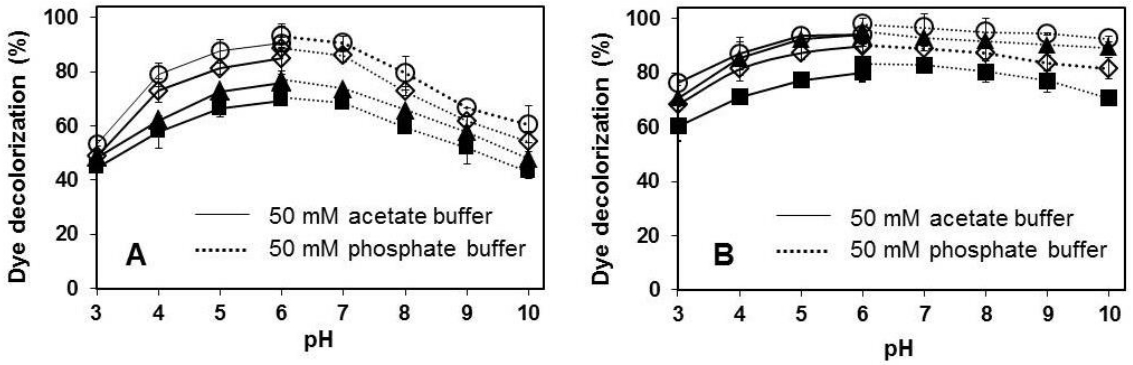


**Fig. 5.** Effects of temperature and pH on RB-5 decolorization by the free laccases (A) and immobilized laccases (B). Reactions were incubated for 6 h with 50 mg.L$^{-1}$ of RB-5 in 50 mM sodium acetate buffer (pH 3.0 to 6.0; solid line) or phosphate buffer (pH 6.0 to 10.0; dot line) at various temperatures including 30 °C (■), 40 °C (▲), 50 °C (○), and 60 °C (◊). The percentage of decolorization was calculated as the differences between the initial and final values of absorbance at 595 nm. Untreated dye solution was used as a control of each buffer (100%). Experiments were performed in triplicate (N = 3), and error bars indicate standard deviations.

The effect of incubation time on dye decolorization was also investigated (Fig. 6). The rate of dye decolorization in both the free and immobilized laccase rapidly increased with increasing incubation time up to 6 h. Further increases in incubation time did not yield any additional dye decolorization. This result was in accordance with the UV/VIS spectrum. The dye was degraded by the immobilized laccase, and there was no significant difference after 6 h of incubation. The untreated dye showed a maximum absorbance ($\lambda_{max}$) at 600 nm, but the decolorized dye in every treatment showed a $\lambda_{max}$ absorbance at 580 nm (Fig. 6B). This shift in spectrum might have been due to an alteration in the structure of the azo dye.

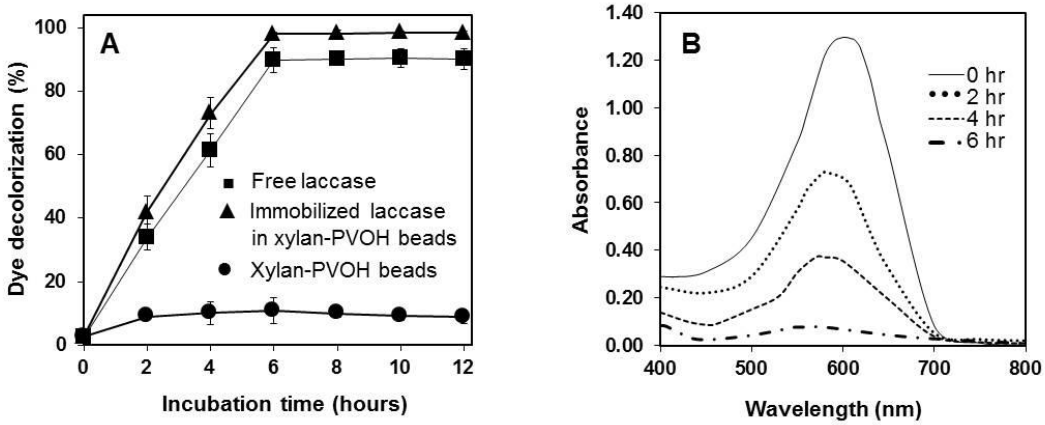


**Fig. 6.** Effects of incubation time on RB-5 decolorization (A) and UV-visible spectra (B) of RB-5 during decolorization by immobilized laccase in xylan-PVOH beads. Reactions were conducted with 50 mg.L$^{-1}$ of RB-5 in 50 mM phosphate buffer (pH 6.0) at 50 °C. Aliquots of the mixture were analyzed for dye decolorization every 2 h for up to 12 h. An untreated dye solution was used as a control (100%). Experiments were performed in triplicate (N = 3), and error bars indicate standard deviations.

