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Production of Indigo by Immobilization of *E. coli* BL21 (DE3) Cells in Calcium-Alginate Gel Capsules*

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Abstract The ability of catalyzing indole into indigo of gene engineering strain expressing P450 BM3 immobilized by entrapment in calcium-alginate gel capsules was examined, and various characteristics of immobilized cells were assessed. Optimum conditions for cells activity were not affected after immobilization, and pH and temperature for both free and immobilized cells were found to be pH 7.5 and 35℃, respectively. The immobilized cells exhibited a markedly improved thermal stability than free cells. After five repeated experiments, the yield of indigo with the immobilized cells retained over 94% of their original activity, which indicated that the operational stability for recycling in batch processes was improved.
Keywords calcium-alginate gel, gene engineering strain, immobilized cells, indigo, P450 BM3

1 INTRODUCTION

Indigo is considered to be the oldest dye[1]. It was generally extracted from various species of plants initially. However, by the end of nineteenth century the commercial synthetic indigo almost completely replaced indigo production from the natural source. More recently, research work has been undertaken to find a way to replace the chemical synthesis of indigo by using bacterial systems[2—8].

The dye indigo is found in mammalian urine[9,10]. P450 enzymes are found throughout nature from archbacteria to humans and can catalyze the monoxygenation of a diverse range of chemicals[11]. It is generally believed that tryptophan is degraded by intestinal bacteria to indole[10,12]. Some research workers considered the hypothesis that indole might be hydroxylated by P450 enzymes and finally carried out oxidation and dimerization to form indigo spontaneously[13,14].

Human cytochrome P450 enzymes expressed in *Escherichia coli* can oxidize indole to indigo[13,15,16]. The wild P450 BM3 enzyme from *Bacillus megaterium* catalyzes subterminal hydroxylation of saturated long-chain fatty acids. However, P450 BM3 enzyme with novel function of converting indole into indigo has been obtained by directed evolution[17,18].

But most P450 enzymes act supported by one or more redox partners. NAD(P)H serves as the electron donor. However, use of whole cell system containing the P450 BM3 monooxygenase can ensure the regeneration of the cofactor NAD(P)H during the bioconversion. But free cells can be used only once in biocatalysis. To ensure reuse of the cells and reduce the cost, immobilization technique is necessary to be applied.

The encapsulation of biocatalysts in calcium alginate capsule is a widespread technique. It can enclose the biocatalysts in an aqueous solution inside a semipermeable membrane capsule[19]. This technique has all the advantages of immobilization in calcium alginate gels: biocompatibility, simplicity, and low cost. Moreover, the main advantage of this technique is that it has a specific liquid core which enables substrate and biocatalyst contact easily[20]. This technique has been applied to immobilize microbial cells, such as *Lactobacillus rhamnosus*[21] and *Lactobacillus casei*[22].

The aim of the current study was to investigate the possible bioconversion of indole into indigo using whole cells immobilized in Ca-alginate gel capsules. The reaction conditions affecting biocatalysis were investigated. The operational stability of immobilized cells was also investigated.

2 MATERIALS AND METHODS

2.1 Chemicals

Sodium-alginate and CMC (sodium carboxy methyl cellulose) were obtained from China Medicine (Group) Shanghai Chemical Reagent Corporation. According to the manufacturer's specifications, the viscosity of 1% (mass concentration) sodium-alginate solution is higher than or equal to 0.02Pa·s at 20℃, and the CMC solution has a viscosity range of 300—800mPa·s. All other chemicals were of analytical grade and commercially available.

2.2 Microorganism and cultivation

The basic system involving the construction of plasmid and the expression of P450 BM3 has been described elsewhere[17,18]. The cultivation was carried out in a 500ml shake flask containing 100ml LB medium supplemented with 30μg·ml^{-1} kanamycin for plasmid selection. The medium was inoculated with 2ml of an overnight culture of recombinant *E. coli* BL21 cells. The shake flask was incubated at 180r·min^{-1} and 37℃. At an optical density (OD_{578nm}) of 1.0, P450 BM3 expression was induced by adding IPTG to a concentration of 0.5mmol·L^{-1} at 35℃ for 6h. Cells

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were harvested by centrifugation at 8000r·min^{-1} for 10min and were washed twice with 0.9% saline solution.

2.3 Cell immobilization

For the immobilization of cells of *E. coli* BL21, 0.15g cells were added into 10ml of 2% (mass concentration) $CaCl_2$ solution which contained 1% (mass concentration) CMC and mixed thoroughly. This mixture was pipetted into a syringe and added dropwise into 50ml of quiescent 1% (mass concentration) sodium-alginate solution, and allowed to react for 5min[23]. The capsules were transferred into fresh 1.5% (mass concentration) $CaCl_2$ and incubated for 20min by agitating to complete gel formation. Then the capsules were rinsed with distilled water to remove excess calcium chloride. The diameter of capsules was (3.5±0.1)mm.

