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Immobilization of *Gluconobacter oxydans* by Entrapment in Porous Chitosan Sponge

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Abstract

The porous chitosan sponge was prepared using $NaHCO_3$ as the porogen and used to immobilize *Gluconobacter oxydans*. Under the optimum conditions, the activity recovery of the immobilized cells reached 92%. The morphology characterization of the immobilized cells revealed that the cells were attached to the surface of the pores (100-400μm) which were well distributed in the chitosan sponge. The valuation of cell activity showed that the immobilized cells displayed enhanced pH and thermal stability compared to free cells. Furthermore, the immobilized cells retained 74% of its origin activity after 12 repeated reaction cycles separated by filtration.

Keywords: Chitosan sponge; Gluconobacter oxydans; Immobilization; Porogen

Introduction

Gluconobacter oxydans have been widely used to incompletely oxidize sugar, alcohol and aldehyde to produce aldehyde, ketone and acid by its dehydrogenases connected to the respiratory chain [1]. However, *G. oxydans* is a very small size bacterial leading to difficulties in the reuse or recycling of the cells for large-scale application.

Whole cell immobilization, which provides cells with easy separation, enhanced stability and reusability, has been proved to be an efficient solution [2]. As the immobilization matrices, natural polymers such as agar, alginate, carrageenan and chitosan have received considerable attention due to their high biocompatibility [3], and a variant of the whole cell immobilization technique have been developed. Chitosan, the second most abundant natural polymer found in the exoskeleton of marine crustaceans, has gained great interest in immobilization technology [4]. It is also supposed to be very interest substance for diverse applications in biomaterial such as in preparing films [5], beads [6], scaffolds [7], hydrogels [8], nanofibers [9] and nanoparticles [10] in the pharmaceutical field due to its biocompatibility, biodegradability and bioactivity.

In this study, we proposed a novel method to immobilize *G. oxydans* in the chitosan spongy material which was synthesized using $NaHCO_3$ as the porogen to react with acetic acid producing CO_2 and forming pores in chitosan sponge. Compared with other porogen such as polyethylene glycol [11] and silica [12,13], $NaHCO_3$ can be removed in mild condition leading to a higher cell activity recovery. After immobilized, the cells were easily separated from the solution by filtering, and their thermal and pH stability and reusability were increased.

Materials and Methods

Microorganism and cultivation conditions

The strain *G.oxydans DSM 2003* was used in this study. The cells were cultured in 500-mL flasks containing 50 mL sterile fermentation medium, which contained 80 g sorbitol/L, 20 g yeast extract/L, 1 g KH_2PO_4/L, 0.5 g $MgSO_4$/L, 0.1 g Glutamine/L. The cells were incubated at 30°C with shaking at 200 rpm for 24 h and collected by centrifugation.

Immobilization of *G. oxydans*

Chitosan (1.5 g) was dissolved in 2% (v/v) acetic acid (100 mL), and then the pH of the solution was adjusted to 5.0 using 2 M NaOH. 5 mL *G. oxydans* suspension (10 g cells/L, dry weight) was added to the above solution followed by the addition of 10 mL the mixture of $NaHCO_3$ (10% w/v) and glutaraldehyde (5% w/v). The reaction mixture was further stirred at room temperature until formatting the porous chitosan sponge with cells embeded. The immobilized cells were filtered and washed several times with phosphate buffer (pH 6.0). The density of free cells in the solution was determined by measuring the optical density of the cell suspension at 600 nm (OD600) with a spectrophotometer (U-2001; Hitachi, Tokyo). The difference in cell density was used to calculate immobilization efficiency. The morphology of the immobilized cells was observed on a Scanning electron microscopy (SEM) (JEOL Japan). Samples were lyophilized and sputter-coated with gold prior to scanning.

Activity assay

The activity of cells was determined by measuring the production of dihydroxy acetone (DHA) from glycerol. The reaction was carried out at 30°C in phosphate buffer (pH 6.0,10 mM) containing 10 g glycerol/L. After reacting for 1 hour, the free cells were separated by centrifugation and the immobilized cells were separated by filtration. The reaction products were analyzed by HPLC using a COREGEL 87H3 column (Transgenomic, USA) with isocratic elution of 4 mM H_2SO_4.