Slight decolorization of dye in the solution was also observed in the modified xylan-PVOH beads, both without enzyme and with inactive laccase, suggesting that dye adsorbed onto the beads. The absorption of reactive dyes has been previously reported, especially in plant derived materials such as activated carbon, cellulosic fiber, hemicellulose, and lignin (Hubbe *et al.* 2012). The color of the modified xylan-PVOH beads slowly turned to bluish within 2 h of incubation in the RB-5 solution (data not shown). This color remained in the beads after it was cross-sectioned and soaked in the 50 mM phosphate buffer (pH 6.0) for 2 h at room temperature. This observation might be due to the adsorption ability of the modified xylan as previously reported by Salam *et al*. (2011) and Deng *et al*. (2014), while no dye adsorption has been reported for other PVOH beads.

## Stability and Reusability of the Immobilized Laccase

Highly stable beads are desirable for applications in wastewater treatment; therefore, the stability of the modified xylan-PVOH beads stored in distilled water was examined. Samples of the immobilized enzyme were stored at two different temperatures, 4 °C and room temperature (28 ± 2 °C), for 20 days prior to dye decolorization evaluation. After incubation for 6 days at 4 °C and room temperature, the efficient of dye decolorization remained at 97.48 ± 3.95 % and 84.91 ± 1.78 %, respectively (Fig. 7A). The immobilized enzyme was more stable at 4 °C than at room temperature, with more than 50% of dye decolorization remaining after incubation for 16 and 10 days, respectively. Notably, the immobilized CBR-04 laccase in the modified xylan-PVOH beads had a higher storage stability than other beads previously reported (Wang *et al*. 2013a; Mirzadeh *et al*. 2014).

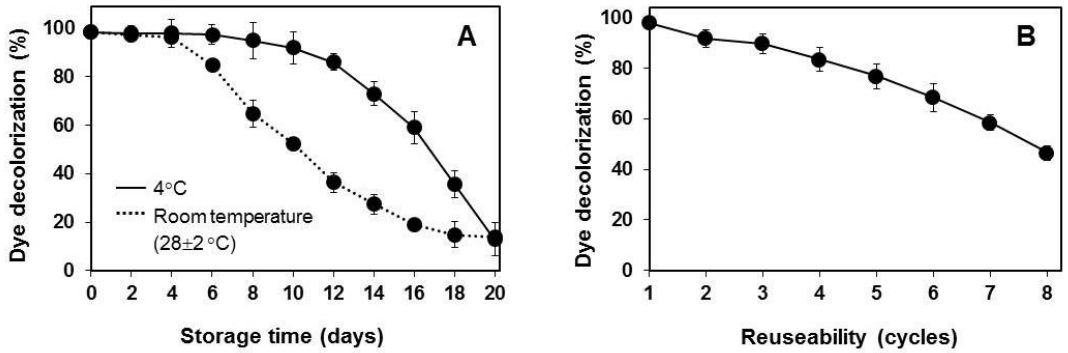


**Fig. 7.** Stability (A) and reusability (B) of the immobilized laccase for RB-5 decolorization. For stability determination, modified xylan-PVOH beads with immobilized laccase were kept in sterilized water for 20 days. Beads were sampled for determination of RB-5 decolorization efficiency under the optimum conditions every two days. One cycle is defined as the incubation of the immobilized laccase in the modified xylan-PVOH beads in a fresh reaction mixture for 6 h. Freshly prepared beads with immobilized enzyme were used in the first cycle, and then the same batch of beads was reused in the following cycles. An untreated dye solution was used as a control (100%). Data are presented as means of triplicates and error bars represent the standard deviation.

Reusability of immobilized enzymes is one of the most important factors for reducing the investment cost for industrial applications. The dye decolorization efficiency of the immobilized enzyme was observed at 40 °C and 6 h per cycle repeatedly until it decreased to about 50% (Fig. 7B). The decolorization efficiency of the immobilized laccase gradually decreased in each cycle and reached 65.84 ± 2.05% at the sixth cycle. At the eighth cycle, the immobilized laccase exhibited slightly lower than 50% RB-5 decolorization (46.48 ± 2.87%).