2.4 Biotransformation

Free cells or encapsules of the same batch were added to 25ml of 50mmol·L^{-1} Tris-HCl buffer (pH 7.5) containing 0.5mmol·L^{-1} indole. The bioconversion was carried out at 35℃ on a rotary shaker at 180r·min^{-1} for 12h.

2.5 Repeated indigo production by reuse of the immobilized cells

The immobilized cells were added into 100ml of 50mmol·L^{-1} Tris-HCl buffer (pH 7.5) containing 0.5mmol·L^{-1} indole. Batch experiments were performed at 35℃ under constant agitation (180r·min^{-1}). After 12h, the immobilized cells were filtered, rinsed twice with sterile deionized water, and transferred into 100ml of fresh Tris-HCl buffer for the next batch reaction.

2.6 Analytical procedure

After biotransformation, encapsules were filtered, filtrates were centrifuged at 8000r·min^{-1} for 10min, the residua of cells and indigo were collected. 25ml *N*,*N*-dimethylformamide (DMF) was added to the residua blue pellet to extract indigo. UV-visible spectra were recorded with an Ultrospec 3300 pro UV/Visible spectrophotometer (Amershan Biosciences, USA) in DMF to determine the absorption at 610nm and estimate the yield of indigo[15].

2.7 Data analysis

Each data point in the figures represent the mean of triplicate samples. All biotransformation experiments were performed in triplicate. The standard deviation is shown by an error bar. Many error bars are smaller than the data point symbols.

3 RESULTS AND DISCUSSION

3.1 Effect of temperature on catalytic reaction

The results of the influence of different temperatures on the indigo yield by free cells and immobilized cells are shown in Fig.1. It was shown that the optimum temperature for the bioconversion by both free cells and immobilized cells of *E. coli* BL21 expressing P450 BM3 was 35℃, and that immobilization could improve the yield of indigo effectively, and that high yield was exhibited over a relatively wider temperature range by immobilized cells than by free cells. Yield sharply decreased at about 40℃ and the bioconversion nearly stopped at 45℃ by free cells. The yield was 41% and 20% by immobilized cells when the conversion performed at 40℃ and 45℃, respectively.

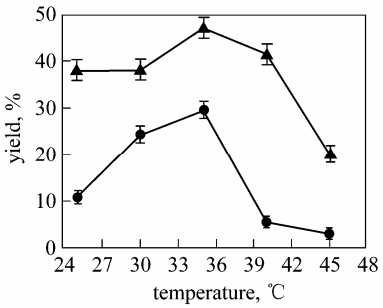

Figure 1 Effect of temperature on the yield of indigo by free and immobilized cells
[Reaction conditions: 0.5mmol·L^{-1} indole in 25ml Tris-HCl buffer (pH 7.5) at different temperatures for 12h]
▲ immobilized cells; ● free cells

3.2 Effect of pH on catalytic reaction

The effect of pH in the range 3—9 on the indigo yield was investigated and the results are shown in Fig.2. At any pH value under investigation, the indigo yield by immobilized cells was higher than that by free cells. It was obvious that the optimal pH was 7.5 by immobilized and free cells. The maximum yield of indigo (47%) was obtained by immobilized cells in comparison with free cells (24%) at pH 7.5. However, high yield was obtained over a relatively narrow pH range. Therefore, the pH of the reaction system is an important factor for indigo formation, and pH during the reaction should be controlled to maintain maximum activity.

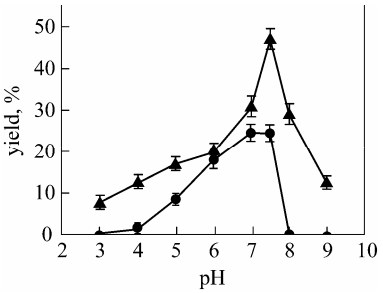

Figure 2 Effect of pH on the yield of indigo by free and immobilized cells
(Reaction conditions: 0.5mmol·L^{-1} indole at 35℃ for 12h, in 25ml Tris-HCl buffer of different pH)
▲ immobilized cells; ● free cells

At pH8.0, the biotransformation could hardly perform with free cells, while the indigo yield was 29% at pH8.0 and the reaction could still carried out at pH9.0 by immobilized cells.

3.3 Effect of substrate concentration on catalytic reaction

Figure 3 shows that indigo yield increased sharply with increasing indole concentration as substrate from 0.1mmol·L^{-1} to 0.5mmol·L^{-1}. The maximum transformation efficiency was obtained at substrate concentration of 0.5mmol·L^{-1}. However, indole added into the reaction system higher than 0.5mmol·L^{-1} resulted in the sharp decrease of indigo yield by both immobilized and free cells. When the indole concentration was 10mmol·L^{-1}, the excessive substrate may be toxic to the cells and the bioconversion stopped.

