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Abstract

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

Keywords: Airlift bioreactor; Copper alginate; Decolorization; Degradation; Immobilization; Laccase

1. Introduction

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

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

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

2. Materials and methods

2.1. Fungal laccase

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

Immobilized enzyme was prepared from the crude enzyme described above in a copper alginate bead. For optimal immobilization conditions, copper-alginate laccase was prepared with 3.6% w/v low mannuronate alginate and 0.15 M $CuSO_4$ following the statistical method of Latin Square Design (Teerapatsakul et al., 2008). There was no leakage of copper ions into the aqueous phase using this method over the period of experiments, 20 days.

2.2. Laccase assay

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

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

2.3. Properties of immobilized and free laccase

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

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

2.4. Capability of the immobilized laccase on dye decolorization

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

resulting in the maximum absorbance (λ_{max}) of each dye are shown in Table 1. The incubation time-related spectra exhibited no absorption contributed from any other substances, thus ensuring the absorbance maximum for each dye used. Control with denatured laccase also entrapped in copper 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 6×10^4 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 decolored. 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.,

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

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

3.2. Decolorization ability of the immobilized laccase on different dyes

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

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

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

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

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

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

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

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

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

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

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

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

3.5. Analysis of metabolic product of Indigo Carmine degradation

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

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

4. Conclusions

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

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Table 1

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

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

[a] Dye decolorization in 12 h.

[b] 100% dye decolorization within 2 h.

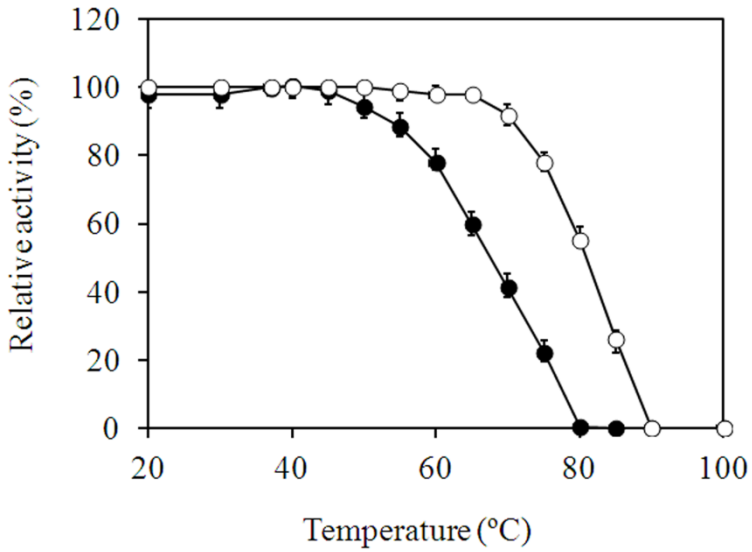

Fig. 1. Thermal stability of (●) free and (o) immobilized laccases from *Ganoderma* sp. KU-Alk4. Values are the average of three independent experiments. Standard deviations of data were ≤ 4%.

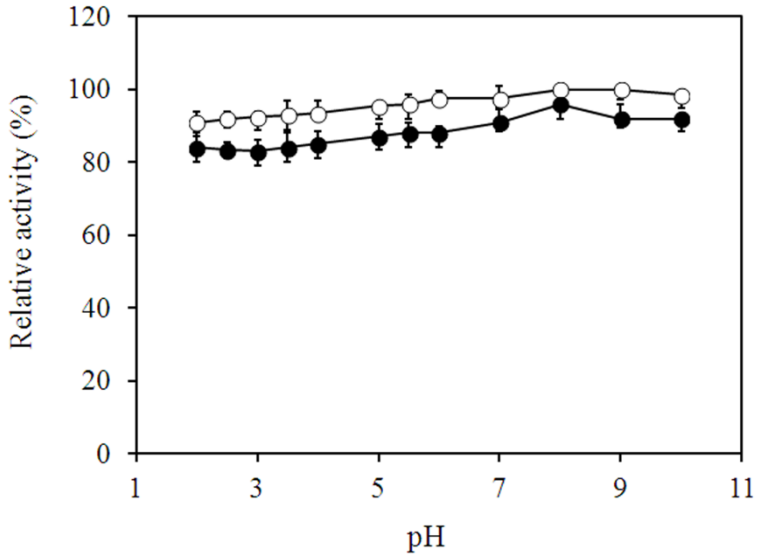

Fig. 2. pH stability of (●) free and (o) immobilized laccase from *Ganoderma* sp. KU-Alk4. Values are the average of three independent experiments. Standard deviations of data were ≤ 4%.