After the eighth cycle, the decolorization efficiency rapidly decreased due to the breaking and physical loss of the carrier (data not shown).

## CONCLUSIONS

1. After esterification with citric acid, xylan extracted from black liquor was copolymerized with PVOH and sodium alginate to form a novel hydrogel bead that could be used for laccase immobilization.
2. The immobilized laccase in the modified xylan-PVOH beads was more tolerant to high temperature and catalyzed better at alkali pH than the free enzyme. Immobilized laccase was highly stable at 4 °C and relatively stable at room temperature. The immobilized laccase beads could be reused up to 8 cycles.
3. Modified xylan-PVOH beads exhibited properties superior to some of the other previously reported hydrogel beads. The results of this study clearly show the great potential of the modified xylan-PVOH hydrogel beads for enzyme immobilization and wastewater treatment.

## ACKNOWLEDGMENTS

This research was performed under the Core-to-Core Program, which was financially supported by the Japan Society for the Promotion of Science (JSPS), National Research Council of Thailand (NRCT), Vietnam Ministry of Science and Technology (MOST), the National University of Laos, Beuth University of Applied Sciences and Brawijaya University, Rachadapisek Somphot Endowment under Outstanding Research Performance Program, and Rachadapisek Sompote Fund for Postdoctoral Fellowship, Chulalongkorn University.

## REFERENCES CITED

Adnan, L. A., Yusoff, A. R. M., Hadibarata, T., and Khudhair, A. B. (2014). "Biodegradation of bis-azo dye Reactive Black 5 by white-rot fungus *Trametes gibbosa* sp. WRF 3 and its metabolite characterization," *Water Air Soil Pollut.* 225(10), 1-11. DOI: 10.1007/s11270-014-2119-2

Arıca, M. Y., Altıntas, B., and Bayramoğlu, G. (2009). "Immobilization of laccase onto spacer-arm attached non-porous poly (GMA/EGDMA) beads: Application for textile dye degradation," *Bioresour. Technol.* 100(2), 665-669. DOI: 10.1016/j.biortech. 2008.07.038

Baldrian, P. (2006). "Fungal laccases–occurrence and properties," *FEMS. Microbiol. Rev.* 30(2), 215-242. DOI: 10.1111/j.1574-4976.2005.00010.x

Banerjee, A., Banerjee, S., and Sarkar, P. (2016). "Statistical design of experiments for optimization of arsenate reductase production by *Kocuria palustris* (RJB-6) and immobilization parameters in polymer beads," *RSC Advances.* 6(55), 49289-49297. DOI: 10.1039/c6ra00030d

Bayramoglu, G., Gursel, I., Yilmaz, M., and Arica, M. Y. (2012). "Immobilization of laccase on itaconic acid grafted and Cu (II) ion chelated chitosan membrane for bioremediation of hazardous materials," *J. Chem. Technol. Biot.* 87, 530-539. DOI: 10.1002/jctb.2743

Bezerra, M. A., Santelli, R. E., Oliveira, E. P., Villar, L. S., and Escaleira, L. A. (2008). "Response surface methodology (RSM) as a tool for optimization in analytical chemistry," *Talanta*. 76(5), 965-977. DOI: 10.1016/j.talanta.2008.05.019

Box, G. E., and Behnken, D. W. (1960). "Some new three level designs for the study of quantitative variables," *Technometrics* 2(4), 455-475. DOI: 10.1080/00401706.1960. 10489912

Cao, X., Peng, X., Zhong, L., and Sun, R. (2014). "Multiresponsive hydrogels based on xylan-type hemicelluloses and photoisomerized azobenzene copolymer as drug delivery carrier," *J. Agric. Food Chem.* 62, 10000-10007. DOI: 10.1021/jf504040s

Chen, Z., Hu, T. Q., Jang, H. F., and Grant, E. (2015). "Modification of xylan in alkaline treated bleached hardwood kraft pulps as classified by attenuated total-internal-reflection (ATR) FTIR spectroscopy," *Carbohydr. Polym.* 127, 418-426. DOI: 10.1016/ j.carbpol.2015.03.084

Clark, C., and Williges, R. C. (1973). "Response surface methodology central-composite design modifications for human performance research," *Proc. Hum. Fact. Ergon. Soc. Annu. Meet.* 15(4), 295-310. DOI: 10.1177/001872087301500402

