Note

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CHITOSANASE PRODUCTION BY *ALCALIGENES FAECALIS* IMMOBILIZED IN CALCIUM ALGINATE GEL BEADS

By Hideo Kusaoka, Tetsuya Takemura, and Hiroumi Ichikawa

(Department of Applied Physics and Chemistry, Fukui Institute of Technology, Fukui 910, Japan)

ABSTRACT: *Alcaligenes faecalis* cells were immobilized in calcium alginate gel beads and employed for production of chitosanase by repeated batch processes. The activity of the immobilized cells increased with extending the culture period, and reached a maximum, 3.6 U/ml in 6 days. This value corresponded to about 1/3 of that of free cells. In repeated batch experiments the activity of the immobilized cells increased gradually in the early cycles, and it decreased slightly after the four cycle. Chitosanase was produced by the immobilized cells in an enzyme production medium containing 0.1% glucose, 0.5% peptone, 0.1% yeast extract, 0.1% KH_2PO_4 , 0.02% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.1% NaCl, 0.5% $\text{CaCl}_2 \cdot \text{H}_2\text{O}$ and 0.5% colloidal chitosan.

1. INTRODUCTION

Enzyme production by immobilized microorganisms is new research area although industrial applications are still restricted¹⁾. The production of extracellular enzymes by immobilized cells has also been reported. Kokubu *et al.*²⁾ made the first attempt to produce α -amylase by polyacrylamide-entrapped *Bacillus subtilis* cells. Similarly, protease³⁾, lytic enzymes⁴⁾, and cellulase⁵⁻⁹⁾ were produced by immobilized cells with promising results. However, enzyme production by immobilized bacteria has not been reported except for the production of α -amylase by *Bacillus subtilis*²⁾.

In our previous papers¹⁰⁻¹⁶⁾, we have reported the investigation of the utilization of chitosan and its derivatives as supports for immobilization of enzymes and microorganism cells. On the other hand, from the standpoint of the biological utilization of chitosan and its related enzymes, first chitosanase-producing bacteria, *Alcaligenes faecalis* IK-5 strain was isolated from microorganisms in soil¹⁶⁾. Chitosanase is an enzyme which hydrolyzates chitosan and its biological application has been noted since several years ago¹⁷⁾. Our interest in production of chitosanase by immobilized living cells is to confirm a new method for continuous production of extracellular enzymes.

In this paper, we would report the immobilization of *A. faecalis* in calcium alginate gel beads and the production

of chitosanase in a batch system with the immobilized cells.

2. EXPERIMENTAL

2.1 Chemicals

The purified chitosan from crab shell powders was kindly supplied by Katakura Chikkarin Co. Ltd. All other materials were of the highest grade commercially available.

2.2 Culture of Microorganism

Alcaligenes faecalis IK-5 (FERM P 9678)¹⁶⁾ was pre-cultured in one liter of a Trypto-soy Broth medium (Eiken Chemical Co. Ltd) consisting of 1.7% tripeptone, 0.3% soypeptone, 0.25% glucose, 0.25% KH_2PO_4 , and 0.5% NaCl (pH 7) in 2 l Erlenmeyer flasks. The cultivation was performed at 30°C for 24 h on a rotary shaker (120 rpm). After the cultivation, cells were harvested by centrifuging at $7800 \times g$ for 15 min, washed with a 0.9% NaCl saline solution, and used for immobilization.

2.3 Immobilization

Immobilized cells were prepared by entrapment in calcium alginate gel beads under aseptic conditions by the method of Gao-Xiang *et al.*⁸⁾ with a little modification. One hundred ml of 1% sodium alginate was autoclaved at 120°C for 15 min and cooled to room temperature. The sterilized alginate was mixed with 1.6 g (wet weight) of cells and extruded dropwise from a No. 18 syringe needles into a gently stirred 0.5 M CaCl_2 solution (400 ml).

The biocatalyst gel beads formed were hardened for 2 h in an ice-cold bath under mixing. The spherical beads obtained were of uniform size of 2-3 mm diameter.

2.4 Enzyme production

For experiments with free cells, 2 ml precultured broth was inoculated into 50 ml of enzyme production medium as shown in Table 1 and incubated at pH 7.0, at 28°C in 200 ml Erlenmeyer flasks on a reciprocating shaker (60 strokes/min), usually for 6 days. Colloidal chitosan was prepared by a little modification of Price and Storck¹⁸. For experiments with immobilized cells, 10 g of gel beads were incubated in 50 ml of the same medium containing 0.5% $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, at pH 7.0 at 28°C in 200 ml flasks as above, usually for 6 days per batch. The immobilized gel beads are broken in the absence of calcium chloride in medium. Thus, 0.5% calcium chloride was put into it. After 6 days growth, the beads were removed, washed three times with sterile 0.3 M CaCl_2 solution and reincubated in fresh production medium.

