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1 Repeated batch for dye degradation in an airlift bioreactor by laccase  
2 entrapped in copper alginate

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14

15 **Abstract**

16 A repeated batch of synthetic dye decolorization was efficiently demonstrated in a 5 L airlift  
17 bioreactor. A laccase from *Ganoderma* sp. KU-Alk4, [degrading commercial aromatic dyes](#) was  
18 selected. The crude enzyme extract expressed laccase activity, and was immobilized under optimal  
19 conditions in copper-alginate beads, 3 IU/bead. The immobilized enzyme showed high efficiency  
20 in degrading various synthetic dyes under non-buffered conditions, in particular the indigoid dye  
21 Indigo Carmine. The immobilized laccase also showed marked increase in stability toward  
22 temperature and pH when compared with free enzyme preparation. Immobilization enhanced its  
23 temperature stability to maintain initial activity up to 55 °C, ten degrees higher than the free  
24 enzyme. The immobilized laccase was stable in the alkaline region up to pH 10.0. The dye  
25 decolorization system in 5 L airlift bioreactor was demonstrated with 25 mg/L Indigo Carmine  
26 dissolved in tap water and a total immobilized laccase activity of 6x10<sup>4</sup> IU. Airflow rate was the  
27 most important factor affecting the number of batch runs and the time for 100% dye degradation.  
28 An optimal airflow rate was of 4 L/min. Fourteen batch runs of complete dye degradation were  
29 successfully completed with only a single enzyme supplementation, and this could be a feasible  
30 system for operation in industry. Total dye degraded by this repeated process at 4 L/min airflow  
31 rate was 1.8 g. Isatin sulfonic acid was a metabolic product of Indigo Carmine degradation  
32 catalyzed by the immobilized laccase. This development of an effective repeatable bioprocess  
33 using enzymes for the treatment of dye-contaminated effluent has potential for implementation on  
34 an industrial scale.

1 *Keywords:* Airlift bioreactor; Copper alginate; Decolorization; Degradation; Immobilization;  
2 Laccase

### 3 **1. Introduction**

4 Synthetic dyes are widely used in the textile and dyeing industries; the chemical synthesis process  
5 is relatively simple and cost-effective, and demonstrate high stability to light, temperature,  
6 detergents, and resistance to microbial attack. However, a substantial amount of liquid effluent is  
7 generated by these industries as a large quantity of water is used in the dyeing processes (Kalyani  
8 et al., 2009). According to one estimate, 280,000 tons of textile dye are released annually in global  
9 textile effluents and cause serious toxic contamination to the surrounding environment (Jin et al.,  
10 2007). Trace amounts of dye in water at 10 – 50 mg/L not only result in visible pollution and  
11 ecological damage, but also represent a public health risk (Chung and Stevens, 1993). The most  
12 serious adverse effect could be cancer.

13 Dye degradation using microbial enzymes has recently received more attention. Most dyes  
14 are formed from a wide variety of aromatic compounds and can be degraded by fungal laccase that  
15 catalyzes oxidation (Campos et al., 2001; Zhang et al., 2006; Couto, 2009; Osma et al., 2010; Singh  
16 et al., 2015). However, one of the main drawbacks of using free enzymes to detoxify textile  
17 effluents is their instability toward thermal and pH denaturation, as well as their non-reusability.  
18 The immobilization process enhances the stability of the enzymes and allows their reuse (Couto et  
19 al. 2004; Delanoy et al. 2005; Daâssi et al., 2014).

20 The logistics for dye degradation concern both effectiveness and cost, as well as practical  
21 operation. In a bioreactor process, the main cost results from the cells or enzyme handling and their  
22 lifespan throughout the operation. Using microbial cells requires knowledge of the best strains to  
23 use and their growth cycles. In biocatalytic processes, the use of enzymes is often more  
24 advantageous than cells in controlling the reactions, but stabilizing the catalyst is an issue. In order  
25 to operate an enzymatic process with high productivity, the system requires enzyme stability with  
26 ease of operation and low cost. Immobilization technology supports reusable enzymes with  
27 improved enzyme stabilization. In the presence of aromatic compounds and EDTA, or without a  
28 stabilizer or activator such as a buffer and metal ions, enzymes can be easily denatured. Moreover,  
29 for individual enzyme reaction, specific factors affecting the enzyme must be considered (Couto,  
30 2009; Solís et al., 2012). For laccase from *Ganoderma* sp. KU-Alk4, copper ion is important as a  
31 reactivator, since it is a crucial component of the enzyme binding site (Durán et al., 2002;  
32 Teerapatsakul et al., 2007a, 2008). An enzyme immobilization using copper alginate beads method  
33 involving entrapment of laccase from this strain, and the optimal conditions for immobilization in  
34 shaken-flask batch systems using statistical experimental design method was reported by  
35 Teerapatsakul et al. (2008). [Here, the potential of implementing an enzyme system using an airlift  
36 bioreactor was reported. Operating conditions that could be used industrially to degrade dye-  
37 contaminated effluents were proposed.](#) Indigo Carmine, the most popular dye in the textile industry  
38 for dyeing blue jeans (Secula et al., 2011) was selected to demonstrate the system. This is the first  
39 description of the development of an effective airlift bioreactor for use of copper alginate laccase  
40 in the dye degradation.