Stability and reusability

Effect of temperature and pH on the activity of immobilized cells were determined from 20-50°C and pH 5-8.5 and compared with free cells. The reusability of the immobilized cells was assessed under the same conditions as described in activity assay section.

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Results and Discussion

Morphology of the biocarrier

Figure 1 showed the SEM images of porous chitosan sponge with and without immobilization of *G. oxydans*. The biocarrier was highly porous and the pores (ranging from 100 to 400μm) were well distributed with an interconnected pore wall structure. The porous structure of the chitosan sponge was beneficial for cell adhesion and substrates diffusion. As shown in Figure 1d, the chitosan sponge surface without cells entrapment is relatively smoother than the one with cells in Figure 1c which revealed the cells attached to the chitosan sponge and covered a majority of the inner surface of the pores. The internal cells distribution of chitosan sponge was confirmed by Laser Scanning Confocal Microscopy (LSCM) (Supplementary Figure S1) and the results displayed that the cells were well distributed inside the chitosan sponge.

Immobilization of *G. oxydans*

The effect of glutaraldehyde concentrations showed the cells' activity decreased with the increase of glutaraldehyde (Supplementary Figure S2). However, the chitosan couldn't form a cross-linked sponge when the concentration of glutaraldehyde was less than 0.5% (w/v), leading to cells leakage. Hence, the optimum glutaraldehyde concentration was 0.5% and the cell activity retained more than 90% at this concentration.

As the porogen, $NaHCO_3$ reacted with acetic acid producing CO_2, which formed pores in chitosan sponge. The porosity of chitosan sponge increased with the increase of the concentration of $NaHCO_3$ (Supplementary Table S1). Figure 2a showed that the activity recovery of immobilized cells increased from 21% to 92% when the concentration of $NaHCO_3$ increased from 0 to 10%, but decreased to 62% as the concentration of $NaHCO_3$ continued to rise to 15%. The improvement of the activity recovery of immobilized cells was attributed to the increase of the porosity of chitosan sponge which was favorable for the diffusion of substrates. However, when the concentration of $NaHCO_3$ was up to 15%, the activity recovery of immobilized cells was decreased quickly due to the alkaline internal condition of chitosan sponge which exceeded the optimum catalytic pH conditions.

The effect of the concentration of chitosan on the activity recovery of immobilized cells was investigated and the result was showed in Figure 2b. Increasing the concentration of chitosan in the range 0 - 1.5% resulted in an increase in the activity recovery from 41% to 87.5%. However, the activity recovery began to decrease above chitosan concentration of 1.5%, which supposed to be due to the decrease of porosity of the chitosan sponge (Supplementary Table S2).

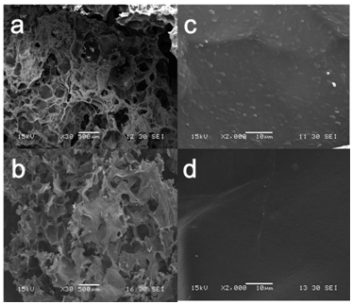

Figure 1: SEM images of porous chitosan sponge. (a) magnification~30x, (b) magnification~30x, (c) magnification~2,000x, (d) magnification~2,000x.

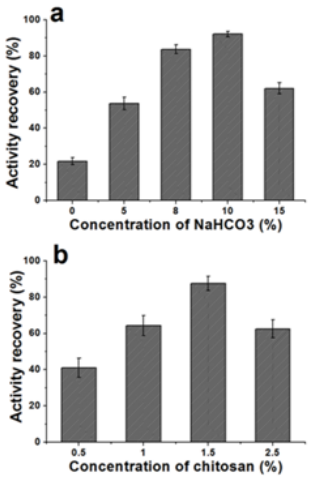

Figure 2: The effect of the concentration of $NaHCO_3$ (a) and chitosan (b) on the activity recovery of immobilized cells. The activity of free cells was defined as 100%.
a: 1.5% (w/v) glutaraldehyde,10% (w/v) $NaHCO_3$, 5 mg (dry weight) cells; b: 1.5% (w/v) glutaraldehyde,1.5% (w/v) chitosan, 5 mg (dry weight) cells.