Table 1 Composition of chitosan production medium

Component	Concentration (g/l)
Colloidal chitosan	5
Glucose	1
Peptone	5
Yeast extract	1
KH_2PO_4	1
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	0.2
NaCl	1

Culture medium was adjusted at pH 7.0 and autoclaved at 120°C for 15 min.

2.5 Assay of chitosanase

Chitosanase activity of culture filtrate was measured according to the slightly modified method of Tominaga and Tsujisaka¹⁹. A substrate solution was prepared by dissolving 1 g of chitosan in 20 ml of 0.1 M acetic acid with stirring. To this solution was added 40 ml of 0.2 M acetate buffer (pH 6.0) and the mixture was then adjusted to pH 5.6 with 0.1 M NaOH and made up to 100 ml with distilled water. The chitosanase activity was assayed by adding 1 ml of culture filtrate to 1 ml of the substrate solution at 40°C for 10 min. The reaction was stopped by keeping in boiling water for 3 min after the addition of 0.1 ml of 1 M KOH. Sugars liberated were

determined by the method of Elson-Morgan²⁰. One unit was defined as the amount of enzyme which produced 1 μmol of the sugars determined as glucosamine per min.

RESULTS AND DISCUSSION

3.1 Chitosanase production of free and immobilized cells

The time course of the enzymatic activity of the chitosanase produced by free and immobilized *A. faecalis* cells are shown in Fig. 1. Chitosanase activity of *A. faecalis* free cells increased rapidly after 3 days, and reached its maximum, 10.4 U/ml, in 6 days. The growth of the *A. faecalis* cells increased with the increase of enzyme production (data not shown). In these experiments with an immobilized biocatalyst, the maximum chitosanase activity of 3.6 U/ml was obtained at the 6th day, increasing gradually after 3 days. If a relatively high chitosanase activity is desired, the cultivation should be continued for about one week under the experimental conditions employed. However, the activity of chitosanase produced by the immobilized *A. faecalis* cells was significantly low, about 1/3 as much as that obtained by free cells. This observation shows that the growth of *A. faecalis* cells is lessened because they are entrapped in calcium alginate gel.

Fig. 1 Enzyme production by free *Alcaligenes faecalis* (○) and *A. faecalis* immobilized in calcium alginate gel beads (●).

3.2 Chitosanase production by the immobilized cells in the repeated batch cycles

The repeated use of the immobilized cells in the batch culture was examined. In repeated batch cultures, the

chitosanase activity of the culture medium was measured after every culture and the culture medium was replaced by fresh medium. The relationship between chitosanase activity and replaced batch culture is shown in Fig. 2. The chitosanase activity increased with repetition of 4 times of batch culture replacing. This indicates that the cells trapped in calcium alginate gel beads grow, and actively produce the enzyme through long culture period. However, the chitosanase activity with the batch culture replaced more than 5 times decreased slightly. This is probably due to the death of cells. But, the aerobic cells of *A. faecalis* immobilized in calcium alginate gel can grow until 5 times through repeated batch cultures, effectively producing the enzymes. Kokubu *et al.* reported that α -amylase activity of the immobilized cells of *Bacillus subtilis* increased gradually, and it reached a steady state after seventh cycle. These results are similar to those observed in our study. It is suggested that the immobilized cells can be used as a reactor of enzyme production on the continuous culture. The chitosanase production with immobilized *A. faecalis* cells in airlift fermentation is in progress.

Fig. 2 The production of chitosanase in repeated 6-day batch cultivations by *A. faecalis* cells immobilized in calcium alginate gel beads.

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アルギン酸カルシウムゲルビーズに固定化された

Alcaligenes faecalis によるキトサナーゼの生産

福井工業大学 草桶秀夫・竹村鉄也・市川広海

Alcaligenes faecalis 菌体をアルギン酸カルシウムゲルビーズに固定化し、くり返しバッチ培養によりキトサナーゼの生産を行った。固定化菌体の活性は、培養時間の増加とともに増加し、6日間の培養後、3.6U/mlとなり、遊離菌体の活性の約3分の1となった。くり返しバッチ培養の実験において、固定化菌体の活性はバッチ回

数の増加とともに増加し、5回目のバッチ以上ではわずかに減少した。固定化菌体によるキトサナーゼの生産は、0.1%グルコース、0.5%ペプトン、0.1%酵母エキス、0.1% KH_2PO_4 、0.02% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 、0.1% NaCl 、0.5% $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ 、および0.5%コロイダルキトサンを含む酵素生産用培地 (pH7.0) を用いて行った。