Daâssi, D., Zouari-Mechichi, H., Frikha, F., Martinez, M. J., Nasri, M., and Mechichi, T. (2013). "Decolorization of the azo dye Acid Orange 51 by laccase produced in solid culture of a newly isolated *Trametes trogii* strain," *3 Biotech.* 3(2), 115-125. DOI: 10.1007/s13205-012-0076-2

Daâssi, D., Rodríguez-Couto, S., Nasri, M., and Mechichi, T. (2014). "Biodegradation of textile dyes by immobilized laccase from *Coriolopsis gallica* into Ca-alginate beads," *Int. Biodeterior. Biodegradation* 90, 71-78. DOI: 10.1016/j.ibiod.2014.02.006

Dave, R., and Madamwar, D. (2006). "Entrapment of lipase in polymer of polyvinyl alcohol-boric acid for esterification in organic media," *Indian J. Biotechnol.* 5(3), 368.

Deng, A., Chen, J., Li, H., Ren, J., Sun, R., and Zhao, L. (2014). "Photo-degradation of methyl orange by polysaccharides/LaFe0. 8Cu0. 2O3 composite films," *BioResources* 9, 2717-2726. DOI: 10.15376/biores.9.2.2717-2726

Dey, G, Bhupinder, S., and Banerjee, R. (2003). "Immobilization of alpha-amylase produced by *Bacillus circulans* GRS 313," *Braz. Arch. Biol. Technol.* 46, 167-176. DOI: 10.1590/ S1516-89132003000200005

Edlund, U., and Albertsson, A-C. (2008). "A microspheric system: Hemicellulose-based hydrogels," *J. Bioact. Compat. Polym.* 23, 171-186. DOI: 10.1177/0883911507088400

El-Tanash, A., El-Baz, E., and Sherief, A. (2011). "Properties of *Aspergillus subolivaceus* free and immobilized dextranase," *Eur. Food Res. Technol.* 233, 735-742. DOI: 10.1007/ s00217-011-1570-1

Ferreira, S. L. C., Bruns, R. E., da Silva, E. G. P., dos Santos, W. N. L., Quintella, C. M., David, J. M., de Andrade, J. B., Breitkreitz, M. C., Jardim, I. C. S. F. and Neto, B. B. (2007). "Statistical designs and response surface techniques for the optimization of chromatographic systems," *J. Chromatogr. A.* 1158(1), 2-14. DOI: 10.1016/j.chroma. 2007.03.051

Forootanfar, H., Moezzi, A., Aghaie-Khouzani, M., Mahmoudjanlou, Y., Ameri, A., Niknejad, F., and Faramarzi, M. A. (2012). "Synthetic dye decolorization by three sources of fungal laccase," *Iran. J. Environ. Health Sci. Eng.* 9, 27. DOI: 10.1186/ 1735-2746-9-27

Galhaup, C., and Haltrich, D. (2001). "Enhanced formation of laccase activity by the white-rot fungus *Trametes pubescens* in the presence of copper," *Appl. Microbiol. Biotechnol.* 56(1-2), 225-232. DOI: 10.1007/s002530100636

Gao, C.-D., Ren, J.-L., Wang, S.-Y., Sun, R.-C., and Zhao, L.-H. (2014). "Preparation of polyvinyl alcohol/xylan blending films with 1,2,3,4-butane tetracarboxylic acid as a new plasticizer," *J. Nanomater.* 2014, 8 DOI: 10.1155/2014/764031

Grigoray, O., Järnström, J., Heikkilä, E., Fardim, P., and Heinze, T. (2014). "Modification of pine pulp during oxygen delignification by xylan self-assembly," *Carbohydr. Polym.*112, 308-315. DOI: 10.1016/j.carbpol.2014.05.074

Güleç, H. A. (2013). "Immobilization of β-galactosidase from *Kluyveromyces lactis* onto polymeric membrane surfaces: Effect of surface characteristics, colloids and surfaces," *Colloids Surf. B.* 104, 83-90. DOI: 10.1016/j.colsurfb.2012.11.039

Hubbe, M. A., Beck, K. R., O'Neal, W. G., and Sharma, Y. C. (2012). "Cellulosic substrates for removal of pollutants from aqueous systems: A review. 2. Dyes," *BioResources* 7(2), 2592-2687. DOI: 10.15376/biores.8.2.3038-3097

