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A Ca-alginate particle co-immobilized with *Phanerochaete chrysosporium* cells and the combined cross-linked enzyme aggregates from *Trametes versicolor*

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HIGHLIGHTS

- The combi-CLEAs of ligninolytic enzymes from *Trametes versicolor* was obtained.
- The *Phanerochaete chrysosporium* cells and the combi-CLEAs were co-immobilized.
- The particles have great decolorizing ability and heavy metal ionic resistibility.

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ABSTRACT

For improving stability of immobilized white-rot fungus to treat various effluents, *Phanerochaete chrysosporium* cells and the combined cross-link enzyme aggregates (combi-CLEAs) prepared from *Trametes versicolor* were co-immobilized into the Ca-alginate gel particles in this paper. The activity yields of obtained combi-CLEAs were 42.7% for lignin peroxidases (LiPs), 31.4% for manganese peroxidases (MnPs) and 40.4% for laccase (Lac), respectively. And their specific activities were 30.2 U/g as combi-CLEAs-LiPs, 9.5 U/g as combi-CLEAs-MnPs and 28.4 U/g as combi-CLEAs-Lac. Further, the present of the combi-CLEAs in the particles extremely improved their ability to degrade the dyes. Compared to the immobilized *Ph. chrysosporium* without the combi-CLEAs, the co-immobilized particles enhanced the decolorized rate of *Acid Violet* 7 (from 45.2% to 93.4%) and *Basic Fuchsin* (from 12.1% to 67.9%). In addition, the addition of the combi-CLEAs improved the adaptability of the white-rot fungal particles to adverse environmental conditions.

1. Introduction

Currently, one of the possible alternatives for the dye treatment is the utilization of white-rot fungi. To data, numerous papers report the capability of the fungi to degrade and detoxify various dyes (Faraco et al., 2009). The white-rot fungi belong to a family of basidiomycetous fungi, which can cause white rot of woods. The dye degradability of these fungi depends on a special ligninolytic enzyme system, namely lignin peroxidases (LiPs), manganese peroxidases (MnPs) and laccase, which are produced and excreted during the secondary metabolic phase (Kokol et al., 2007). The enzyme system takes advantage of various electron carriers to oxidative aromatic and xenobiotic compounds (Camrero et al., 2005). As a result of the indirect contact of the enzymes with the pollutions, the white-rot fungi are able to resist the toxicity of the dyes.

However, there are many problems associated with the cultivation of suspensions of fungi in practical engineering situations, including low efficiency, poor stability of the enzyme system, strict temperature, pH requirements, and etc. (Iqbal and Saeed, 2007). Although utilization of immobilization could improve these problems, the turbulent nature of the enzyme system leads to instability of the fungal equipment to treat the pollutions The activity of the enzymes remain extremely volatile even in an optimal enzyme producing condition (Alam et al., 2009; Gill and Arora, 2003), which resulted in a detention in the retention time of industrial effluents. In addition, the production and excretion of the enzyme system require a high carbon–nitrogen ratio of effluents and are influenced by the wastewater composition. These cause limit application area of the white-rot fungi in the controlling the treatment of various industrial effluents.

It seems more preferable to use directly the ligninolytic enzyme system to treat the wide range of dyes in industrial wastewaters. However, the poor solubility of the free enzymes in aqueous phase makes them difficult to maintain in a reactor. Besides, their

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stability towards chemicals or thermal denaturation is also extremely poor which further reduces their potential (Eibes et al., 2007; Mielgo et al., 2003). Moreover, the direct utilization of the ligninolytic enzyme system requires the addition of hydrogen peroxide or glucose which is able to transform hydrogen peroxide by glucose oxidase, because hydrogen peroxide is the terminal electron acceptor of LiPs and MnPs biocatalysts (Kokol et al., 2007; Camrero et al., 2005; Alam et al., 2009; Gill and Arora, 2003). The latter greatly affects the cost of such enzyme-based processes.

Immobilized enzymes have the characters of reusability, stability and practicability. One increasingly popular immobilization method is cross-linked enzyme aggregates (CLEAs). The formation of cross-linked enzyme aggregates (CLEAs) is a rapid, gentle and cost effective method for the production of carrier insolubilized catalytically active enzymes (Sheldon, 2011). The enzymes as CLEAs are insolubilized through the precipitation of the active protein followed by its cross-linking. Compared with free biocatalysts, the insolubilized counterparts have shown a considerably high enzyme activity per unit volume with improved properties, such as stability, kinetics and reusability (Reshmi and Sugunan, 2013; Ju et al., 2013). What's more, the CLEAs provide a way to co-immobilize different enzyme, namely combined cross-linked enzyme aggregates (combi-CLEAs), to achieve multiple biocatalysis. For example, the combi-CLEAs of laccase, versatile peroxidase and glucose peroxidase realized a cascade reaction in the transform of pharmaceutically active compounds (Touahar et al., 2014).

Co-immobilization of the ligninolytic enzyme system through combi-CLEAs comes as an alternative way to improve the practical applicability of the enzymes. However, besides the hydrogen peroxide needed property of the ligninolytic enzyme system, the CLEAs are not mechanically resistant, and they are considered to be too soft for many industrial applications in almost any kind of reactor configuration (Garcia-Galan et al., 2011). In addition, the particle size of CLEAs is usually small (below 10 μm) (Cui et al., 2014), and they are easy to form increased clumps by centrifugation and filtration treatments. The clumps would hamper CLEAs to disperse again in solution and thereby cause low catalytic efficiency (Cui and Jia, 2015).

