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Construction of an integrated enzyme system consisting azoreductase and glucose 1-dehydrogenase for dye removal

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HIGHLIGHTS

- NADH regeneration system was created by azoreductase and glucose 1-dehydrogenase.
- 1 U azoreductase:10 U glucose 1-dehydrogenase was the most suitable enzyme ratio.
- Artificial neural network could satisfactory fit for integrated enzyme system.
- All variables have strong effects on dye removal of integrated enzyme system.
- Batch results indicated integrated enzyme system was potential for dye removal.

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ABSTRACT

Azo dyes are toxic and carcinogenic and are often present in industrial effluents. In this research, azoreductase and glucose 1-dehydrogenase were coupled for both continuous generation of the cofactor NADH and azo dye removal. The results show that 85% maximum relative activity of azoreductase in an integrated enzyme system was obtained at the conditions: 1 U azoreductase:10 U glucose 1-dehydrogenase, 250 mM glucose, 1.0 mM NAD^+ and 150 μM methyl red. Sensitivity analysis of the factors in the enzyme system affecting dye removal examined by an artificial neural network model shows that the relative importance of enzyme ratio between azoreductase and glucose 1-dehydrogenase was 22%, followed by dye concentration (27%), NAD^+ concentration (23%) and glucose concentration (22%), indicating none of the variables could be ignored in the enzyme system. Batch results show that the enzyme system has application potential for dye removal.

1. Introduction

Industrial wastewater such as effluents from dye and textile industries has become a great concern (Yang et al., 2011b). Dyes are complex aromatic compounds and recalcitrant. Some dyes are toxic, carcinogenic and harmful to human health (Forgacs et al., 2004). In addition, dyes could be highly visible at low concentration (1 mg/L), which could cause an aesthetic pollution and disturbance to the aquatic ecosystem for reduction of oxygen levels (Vimonses et al., 2010). Treatment of dye-based effluents remains to be a challenge for environmental scientists. Therefore, many different treatment methods have been developed and employed for dye removal, including chemical precipitation, reverse osmosis, ozonation, and membrane filtration. High operational costs and secondary sludge generation have hindered applications of these methods (Yang et al., 2012).

Biotreatment offers an economical and environmentally friendly alternative for color removal in textile effluents (Chacko and Subramaniam, 2011). Microorganisms being capable of adapting a variety of environmental conditions are selected to degrade and mineralize dyes (Cai et al., 2012; Chi et al., 2009; Khalid et al., 2009; Saratale et al., 2009; Singh et al., 2012). In the studies of biological degradation of dyes, an effort has been made in order to identify, isolate and test the enzymes responsible for the decolorization. Enzyme-based methods have been developed for the detoxication of organic pollutants in the recent years. These treatments have a minimal impact on ecosystems, as they present no risk of biological contamination and reduce the possible chances for the release of exogenous genes from engineered bacteria into

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the environment (Garcia-Arellano et al., 2004). Furthermore, enzymes also present some interesting properties, such as low energy requirements, easy process control and operation over a wide range of pH, temperature and ionic strength (Torres et al., 2003).

Azoreductases that catalyze the cleavage of azo bond (−N=N−) with NAD(P)H as cofactor have been isolated and tested for dye removal (Bafana and Chakrabarti, 2008; Bafana et al., 2008; Nachiyar and Rajakumar, 2005). Therefore, an efficient and economical cofactor regeneration is indispensable for the dye removal using azoreductases, because cofactors such as NAD(H) and NADP(H) are complex, unstable and quite expensive. In our previous studies, an efficient azoreductase from *Shewanella oneidensis* MR-1 and glucose1-dehydrogenase from *Lysinibacillus sphaericus* G10 (Ding et al., 2011) were successfully expressed in *Escherichia coli*. To avoid potential risks of engineered bacteria to ecological systems, purified glucose1-dehydrogenase and azoreductase were used to construct an integrated enzyme system for removal of methyl red in the present research. The factors influencing the performance of the enzyme system were investigated in details. The effects of the operational parameters were also analyzed by using artificial neural network (ANN). Batch experiments were carried out to test the feasibility of the integrated enzyme system for dye removal.