### 41 **2. Materials and methods**

#### 42 *2.1. Fungal laccase*

1 Laccases of a *Ganoderma* sp., designated as 'KU-Alk4', was used in the form of  
2 immobilized enzyme. The enzyme was obtained from a cell suspension of the fungus grown  
3 aerobically in a pH 8.0, modified Kirk's medium, with 1% glucose as the sole carbon source (Tien  
4 and Kirk, 1988). The crude enzyme was composed of three dominant proteins which all exhibited  
5 laccase activity. One protein was reported as a new laccase (Teerapatsakul et al., 2007a).

6 Immobilized enzyme was prepared from the crude enzyme described above in a copper  
7 alginate bead. For optimal immobilization conditions, copper-alginate laccase was prepared with  
8 3.6% w/v low mannuronate alginate and 0.15 M CuSO<sub>4</sub> following [the statistical method of Latin](#)  
9 [Square Design](#) (Teerapatsakul et al., 2008). There was no leakage of copper ions into the aqueous  
10 phase using this method over the period of experiments, 20 days.

### 11 2.2. Laccase assay

12 Laccase activity was determined using a spectrophotometer (Lambda 25; PerkinElmer,  
13 Waltham, MA, USA), by the oxidation of 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonate)  
14 (ABTS) at 415 nm ( $\epsilon = 3.6 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$ ). The assay mixture consisted of 2.5 mM ABTS and 0.1  
15 M sodium tartrate buffer (pH 3.5). One international unit (IU) of laccase activity was defined as  
16 the amount of enzyme that was required to oxidize 1  $\mu\text{mol}$  of ABTS per min at 25 °C  
17 (Teerapatsakul et al., 2007b).

18 Laccase from the immobilized bead was determined by recovering the bead from the  
19 reaction mixture, washing with distilled water and dissolution by incubating in 0.1 M malonate  
20 buffer pH 3.5 for 15 min at 4 °C. Residual activity was assayed and compared with the starting  
21 activity.

### 22 2.3. Properties of immobilized and free laccase

23 Effects of both pH and temperature on the activity of free and immobilized laccase were  
24 studied. ABTS was used as a substrate to determine optimal pH. Reaction mixtures of the enzymes  
25 with 2.5 mM ABTS were incubated in 0.1 M buffers at various pH values. The buffers were  
26 hydrochloric acid-potassium chloride at pH 2.0 – 2.5, glycine-HCl at pH 2.5 – 3.5, malonate at pH  
27 3.0 – 4.5, citrate phosphate at pH 3.0 – 7.0, phosphate at pH 6.0 – 8.0,  
28 Tris(hydroxymethyl)aminomethane at pH 7.5 – 9.0, and glycine-NaOH at pH 9.0 – 10.0. The pH  
29 stability was examined after the enzymes were incubated in the buffers of different pH at 25 °C for  
30 1 h. The residual activity was measured as described in section 2.2.

31 Optimum temperature was determined in the range of 20 – 95 °C using a spectrophotometer  
32 (Libra S12, Biochrom). Temperature stability was investigated by incubating the enzyme solution  
33 at various temperatures, 20 – 100 °C for 1 h. The residual activity was measured as described in  
34 section 2.2.