Therefore, the paper designed a new type of white-rot fungal gel-particles. The ligninolytic enzyme system is prepared to a combined cross-linked enzyme aggregates (combi-CLEAs), and then the combi-CLEAs is co-immobilized with white-rot fungal cells into the Ca-alginate gel-particles. In the co-immobilized system, the enzymes catalyze refractory pollutions to small molecular organics which can be further degraded by the cells. At the same time, the cells utilize the small molecular organics to secrete hydrogen peroxide for the enzymes. Moreover, because of the formation of combi-CLEAs, the enzymes are able to resist the decomposition of the fungal cells, and retain the stable activity to be continued a relatively long time (Mateo et al., 2006). In addition, the co-immobilization of white-rot fungi cells and ligninolytic enzymes from different origin makes it possible to build more efficient systems of white-rot fungal reactor involving specific industrial wastewater.

In this study, *Phanerochaete chrysosporium* cells are associated with combi-CLEAs prepared from *Trametes versicolor*. Contrasted with the traditional gel-particles of white-rot fungi, the resulting new type of fungal particles shows extremely high activity for the dye degradation and better capability to defense the heavy metal toxicity.

2. Methods

2.1. Chemicals

Azo dyes and triphenylmethane dyes are widely used in the dyeing and contribute more than 99% of the commercial dyes. *Acid Violet* 7 (CAS 4321-69-1) and *Basic Fuchsin* (CAS 632-99-5) obtained from Tianjin Yadong Chemical Dyestuff Factory of China were used in the study. All other chemicals used were of analytical grade.

2.2. Fungal strain and growth condition

The *Ph. chrysosporium* (BKM-F-1767) was obtained from the Guangdong Microbiological Culture center, and the *T. versicolor* (CICC 50001) was obtained from China Center of Industrial Culture Collection. The growth medium was prepared according to the method of Tien and Kirk, with 10 g/L glucose as a carbon source. However, dimethyl succinate was replaced with 20 mM acetate buffer (pH 4.5). Nitrogen in the growth medium was limited to a soluble carbon–nitrogen ratio (C/N) of 56/8.7 (in mM).

2.3. Activity assays of the enzymes

Activity of lignin peroxidases was determined by monitoring the absorbance change at 651 nm related the rate of oxidation of 32 μM Azure B to its cation radical in 50 mM Na-tartaric buffer (pH 4.5) at 30 °C (Archibald, 1992). Activity of manganese peroxidases was measured at 450 nm by oxidizing 0.4 mM guaiacol in 0.2 mmol L^{-1} $MnSO_4$ and 50 mM Na-acetate buffer (pH 4.5) (Paszczynski et al., 1988). Both of LiPs and MnPs were activated by 0.01 mM hydrogen peroxide. Activity of laccase was measured at 450 nm by oxidizing 0.4 mM guaiacol without hydrogen peroxide. And the oxidization was performed in 50 mM Na-tartaric buffer (pH 4.5) at 30 °C (Arora and Sharma, 2009). One unit of LiPs or MnPs or Lac activity was defined as the amount of enzyme that leads to the oxidation of 1 μmol of substrate per minute.

2.4. Preparation of combi-CLEAs and activity yields

Combi-CLEAs of LiPs, MnPs and Lac were produced using a protocol adapted from Taboada-Puig et al. (2011) by successive aggregation and cross-linking of the enzyme. Firstly, *T. versicolor* was inoculated in the growth medium at 25 °C and 100 rpm for 5 days, and the growth medium was dialyzed and concentrated by a semipermeable membrane (MD 44 mm, MW 8000–14000, USA). The concentrated medium was then freeze dried to obtain enzyme powder. Activities of the enzyme powder prepared by the crude solution from *T. versicolor* after the freeze drying were 41.0 U/g for LiPs, 30.4 U/g for MnPs and 70.3 U/g for Lac. 1.5 g of the obtained powder was dialyzed in 10 mL 20 mM phosphate buffer (pH 7.5) for 30 min. Secondly, the enzyme solution was precipitated with ammonium sulfate to obtain an 800 g/L of solution, precipitation lasted 30 min at room temperature. And then cross-linking was started by adding 0.25% glutaraldehyde (w/v) as cross-linking agent. The solution was then stored for 24 h at 30 °C and 100 rpm. After the cross-linking reaction, 1 mL of the suspension was taken for activity test and the rest of the suspension was filtered through a filter paper (0.2 μm). The combi-CLEAs were collected on the filter paper and washed repeatedly by the sterilized water. Eventually, the floccules of the combi-CLEAs were freeze-dried and stored at 4 °C until further use.

The suspension was diluted 10 times, and the enzyme activity of the diluted suspension before and after centrifugation (5870g, 20 min) was tested. Activities of the combi-CLEAs were calculated by subtracting the activities in the supernatants after centrifugation. Conversion yields were calculated by dividing the activities of combi-CLEAs by the initially applied activities.

2.5. Biochemical enzyme characterization

The apparent Michaelis–Menten kinetic parameter, K_m and the catalytic rate constant, K_{Cat}, of the Combined CLEAs and the