Note

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Utilization of Chitosan Gel Beads as Supports of Fungus *Trichoderma viride* Cells for Cellulase Production

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Abstract: Cells of *Trichoderma viride*, a cellulolytic fungus, were immobilized in chitosan gel beads and employed for producing cellulase by a repeating batch procedure. The activity of the immobilized cells increased with extending culture period, and reached a maximum, 37 μg of reducing sugars per mL of culture filtrate, in 6 days. This value was about 1/4 of that for free cells. The enzyme activity of chitosan gel-immobilized cells in the repeated, batch culture retained about 50 % of the initial activity after ten times of batch culture.

1. Introduction

Enzyme production by immobilized microbial cells is an active research area despite the fact that industrial applications are still limited [1]. Immobilized cells have unique properties as a biocatalyst and can provide the basis for a continuous process. Methods for immobilizing intact cells have been confined to the entrapment in a hydrophilic polymer matrix such as polyacrylamide, sodium alginate, and carrageenan [2-6]. As a result of immobilization, the rate of the desired enzyme reaction is often suppressed by the diffusion barrier presented to the substrate or oxygen by the polymer matrix.

Aerobic bacterium, *Bacillus* sp., was first immobilized several years ago using polyacrylamide [7] and carrageenan [8] gels. In our previous paper [9], we reported that chitosanase production could be performed by immobilized bacterial cells, *Alcaligenes faecalis*, using calcium alginate gels. We have also reported the investigation on utilization of chitosan derivatives as supports for immobilization of microorganism cells [10] and enzymes.

In this work, the immobilization of an aerobic filamentous fungus *Trichoderma viride*, has been studied using chitosan gel beads. Kumakura et al. [11] reported that aerobic cells such as *Trichoderma reesei* were immobilized with fibrous substances, the fibrils of which were attached by radiation polymerization of hydroxyethyl methacrylate and that the immobilized cells grew on the surface of the fibrils. Seo [12] reported that the gel strength of the chitosan gel beads was higher than that of polysaccharides such as agarose and dextran. A

chitosan bead, which is spherical in shape and has more than 100 m^2/g of specific surface area, forms a micro-pore structure with 0.1 ~ 0.2 μm of pore diameter. Therefore, the use of chitosan beads as a support is expecting for the effective enzyme production by the immobilized cells.

Recently, the enzymatic hydrolysis of cellulosic wastes with cellulase has been studied with the objective of biomass conversion and many works have focused on the use of cellulase from *Trichoderma viride* grown aerobically on cellulose. Cellulase production by *T. viride* in a continuous culture is very important for reducing the cost of the cellulase production.

2. Experimental

2.1 Chemicals

The purified chitosan (degree of deacetylation, 97%) from crab powders was kindly supplied by KATAKURA CHIKKARIN Co. Ltd. All other materials were of the highest grade commercially available.

2.2 Culture of Microorganism

Trichoderma viride (IFO 31137) was cultured in a medium containing 2.0 g of malt extract, 2.0 g of glucose, and 1.0 g of peptone in 1L of deionized water in a 2L Erlenmeyer flask. The pH was adjusted to 6.0 with 1 N HCl. The cultivation was carried out aerobically at 28 °C for 48 h on a rotary shaker (120 rpm). After the cultivation, cells were harvested by centrifugation at 7800 $\times g$ for 15 min, washed with a 0.9% NaCl solution, and used for immobilization.

Table 1 Composition of Cellulase Production Medium^{a)}

Component	Concentration (g/L)
Microcrystalline cellulose	7.5
Peptone	0.75
Tween 80	2.0
Urea	0.3
KH ₂ PO ₄	2.0
(NH ₄) ₂ SO ₄	1.4
MgSO ₄ · 7H ₂ O	0.6
CaCl ₂ · 2H ₂ O	0.4
FeSO ₄ · 7H ₂ O	5.0×10^{-3}
MnSO ₄ · 4H ₂ O	2.2×10^{-3}
ZnSO ₄ · 4H ₂ O	1.4×10^{-3}
CoCl ₂ · 6H ₂ O	3.6×10^{-3}

a) The medium containing culture components except urea was adjusted to pH 7.0 and was sterilized by autoclaving at 121 °C for 15 min. Urea was filter-sterilized and added to the culture medium.

Fig. 1 Enzyme production by free *Trichoderma viride* (○) and *T. viride* immobilized in chitosan gel beads (●).

2.3 Immobilization

The pH of 2% (w/v) chitosan hydrochloride solution was adjusted to 6.0 with 4 N HCl. Wet *T. viride* cells (1g) were suspended in 40 mL of the chitosan solution. This suspension was dropped into a 1.5% (w/v) Na-tri-

polyphosphate solution (pH 8.3) with a No. 18 syringe needle under slow stirring. After shrinking and hardening for 2 h, the biocatalyst gel beads were washed with 0.1 M phosphate buffer (pH 7.2). Gel beads were immersed in a 0.05% glutaraldehyde solution for 15 min at room temperature and washed with distilled water. The beads were uniform in diameter (2-3 mm).