### 35 2.4. Capability of the immobilized laccase on dye decolorization

36 The decolorization ability of the laccase immobilized in copper alginate was also performed  
37 with eight commercial synthetic dyes of different structural patterns: Indigo Carmine, Remazol  
38 Brilliant Blue R (RBBR), Bromophenol Blue, Crystal Violet, Malachite Green, Congo Red, Direct  
39 Blue 15, and Direct Red 23. The dyes were obtained from DyStar Thai Ltd. Immobilized laccase  
40 of 90 IU was added into 3 mL of 25 mg/L dye dissolved in tap water. The reaction mixtures were  
41 incubated at room temperature ( $25 \pm 2$  °C), and stirred at 200 rpm for 12 h. Decolorization of the  
42 dye was determined as the decrease of each dye from its maximum absorbency using a JASCO V-  
43 670 UV-visible spectrophotometer (Jasco Inc., MD, USA). The dye solutions were scanned in the  
44 spectrum mode from 800.0 to 200.0 nm to monitor each absorption spectrum. Wavelengths

1 resulting in the maximum absorbance ( $\lambda_{\text{max}}$ ) of each dye are shown in Table 1. The incubation time-  
2 related spectra exhibited no absorption contributed from any other substances, thus ensuring the  
3 absorbance maximum for each dye used. Control with denatured laccase also entrapped in copper  
4 alginate was run in parallel under identical conditions. All reactions were performed in triplicate.

## 2.5. Enzyme reactor

The bioreactor was a 5 L airlift reactor (BioLab; B. Braun, Germany) comprised of a vessel 1,000 mm high and 80 mm in diameter, expanding at the top to 160 mm and 250 mm in diameter. The working volume was 4 L, and the diameter of the air sparger was 60 mm. Air was supplied by an air pump. Indigo Carmine was selected for the repeated system, using the immobilized laccase of *Ganoderma* sp. KU-Alk4 in the upscaling 5 L bioreactor. The reactor was filled with 3.6 L of 25 mg/L Indigo Carmine dissolved in tap water. Immobilized laccase at  $6 \times 10^4$  IU in 400 mL tap water was added and used repeatedly. After decolorization of each batch was completed, the liquid phase was drained, and then supplemented with fresh aliquots of dye solution. The experiment was run in two replicates with the bioreactor at room temperature. Control was containing dye with the immobilized denatured laccase in another bioreactor. Rate of airflow was investigated by purging zero, 4, 7, and 10 air/4 L reaction mixture/min continuously until the dye was completely decolorized. Four milliliter samples were drawn simultaneously to monitor the efficiency of decolorization compared with the control. Quantitative analysis of Indigo Carmine in the bioreactor was performed using a JASCO V-670 UV-visible spectrophotometer. The dye solution was scanned in the spectrum mode from 800.0 to 200.0 nm to monitor the absorption spectrum (Kandelbauer et al., 2004; Yavuz et al., 2014). The 610 nm wavelength indicated maximum absorbance of Indigo Carmine. Initial and remaining concentrations of Indigo Carmine in the bioreactor were calculated from the standard curve which showed a linear relation in the concentration range 0 – 75 mg/L ( $R^2 = 0.998$ ).

Laccase activity leakage into the solution was determined. Laccase activity of the immobilized bead was measured daily. The time taken to complete Indigo Carmine decolorization for each batch and the number of batches with complete decolorization were recorded. The best system was defined as one showing complete decolorization in one day, the high number of repeated batch runs, and the immobilized enzyme remained stable in the beads throughout the repeated batch experiments.

## 2.6. Analysis of metabolic product of Indigo Carmine degradation

The final reaction product of Indigo Carmine decolorization was analyzed by UV-VIS and HPLC. UV-VIS analysis was carried out using a JASCO V-670 UV-visible spectrophotometer (Jasco Inc., MD, USA), and absorption spectra were recorded from 800 to 200 nm (Kandelbauer et al., 2004; Cano et al., 2011). HPLC analysis was performed using an 1100 series Hewlett-Packard (HP 1100) with a diode array detector (Agilent Technologies, MA, USA), and a C18 reversed phase column (Campos et al., 2001; Cano et al., 2011). Standard Isatin sulfonic acid, the primary degradation product of Indigo Carmine (Campos et al., 2001), was purchased from Sigma-Aldrich, USA.

## 2.7. Statistical analysis

All experiments, except the enzyme reactor, were performed in triplicate with results analyzed by SAS statistical software.