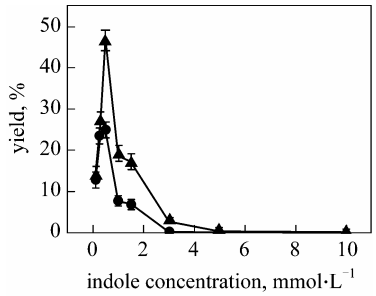

Figure 3 Effect of substrate concentration on the yield of indigo by free and immobilized cells
[Reaction conditions: indole of various concentrations in 25ml Tris-HCl buffer (pH 7.5) at 35℃ for 12h]
▲ immobilized cells; ● free cells

3.4 Thermal stability

Figure 4 reveals the relationship between residual cells activity and temperature for free and immobilized cells stored at pH 7.5 at different temperatures for 1h. Decrease of P450 BM3 activities in free cells began at temperature in excess of 50℃. The enzyme activity in immobilized cells did not decrease at the temperature of lower than 60℃, and the enzyme activity in immobilized cells remained 15% of the initial activity at 80℃, at which the enzyme activity of free cells was nearly undetected. The results indicated the immobilized cells in Ca-alginate gel capsules could prevent enzyme deactivation at high temperature.

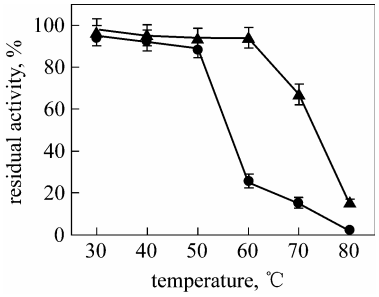

Figure 4 Thermal stability of free and immobilized cells of *E.coli* BL21 expressing P450 BM3
(After stored at pH 7.5 at different temperatures for 1h, reactions were carried out utilizing conditions described in Section 2. The residual activity is expressed as percentage of maximum indigo yield obtained with the no-heat-treated immobilized or free cells under the experimental conditions described in Section 2.)
▲ immobilized cells; ● free cells

3.5 Time course of transformation of indole to indigo by immobilized cells

Figure 5 demonstrates typical transformation of indole to indigo by immobilized cells of *E. coli* BL21. The indigo synthesis showed a linear increase during the initial four hours. The indigo concentration could reach 105μmol·L^{-1} by immobilized cells, which was much higher than that by free cells.

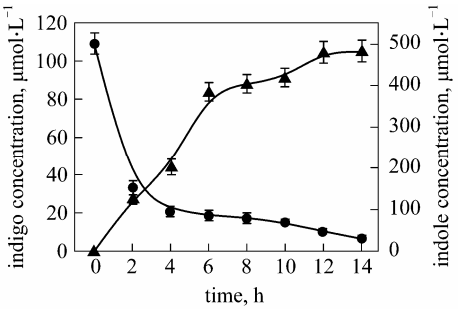

Figure 5 Time course of indigo synthesis by immobilized cells
[Reaction conditions: 0.5mmol·L^{-1} indole in 25ml Tris-HCl buffer (pH 7.5) at 35℃]
▲ indigo; ● indole

3.6 Reusability of immobilized *E.coli* BL21 cells

The reusability of immobilized cells in Ca-alginate gel capsules was studied. The results presented in Fig.6 indicate that the transformation capacity decreased slightly by repeating the use of immobilized cells for five times. Reuse of immobilized cells from 6 to 10 cycles was accompanied by a gradual reduction in transformation activity.

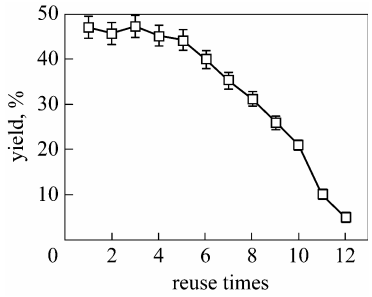

Figure 6 Repeated indigo production by reuse of the immobilized cells

The *E. coli* BL21 immobilized cells in Ca-alginate gel capsules can be reused five times and this is significant from an economical viewpoint. Compared with free cells, the immobilized cells would be shown much more stable in the batch process.

The Ca-alginate gel protected the cells from the influence of external conditions and decreased the sensitivity of the cells and enzymes to the external conditions, which increased the stability and bioconversion activity of cells.

4 CONCLUSIONS

E. coli BL21 cells have been successfully immobilized in Ca-alginate gel capsules. In comparison

with the free cells, immobilized cells were less sensitive to variations in incubation temperature, and exhibited a notably improved thermal stability. Treatment procedure with immobilization did not change the optimal pH and temperature of the biotransformation. Furthermore, the immobilized cells held better stability in repeated operations. In a word, immobilizing *E. coli* BL21 cells in Ca-alginate gel capsules can successfully increase the stability and bioconversion activity of P450 BM3 expressed in *E. coli* BL21.

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