2. Methods

2.1. Materials and regents

Azoreductase from *S. oneidensis* MR-1 and glucose1-dehydrogenase from *L. sphaericus* G10 were expressed in *E. coli*, purified as Ding et al. (2011) and stored in glycerol buffer. Methyl Red (C.I. 13020, $C_{15}H_{14}N_3NaO_2$, Molecular weight: 291.28) with 95% dye content was ACS reagent obtained from Sigma–Aldrich Co. LLC. NAD^+ and NADH were purchased from Oriental Yeast Co., Ltd, Japan. Glucose was purchased from Sangon Biotech (Shanghai) Co., Ltd.

2.2. Enzyme assays and metabolic products analysis

Azoreductase activities were tested in 4 mL cuvettes containing 50 mM citrate–phosphate buffer (pH 6.5); 150 μM methyl red; 1.0 mM NADH and an appropriate amount of enzyme (1–10 μg). A unit (U) of azoreductase activity was defined as the amount of enzyme required to reduce 1 μmol per min of methyl red (Punj and John, 2009). The extinction coefficient for methyl red used was 23360 M^{-1} cm^{-1}. The relative enzyme activity of azoreductase was employed to describe the performance of cofactor regeneration reaction, which was calculated as following:

$$\text{Relative activity}(\%) = \frac{\text{Azoreductase activity unit determined in integrated enzyme system}}{\text{Azoreductase activity unit added in integrated enzyme system}} \times 100\%$$

2.3. Effect of parameters on the performance of integrated enzyme system

Azoreductase and glucose 1-dehydrogenase were used to construct integrated enzyme system for both continuous generation of the cofactor NADH and azo dye removal. Effects of parameters on the performance of integrated enzyme system were carried out as following:

(1) Enzyme ratio: The glucose 1-dehydrogenase was set at 1500 U in the 4 mL tested cuvettes containing 50 mM citrate–phosphate buffer (pH 6.5); 150 μM methyl red; 1.0 mM NAD^+, 50 mM glucose and an appropriate amount of azoreductase from 75 U to 900 U.
(2) NAD^+ concentration: NAD^+ of different initial concentrations, ranging from 0.25 to 2.0 mM, was added to the tested cuvettes for methyl red removal at an appropriate enzyme ratio.
(3) Glucose concentration: Glucose concentrations in a range from 10 mM to 200 mM were investigated for the influence of glucose on the dye removal efficiency of integrated enzyme system.
(4) Dye concentrations: The dye concentrations of methyl red were tested in the range of 50–200 μM in the 4-ml cuvettes containing 50 mM citrate–phosphate buffer (pH 6.5), 1.0 mM NAD^+, 50 mM glucose at an appropriate enzyme ratio.

Each experiment was carried out in triplicate.

2.4. Statistical analysis and artificial neural network modeling

To discover the relative importance of parameters influencing the integrated enzyme system, sensitivity analysis was carried out by ANN. In this study, 81 sets of data obtained from experiments were used to train and test a back propagation ANN model. The data sets were randomized and divided into training, validation and test subsets, which included 41, 20, and 20 samples, respectively. All samples were normalized in the range of 0.1–0.9 using the following equation:

$$A_i = 0.8\left(\frac{X_i - \min(X_i)}{\max(X_i) - \min(X_i)}\right) + 0.1$$

where $\min(X_i)$ and $\max(X_i)$ were the extreme values of variable X_i (Khataee et al., 2010). The mean square error term was used to determine the structure of the three-layered feed forward back propagation neural network ANN. The number of hidden nodes of ANN was finally determined as 4 in this research.

A SPSS software package was used for significance analysis. A *P*-value at 0.05 was used to determine the significance between different treatments. MATLAB (Version R2009a, USA) software was used to model the performance of integrated enzyme system by artificial neural network.

2.5. Batch experiment of integrated enzyme system for dye removal

To test the potential use of integrated enzyme system for dye removal, batch experiments were carried out in 10-ml flasks mixing with magnetic stirrer. Dyes were added in batch flasks when the color disappeared. The time interval was recorded. The products of methyl red after enzyme catalysis were assayed according to a HPLC method (Moutaouakkil et al., 2003).

3. Results and discussion

3.1. Effect of enzyme ratio on the performance of integrated enzyme system

Enzyme-coupled NADH regeneration system has advantages of high selectivity, high rates and easy monitoring of reaction