# 3. Results and discussion

## 3.1. Properties of immobilized and free laccases from *Ganoderma* sp. KU-Alk4

The properties of the immobilized laccases of *Ganoderma* sp. KU-Alk4 entrapped in copper-alginate beads were studied. The immobilized laccase was prepared according to the optimal conditions suggested by Latin square design as previously reported (Teerapatsakul et al.,

1 2008). Optimal temperature and thermal stability of the immobilized laccase were compared with  
2 those of the free laccase. Optimal temperature for the laccase activity did not change from 37 °C  
3 after immobilization with the copper alginate under the suggested conditions. However,  
4 immobilization enhanced its temperature stability to maintain initial activity up to 55 °C for 1 h  
5 (Fig. 1), ten degrees higher than the free enzyme. This increase is a fundamental requirement for  
6 practical enzyme implementation. Moreover, the immobilized enzyme maintained 98% of its initial  
7 activity after 1 h of incubation at temperatures up to 65 °C. The activity of immobilized laccase  
8 was determined at 55% after incubation at 80 °C for 1 h. In general, the immobilization provided  
9 protective support at high temperatures up to 60 °C. This was in agreement with the results of  
10 laccase from *Coriolopsis gallica* immobilized in calcium alginate beads (Daâssi et al., 2014).

11 Optimum pH of the laccase after immobilization increased from 3.5 and 4.5 (data not  
12 shown). Stability of the immobilized laccase was similar to free laccase, and both showed stability  
13 for 1 h in the alkaline region up to pH 10.0 (Fig. 2). Stability of the immobilized laccase for every  
14 pH measurement was about 10% higher than the free enzyme.

### 15 3.2. Decolorization ability of the immobilized laccase on different dyes

16 The ability of immobilized laccase from *Ganoderma* sp. KU-Alk4 to decolorize synthetic  
17 aromatic dyes was tested in the tap water, under non-buffered condition (Table 1). An indigoid dye  
18 Indigo Carmine was rapidly decolorized to 100% by 90 IU of the immobilized laccase within 2 h  
19 under stirring at 200 rpm. The immobilized laccase was also highly effective against the  
20 anthraquinone dye RBBR, removing 100% color after 2 h. Dye decolorization of the immobilized  
21 laccase was also found toward the triphenylmethane dyes Malachite Green, Bromophenol Blue and  
22 Crystal Violet at 82, 64.4, and 48.5%, respectively. More abilities in dye decolorization were also  
23 observed toward the azo dyes Congo Red, Direct Blue 15, and Direct Red 23 by 64, 54, and 22%,  
24 respectively. Compared with the direct use of fungal cells of laccase producing *Pycnoporus*  
25 *sanguineus*, decolorization of the triphenylmethane dyes was also successful, but at a slower rate  
26 of decolorization. Complete decolorization of Malachite Green and Bromophenol Blue was  
27 observed in 12 days with the fungal cells, while for Crystal Violet was 80% decolorization  
28 (Pointing and Vrijmoed, 2000). Using the immobilized laccase of *Ganoderma* sp. KU-Alk4 had  
29 advantages in time saving over catalysis using the fungal cells. However, these experiments were  
30 carried out in batch system. So a repeated dye decolorization process was implemented with the  
31 potential to meet industrial requirements. The use of immobilized enzyme improves ease of  
32 operation compared to immobilized living cells as there is no release of biomass into the  
33 environment causing processing problems. Moreover, the system using the immobilized laccase of  
34 *Ganoderma* sp. KU-Alk4 reduced costs due to its operation in water.

### 35 3.3. Running a repeated system for dye decolorization using the immobilized laccase with a 5 L 36 airlift bioreactor

37 A dye decolorization system with the immobilized laccase scaled up in a 5 L airlift  
38 bioreactor for repeated batch process with the immobilized enzyme was designed and constructed.  
39 The repeated system was successful with complete dye decolorization under the non-buffered  
40 condition with a number of treated batches of the reusable immobilized enzyme. The airflow rate  
41 was fixed at 10 L air/min to ensure good circulation of the copper alginate beads within the  
42 bioreactor. There was no bead breakage to release enzyme and more importantly, copper ion. The  
43 beads were used over a 20 day period without any breakage. Neither significant enzyme release  
44 nor adsorption of dye by the gel was detected during the experiment. Under these conditions,