Jesionowski, T., Zdarta, J., and Krajewska, B. (2014) "Enzyme immobilization by adsorption: A review," *Adsorption* 20, 801-821. DOI: 10.1007/s10450-014-9623-y

Jönsson, A.-S., Nordin, A.-K., and Wallberg, O. (2008). "Concentration and purification of lignin in hardwood kraft pulping liquor by ultrafiltration and nanofiltration," *Chem. Eng. Res. Des.* 86, 1271-1280. DOI: 10.1016/j.cherd.2008.06.003

Kandelbauer, A., Maute, O., Kessler, R. W., Erlacher, A., and Gübitz, G. M. (2004). "Study of dye decolorization in an immobilized laccase enzyme-reactor using online spectroscopy," *Biotechnol. Bioeng* 87, 552-563. DOI: 10.1002/bit.20162

Karaaslan, M. A., Tshabalala, M. A., and Buschle-Diller, G. (2010). "Wood hemicellulose/ chitosan-based semi-interpenetrating network hydrogels: Mechanical, swelling and controlled drug release properties," *BioResources* 5(2), 1036-1054. DOI: 10.15376/biores. 5.2.1036-1054

Kumar, S., Negi, Y. S., Ghosh, M. K., and Choure, K. (2015). "Renewable biopolymer-based excipients for colon drug delivery system: An overview," *J. Pharm. Sci. & Res.* 7 (3), 166-174.

Kuzmenko, V., Hägg, D., Toriz, G., and Gatenholm, P. (2014). "*In situ* forming spruce xylan-based hydrogel for cell immobilization," *Carbohydr Polym.* 102, 862-868. DOI: 10.1016/j.carbpol.2013.10.077

Li, Y., Gao, F., Wei, W., Qu, J. B., Ma, G. H., and Zhou, W. Q. (2010). "Pore size of macroporous polystyrene microspheres affects lipase immobilization," *J. Mol. Catal. B-Enzym.* 66, 182-189. DOI: 10.1016/j.molcatb.2010.05.007

Lisboa, S., Evtuguin, D., Neto, C. P., and Goodfellow, B. (2005). "Isolation and structural characterization of polysaccharides dissolved in *Eucalyptus globulus* kraft black liquors," *Carbohydr Polym.* 60, 77-85. DOI: 10.1016/j.carbpol.2004.11.024

Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951). "Protein measurement with the Folin phenol reagent," *J. Biol. Chem.* 193: 265-275.

Mirzadeh, S-S, Khezri, S-M, Rezaei, S., Forootanfar, H., Mahvi, A.H. and Faramarzi, M. A. (2014). "Decolorization of two synthetic dyes using the purified laccase of *Paraconiothyrium variabile* immobilized on porous silica beads," *J. Environ. Health Sci. Eng.* 12, DOI: 10.1186/2052-336x-12-6

Mohajershojaei, K., Khosravi, A. and Mahmoodi, N. (2014). "Decolorization of dyes using immobilized laccase enzyme on zinc ferrite nanoparticle from single and binary systems," *Fiber Polym.* 15(10), 2139-2145. DOI: 10.1007/s12221-014-2139-y

Peng, X.-W., Ren, J.-L., Zhong, L.-X., Peng, F., and Sun, R.-C. (2011). "Xylan-rich hemicelluloses-graft-acrylic acid ionic hydrogels with rapid responses to pH, salt, and organic solvents," *J. Agric. Food Chem.* 59, 8208-8215. DOI: 10.1021/jf201589y

Popescu, C.-M., Popescu, M.-C., Singurel, G., Vasile, C., Argyropoulos, D. S., and Willfor, S. (2007). "Spectral characterization of eucalyptus wood," *Appl . Spectrosc*. 61, 1168-1177. DOI: 10.1366/000370207782597076

Rakmai, J., Cheirsilp, B., and Prasertsan, P. (2015). "Enhanced thermal stability of cyclodextrin glycosyltransferase in alginate–gelatin mixed gel beads and the application for β-cyclodextrin production," *Biocatal. Agric. Biotechnol*. 4(4), 717-726. DOI: 10.1016/j.bcab.2015.10.002

Salam, A., Venditti, R. A., Pawlak, J. J., and El-Tahlawy, K. (2011). "Crosslinked hemicellulose citrate–chitosan aerogel foams," *Carbohydr Polym.* 84, 1221-1229. DOI: 10.1016/ j.carbpol.2011.01.008