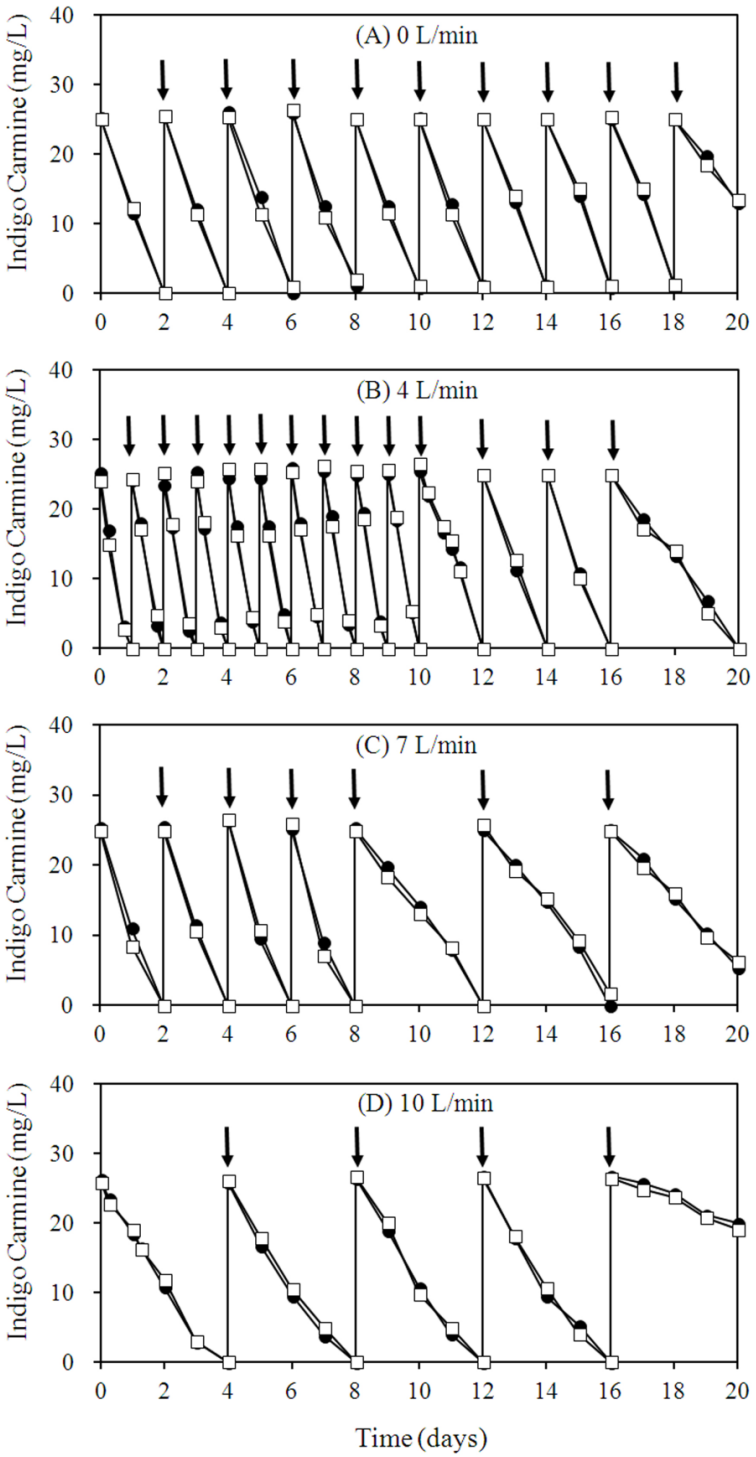

Fig. 3. Comparison of Indigo Carmine decolorization by Cu-alginate immobilized laccase of *Ganoderma* sp. KU-Alk4 in a 5 L airlift bioreactor system with different airflow rates: (A) 0, (B) 4, (C) 7, and (D) 10 L air/4 L reaction mixture/min. Tap water was used to dissolve Indigo Carmine. For each batch: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

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

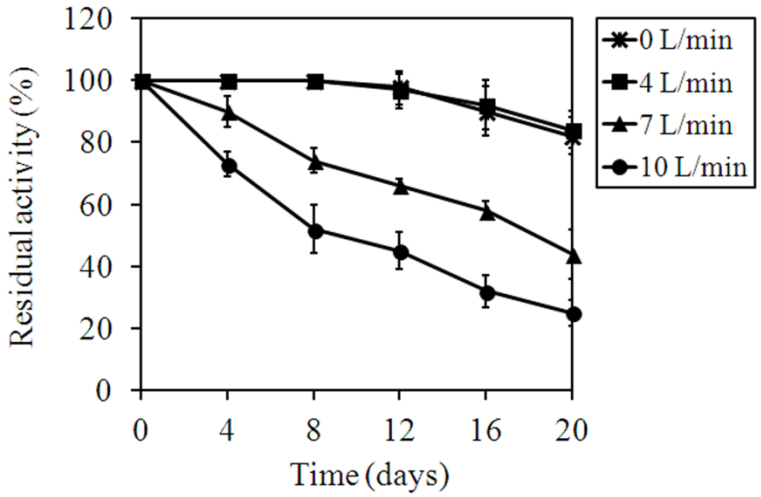

Fig. 4. Percent activity retained by *Ganoderma* sp. KU-Alk4 laccase immobilized in Cu-alginate beads during dye decolorization in a 5 L airlift reactor with different airflow rates. Values are the average of six Cu-alginate beads from two replicate bioreactors. Standard deviations of data were ≤ 8%.