1 decolorization efficiency toward Indigo Carmine of the immobilized laccase remained 100% after  
2 completion of 4 cycles with 4 days for each cycle (Fig. 3D). After the fifth repeated batch, i.e. after  
3 20 days, only 20% of the dye was degraded. At the end of 20 days incubation, the total amount of  
4 dye removed by the immobilized laccase in the airlift bioreactor was 104 mg/L. At this flow rate  
5 there was no shear effect on the beads, and also no degradation of the alginate gel through chelating  
6 of copper. This repeated system was successful for 4 cycles, however, the time to achieve 100%  
7 decolorization of each batch was twice that of the small-scale batch shaking system using a rotary  
8 shaker at 200 rpm as previously reported (Teerapatsakul et al., 2008). The inferior performance of  
9 the system occurred due to unsuitable oxygen transfer in the airlift bioreactor, resulting in an  
10 inappropriate number of oxygen molecules, required as a co-substrate in the catalytic mechanism  
11 of laccase (Yaropolov et al., 1994; Hatakka, 2001). However, excess oxygen molecules act as an  
12 oxidant and denature the enzyme. Therefore, the concentration of oxygen transfer in the airlift  
13 bioreactor was considered to be optimized.

#### 14 *3.4. Optimization of oxygen transfer on efficiency of the immobilized enzyme in an airlift bioreactor*

15 Results of Indigo Carmine decolorization differed in the airlift bioreactor when the airflow  
16 rates were varied between 0 – 10 L air/min. With no airflow through the bioreactor, decolorization  
17 efficiency toward the dye remained 100% over a period of 9 cycles, 2 days for each cycle (Fig.  
18 3A). After 20 days, only 35% of the dye was degraded. The total amount of dye removed at the  
19 end of 20 days incubation was 237 mg/L, which was 2.3 times greater than that obtained when 10  
20 L air/min oxygen transfer was used (Fig. 3D).

21 When airflow rate was reduced from 10 to 7 L air/min, decolorization efficiency was still  
22 100% after completion of 6 cycles (Fig. 3C), 2 complete decolorized cycles. The first 4 cycles  
23 consisted of 2 days for each cycle, and then decolorization time was increased to 4 days for the  
24 remaining cycles. For the last cycle, the 7<sup>th</sup>, the dye was 80% decolorized. The total amount of dye  
25 removed at the end of 20 days incubation was 169 mg/L, 1.6 times better than that of the system  
26 using 10 L airflow rate.

27 When the airflow rate was adjusted to 4 L air/min, efficiency of the system using the  
28 immobilized laccase was the highest. Indigo Carmine decolorization was 100% completion for 14  
29 cycles (Fig. 3B). The complete decolorization rate of the first 10 cycles was 1 day for each cycle.  
30 Decolorization time exponentially increased after the 10<sup>th</sup> cycle to 2 days for the remaining 4 cycles  
31 with complete decolorization. Four cycles of complete decolorization, although lasting 2 days each,  
32 could be obtained for up to 20 days. The total amount of dye removed at the end of 20 days  
33 incubation was 350 mg/L, which was 3.4, 2.1, and 1.5 times better than when 10, 7, and 0 L/min  
34 airflow rates were used, respectively.

35 The results showed that dye decolorization using the enzyme entrapped within copper  
36 alginate beads was optimal when the airflow rate in the 5 L airlift bioreactor was adjusted to 4  
37 L/min. The total amount of dye removed at the end of 20 days incubation was as high as 1.8 g. A  
38 dramatic loss of efficiency of the immobilized laccase was observed when the airflow rate was  
39 increased to more than 4 L/min, although a high flow rate produced good circulation of beads in  
40 the bioreactor.

41 The residual laccase activity found after dissolution of the beads is shown in Fig. 4. Copper  
42 alginate beads from airflow rates of 0, and 4 L/min retained 100% relative activity for 8 days of  
43 incubation. In the system with higher airflow rates of 7 L/min and 10 L/min, residual activity  
44 dropped dramatically with successive incubation cycles. The residual activity at 4 days of  
45 incubation of the optimal system, 4 L/min, was 100%, which was 1.1 and 1.4 times higher than

1 those of the 7 L/min system and 10 L/min system, respectively. These results showed that laccase  
2 could not retain its activity in the presence of excess oxygen. Aeration increase has negative effect  
3 on activity. This is the first report of immobilized laccase in copper alginate for the decolorization  
4 of dyes in an airlift bioreactor system. The airlift bioreactor operation was optimized for dye  
5 decolorization based on aeration rate representing the effect of oxygen in the bioreactor.