2.4 Enzyme Production

For experiments with free cells, 2 mL of broth precultured for 18 h at 28 °C was inoculated into 50 mL of an enzyme production medium (Table 1) and incubated at 28 °C in a 200 mL Elenmeyer flask on a reciprocating shaker (85 strokes/min), usually for 6 days. For experiments with immobilized cells, 1 g of wet gel beads was incubated in 50 mL of the same medium in a 200 mL Elenmeyer flask as above at 28 °C pH 7.0, usually for 6 days per one batch. After 6 days the beads were removed, washed three times with 0.1 M phosphate buffer (pH 7.2) and reincubated in a fresh production medium.

2.5 Assay of Cellulase

Cellulase activity were determined by the amount of reducing sugars produced from cellulose. Cellulose (50 mg) was added to the solution composed of 5.0 mL of 0.1 M acetate buffer (pH 4.6) and 0.5 mL culture filtrate. The mixture was incubated at 50 °C for 1 h. The reducing sugars produced in the mixture were assayed by the Nelson-Somogyi method [13].

3. Results and Discussion

3.1 Cellulase Production of Free and Immobilized Cells

Fig. 1 shows the time course of the enzyme activity for cellulase produced by free and immobilized *T. viride* cells. The unit of cellulase activity was defined as mg of reducing sugars produced per mL of reaction solution. Cellulase activity of free cells of *T. viride* increased gradually with the increase in culture time, and was 127 μg/mL after 6 days. On the other hand, the cellulase activity of immobilized cells increased rapidly after 4 days and was 39 μg/mL after 6 days. If a relatively high cellulase activity were desired, the cultivation should be continued for about one week under the experimental conditions employed. However, the activity of cellulase by the immobilized cells was significantly low, about 1/4 of that for free cells. At the culture of 6 days, dry weight of free cells was 30 mg per 50 mL culture medium, and that of cells immobilized in gels was 0.7 mg. This

observation shows that the growth of *T. viride* cells was restricted because they were entrapped in chitosan gel beads.

Leakage of the cells from the gel beads was observed with the increase in the amount of immobilized cells. This indicates that the apparent bulk of the immobilized cells increased during culture as the results of the active growth of the cells. To protect the diffusion of cells into medium, cells immobilized in chitosan gel beads were treated with 0.05% glutaraldehyde. Fig. 2 shows the time

Fig. 2 Enzyme production by *T. viride* immobilized in chitosan gel beads linked with glutaraldehyde.

course of the enzyme activity of the cellulase by cells immobilized in gel beads linked with glutaraldehyde. Cellulase was produced on culture over 4 days, and reached a maximum, 37 μ g/mL, after 6 days without leakage of the cells into the medium. With the increase in the culture time to over 6 days, contents of reducing sugars slightly decreased. Cellulase production by the immobilized cells may be reduced after 6 days, for the swelling of the gel was observed for the culture after 6 days and aliquotes of the immobilized cells leaked out of gel into medium. The induction period in enzyme production by the immobilized cells was longer than that for free cells (Figs. 1 and 2). It seems that, in the immobilized cells, the rate of the incorporation of nutrients into the immobilized cells was slower than that into the free cells because of the trapping of the cells in the gels.

3.2 Cellulase Production by the Immobilized Cells in the Repeated Batch Cycles.

The repeating use of the immobilized cells in the batch culture was examined. In the repeated batch culture, cellulase activity of the culture medium was measured after every culture and the culture medium was replaced by a new medium. Fig. 3 shows the relationship between cellulase activity and repetition number of batch culture. Cell survival was almost constant for repeated culture, about 50 mg as wet weight of cells per 1g wet gel beads

Fig. 3 The production of cellulase in repeated 6-day batch culture by *T. viride* cells immobilized in chitosan gel beads. Experimental conditions are described in 2.4.

at each batch culture. Thus the cells of *T. viride* immobilized in gel beads seem to be viable throughout the repeated batch culture, effectively producing the enzyme. After the batch culture of 4 times, however, the enzyme activity went down to about 50% of the beginning value. Thereafter it showed no significant drop in activity even after the repeating use over 10 times. This indicates that gel beads were slightly loosened and that aliquotes of cells in gell beads leaked into the medium during repeated batch culture over 5 times (Fig. 2).

In conclusion, continuous culture of the immobilized cells for cellulase production is possible and the immobilized cells can be used for a reactor of enzyme production in continuous culture. Chitosan gel beads is useful for a cell-immobilization support for a bioreactor.

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