Sathishkumar, P., Kamala-Kannan, S., Cho, M., Kim, J. S., Hadibarata, T., Salim, M. R., and Oh, B-T. (2014). "Laccase immobilization on cellulose nanofiber: The catalytic efficiency and recyclic application for simulated dye effluent treatment," *J. Mol. Catal. B-Enzym.* 100, 111-120. DOI: 10.1016/j.molcatb.2013.12.008

Silva, C., Silva, C. J., Zille, A., Guebitz, G. M., and Cavaco-Paulo A. (2007). "Laccase immobilization on enzymatically functionalized polyamide 6, 6 fibres," *Enzyme Microb. Technol.* 41(6), 867-875. DOI: 10.1016/j.enzmictec.2007.07.010

Silva, T. C. F., Habibi, Y., Colodette, J. L., and Lucia, L. A. (2011). "The influence of the chemical and structural features of xylan on the physical properties of its derived hydrogels," *Soft Matter* 7, 1090-1099. DOI: 10.1039/C0SM00868K

Singh, R., Singh, P., and Singh, R. (2015). "Enzymatic decolorization and degradation of azo dyes – A review," *Int. Biodeterior. Biodegradation* 104 (2015), 21-31. DOI: 10.1016/j.ibiod.2015.04.027, 21-31.

Solís, M., Solís, A., Pérez, H. I., Manjarrez, N., and Flores, M. (2012). "Microbial decolouration of azo dyes: A review," *Process Biochem.* 47, 1723-1748. DOI: 10.1016/j.procbio.2012.08.014

Tanodekaew, S., Channasanon, S., and Uppanan, P. (2006). "Xylan/polyvinyl alcohol blend and its performance as hydrogel," *J. Appl. Polym. Sci*. 100(3), 1914-1918. DOI: 10.1002/app.22919

Venugopal, J. R., Sridhar, S., and Ramakrishna, S. (2014). "Electrospun plant-derived natural biomaterials for tissue engineering," *PST* 1(3), 151-154. DOI: 10.14719/pst.2014.1.3.65

Wang, F., Guo, C., Yang, L.-R., and Liu C.-Z. (2010). "Magnetic mesoporous silica nanoparticles: fabrication and their laccase immobilization performance," *Bioresour. Technol.* 101(23), 8931-8935. DOI: 10.1016/j.biortech.2010.06.115

Wang, P., Fan, X., Cui, L., Wang, Q., and Zhou, A. (2008). "Decolorization of reactive dyes by laccase immobilized in alginate/gelatin blend with PEG," *J. Environ. Sci.* 20, 1519-1522. DOI: 10.1016/S1001-0742(08)62559-0

Wang, Q., Peng, L., Li, G., Zhang, P., Li, D., Huang, F., and Wei, Q. (2013a). "Activity of laccase immobilized on $TiO_2$-montmorillonite complexes," *Int. J. Mol. Sci.* 14, 12520-12532. DOI: 10.3390/ijms140612520

Wang, S., Li, H., Ren, J., Liu, C., Peng, F., and Sun, R. (2013b). "Preparation of xylan citrate: A potential adsorbent for industrial wastewater treatment," *Carbohydr Polym.* 92, 1960-1965. DOI: 10.1016/j.carbpol.2012.11.079

Yang, J., Hu, Y., Jiang, L., Zou, B., Jia, R., and Huang, H. (2013). "Enhancing the catalytic properties of porcine pancreatic lipase by immobilization on SBA-15 modified by functionalized ionic liquid," *Biochem. Eng. J.* 70, 46-54. DOI: 10.1016/j.bej.2012. 09.016

Yang, J., Yang, X., Lin, Y., Ng, T. B., Lin, J., and Ye, X. (2015). "Laccase-catalyzed decolorization of malachite green: Performance optimization and degradation mechanism," *PloS one*, 10(5), e0127714. DOI: 10.1371/journal.pone.0127714

Zhan, J., Jiang, S., and Pan, L. (2013). "Immobilization of phospholipase a1 using a polyvinyl alcohol-alginate matrix and evaluation of the effects of immobilization," *Braz. J. Chem. Eng.* 30, 721-728. DOI: 10.1590/S0104-66322013000400004

Article submitted: March 11, 2016; Peer review completed: May 31, 2016; Revised version received and accepted: June 16, 2016; Published: July 7, 2016.
DOI: 10.15376/biores.11.3.6984-7000