6 Results determined that the most efficient operation of this enzymatic dye decolorization  
7 system was in aerobic conditions with copper alginate immobilized laccase of *Ganoderma* sp. KU-  
8 Alk4, using a 5 L airlift bioreactor with 4 L working volume of 25 mg/L dye effluent and  $6 \times 10^4$   
9 IU of immobilized laccase. A 4 L airflow rate/min gave the best results for dye decolorization with  
10 immobilized laccase completely removing the dye within one day. This method has the potential  
11 to be used in industrial wastewater treatment operations. The repeated batch system could be run  
12 without any further addition of immobilized enzyme or buffer solution for 20 days, and removed  
13 the highest total amount of dye at 1.8 g. The dye-effluent treatment was low cost, and there were  
14 no breakages of beads with contamination of copper ions into the environment.

### 15 3.5. Analysis of metabolic product of Indigo Carmine degradation

16 A previous report detailed a pathway for Indigo Carmine degradation under purified fungal  
17 laccase catalysis to give Isatin sulfonic acid as a harmless product, and further decarboxylation to  
18 anthranilic acid as the final stable harmless product (Campos et al., 2001). Anthranilic acid may  
19 enter into the tryptophan biosynthetic pathway as an intermediate. The time-course of the UV-Vis  
20 spectrum during Indigo Carmine transformation by the copper alginate laccase of *Ganoderma* sp.  
21 KU-Alk4 was monitored. The chromophore peak at 610 nm decreased over time during the  
22 reaction. New peaks in the UV region were formed in parallel at 212 and 240 nm, indicating the  
23 generation of Isatin sulfonic acid as a degradation product (Kandelbauer et al., 2004; Cano et al.,  
24 2011).

25 The reaction product of Indigo Carmine degradation from the best operational system was  
26 analyzed by UV-VIS and HPLC to prove the presence of metabolic products. The identity of the  
27 metabolite was confirmed using authentic standards. The UV-VIS spectrum of the reaction mixture  
28 at the end of each cycle showed two characteristics peaks with maximum absorbance at 212 and  
29 240 nm, whereas the Isatin sulfonic acid standard solution also detected two peaks at 214 and 242  
30 nm. This suggested that the reaction product contained Isatin sulfonic acid. Results of HPLC  
31 analysis found that only Isatin sulfonic acid peak was observed in the chromatogram of final  
32 reaction product, and no other HPLC peaks appeared. Thus, Isatin sulfonic acid was confirmed as  
33 the major product of Indigo Carmine degradation catalyzed by the copper alginate laccase of  
34 *Ganoderma* sp. KU-Alk4.

## 35 4. Conclusions

36 This study has provided the knowledge about a cheap immobilized biocatalyst production.  
37 The biocatalyst was efficient at low concentrations without the need for any buffer. The developed  
38 scalable bioreactor system may prove useful for industrial applications regarding dye  
39 decolorization and degradation.

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1 **Table 1**

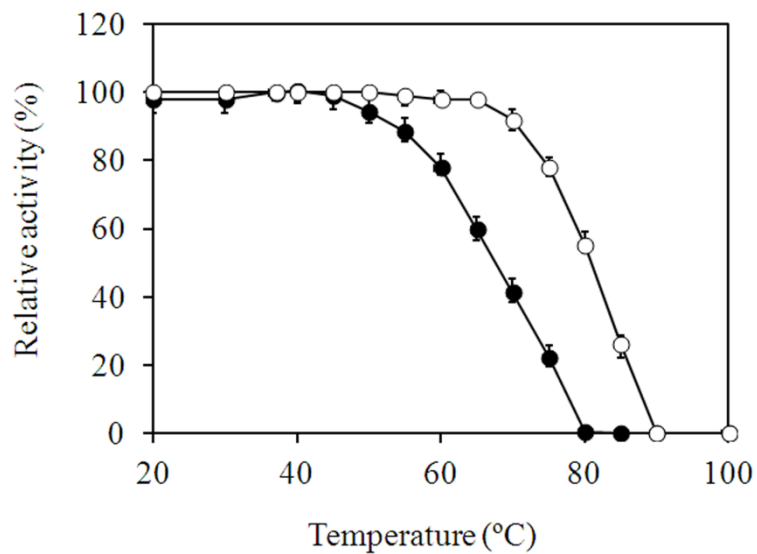
2 Decolorization of various dyes by the immobilized laccases of *Ganoderma* sp. KU-Alk4 cultivated  
 3 in Kirk's medium (pH 8.0) with 1% glucose. Total activity of laccase = 90 IU; dye = 25 mg/L  
 4 dissolved in tap water; total volume 3 mL. Values are the average of three independent  
 5 experiments.

