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

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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 6×10^4 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.

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

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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 1000 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

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.