Effect of pH and temperature

Figures 3a and 3b showed that the optimal temperature and pH for both free and immobilized cells to achieve the highest activity were 30°C and pH 6.0. Compared to the free cells, the immobilized cells retained higher thermal and pH stability after incubated at various temperatures (Figure 3c) and pH (Figure 3d). Because the enzyme responsible for DHA synthesis was a membrane bound dehydrogenase, the enhanced stability of immobilized cells may be attributed to the decrease damaged of cell's membrane after the cells were covalently linked to the chitosan sponge by glutaraldehyde.

Reusability

The reusability of immobilized cells is a very important property in their application. Compared to the free cells separated by centrifugation, the chitosan sponge entrapped cells can be easily separated by filtration and applied for another cycle. As shown in Figure 4, the immobilized cells retained 74% of its initial activity after 12 cycles, whereas the free cells only retained 50% of its initial activity. The decrease of activity was caused by the cell inactivation, but not the cell leakage (no cell loss was observed in this process). Furthermore, the cells were hardly leaked from the chitosan sponge even after shaking at 200 rpm for 3 days (data not shown). It was suggested that the cells were covalently linked with chitosan sponge by glutaraldehyde which prevented cells detaching from the carrier.

Conclusion

Gluconobacter oxydans was immobilized on the porous chitosan sponge with a 92% activity recovery after optimization. The immobilized cells displayed higher thermal and pH stability than the free cells. More importantly, the immobilized cells can be easily separated and reused, and retained 74% of the initial activity even after 12 cycles. These results make the porous chitosan sponge a promising material in cells immobilization applications.

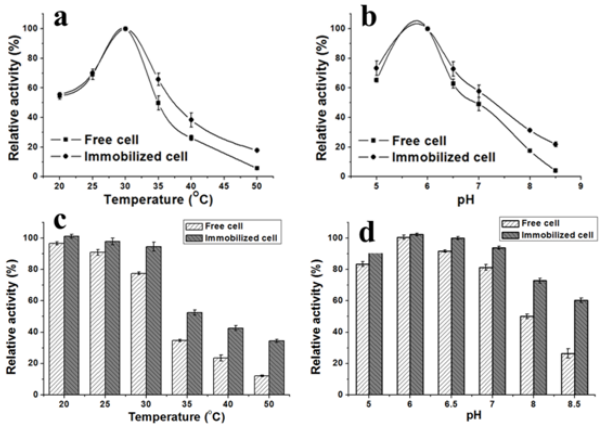

Figure 3: Effects of temperature (a) and pH (b) on the activity of the free and immobilized cells, Thermal (c) and pH (d) stability of the free and immobilized cells.
a: 5 mg cells, 10 g glycerol/l, sodium phosphate buffer (pH 6), temperature ranging from 20-50°C; b: 5 mg cells, 10 g glycerol/l, 100 mM sodium phosphate buffer (pH from 5to 8.5), at 30°C; c: The samples were pre-incubated at temperature ranging from 25 to 50°C for 12 hours and the residual activity was measured at pH 6.0 at 30°C; d: The samples were pre-incubated at pH ranging from 5 to 8.5 for 12 hours and the residual activity was measured at pH 6.0 at 30°C.

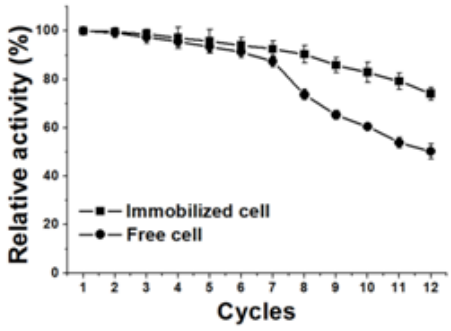

Figure 4: Reusability of the immobilized cells. Reaction conditions: 5 mg cells, 100 mM sodium phosphate buffer (pH 6), at 30°C. After each run, the immobilized cells were separated by filtration and the free cells were separated by centrifugation (10,000 rpm), washed with phosphate buffer (pH 6.0) for 2 times. The initial activities of the free and immobilized cells were defined as 100%.

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