6

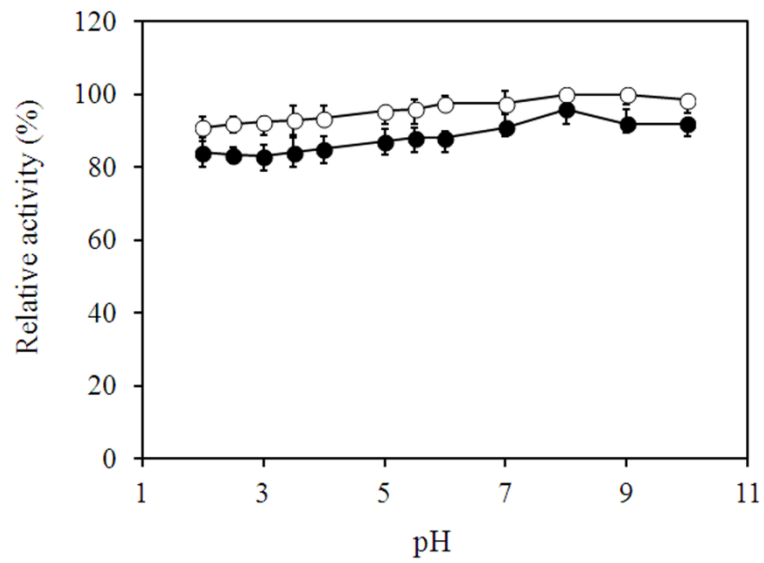
Dyes	$\lambda_{\max}$ (nm)	Dye decolorization <sup>a</sup> (%)
<b>Indigoid dye</b>		
Indigo Carmine	610	100 <sup>b</sup>
<b>Anthraquinone dye</b>		
Remazol Brilliant Blue R	595	100 <sup>b</sup>
<b>Triphenylmethane dye</b>		
Bromophenol Blue	590	64.4
Crystal Violet	630	48.5
Malachite Green	650	82
<b>Azo dye</b>		
Congo Red	570	64
Direct Blue 15	615	54
Direct Red 23	560	22

7 <sup>a</sup> Dye decolorization in 12 h.

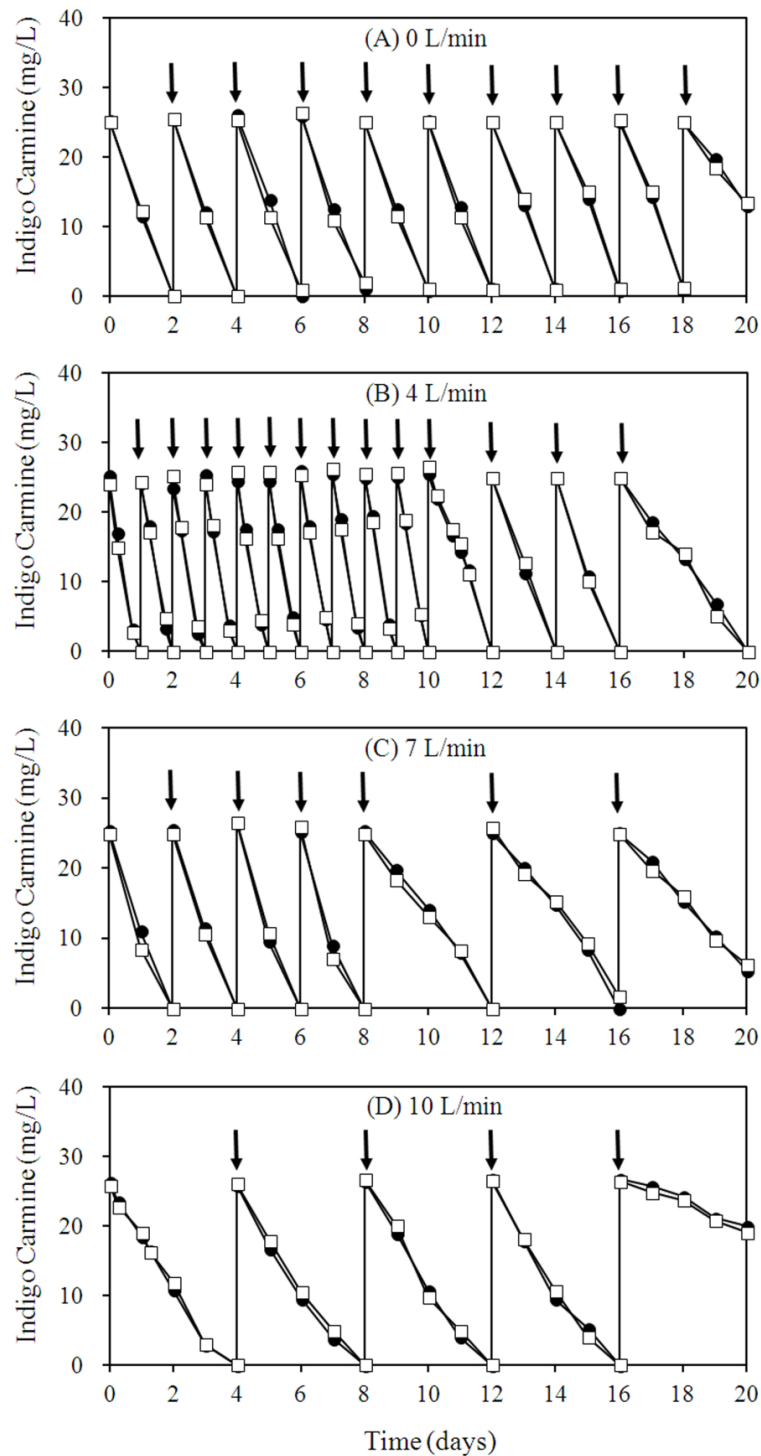
8 <sup>b</sup> 100% dye decolorization within 2 h.



- 1 **Fig. 1.** Thermal stability of (●) free and (○) immobilized laccases from *Ganoderma* sp. KU-Alk4.
- 2 Values are the average of three independent experiments. Standard deviations of data were  $\leq 4\%$ .



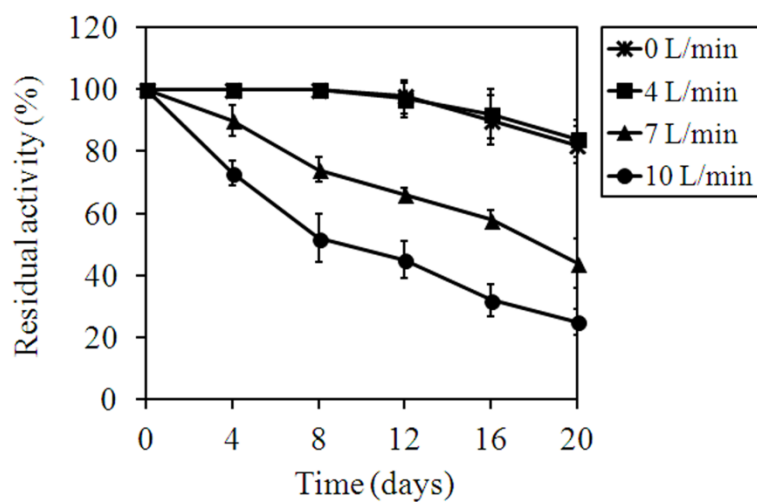
1 **Fig. 2.** pH stability of (●) free and (○) immobilized laccase from *Ganoderma* sp. KU-Alk4. Values  
 2 are the average of three independent experiments. Standard deviations of data were  $\leq 4\%$ .  
 3



1 **Fig. 3.** Comparison of Indigo Carmine decolorization by Cu-alginate immobilized laccase of  
2 *Ganoderma* sp. KU-Alk4 in a 5 L airlift bioreactor system with different airflow rates: (A) 0, (B)  
3 4, (C) 7, and (D) 10 L air/4 L reaction mixture/min. Tap water was used to dissolve Indigo Carmine.  
4 For each batch: Indigo Carmine = 25 mg/L; immobilized Cu-alginate = 3 U/bed; total units = 6 ×



- 1  $10^4$  IU. Arrows indicate the addition of the fresh dye solution. Data shown are the actual values
- 2 collected from two replicate bioreactors (□, bioreactor 1; □, bioreactor 2).



1 **Fig. 4.** Percent activity retained by *Ganoderma* sp. KU-Alk4 laccase immobilized in Cu-alginate  
 2 beads during dye decolorization in a 5 L airlift reactor with different airflow rates. Values are the  
 3 average of six Cu-alginate beads from two replicate bioreactors. Standard deviations of data were  
 4  $\leq 8\%$ .