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Invertase Production by *Bacillus macerans* Immobilized on Calcium Alginate Beads

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Abstract: *Bacillus macerans* cells were immobilized in calcium alginate and used for the production of invertase. The influence of alginate concentration, cation concentration, cell/ alginate ratio, initial cell loading, curing time and bead diameter on conversion of sucrose to inverted syrup were investigated. Repeated batches fermentation with immobilized cells in shake flask were carried out with the optimized parameters such as 3% (w/v) sodium alginate, 3% (w/v) calcium chloride with 2 h curing time, 200 alginate beads/ flask with 2 mm bead diameter. The immobilized cells of *Bacillus macerans* in alginate beads are more efficient for the production of invertase and can be reused for seven cycles (336 h) without any loss in their activity and 12 cycles with 72% residual activity.

Key words: *B. macerans*, immobilized cells, invertase, repeated batch.

INTRODUCTION

Invertases (EC.3.2.1.26) are special kind of enzymes that catalyze the hydrolysis of sucrose into a mixture of glucose and fructose named inverted sugar. Sucrose hydrolysis may carried out either by hydrochloric acid at 75-80^{o}C or by invertase enzyme at 35-45^{o}C with a notorious advantage for an enzymatic over acid processes in terms of energy economy, environmental safety and low formation of by-products [15]. Generally, enzymatic invert sugar is a completely healthy sweetener. Invertase finds uses in the production of confectionery with liquid or soft centers, fermentation of cane molasses into ethanol, in calf feed preparation and also in manufacture of inverted sugars as food for honeybees[13]. Microbial products are usually produced either by free or immobilized cells. Immobilized cells have been used in a variety of applications such as biotransformation, biosensors, production of ethanol, degradation of phenol etc[6,12]. At present, the immobilization technology is often studied for its potential to improve fermentation processes and bioremediation[1]. Cell immobilization has some advantages when compared with free cell culture. The reaction speed can be accelerated, it is less susceptible to the effect of inhibitory compounds a nutrient depletion[8], protect the cells against damage and reduced susceptibility to contamination[12]. Also, cell immobilization increase productivity and stability, ease of separation, repeated use etc[5,6]. Among different immobilization methods, gel entrapment is the most common[11]. The major limitations which may need to be addressed while using such cells are dispersion of cells, flow of nutrients away from cells, diffusion of substrate and products through the cell wall and unwanted side reactions due to the presence of other enzymes[6]. Entrapment of cells in alginate is one of the simplest, cheapest and non-toxic that most frequently used method of immobilization[2]. It also provides mild and physiological conditions for cell entrapment[9]. Thus, the present work aims to optimize the production of invertase enzyme by immobilized *B. macerans* cells. The reusability of immobilized cells for invertase production was also investigated. This study represents the first report on invertase production by *B. macerans* cells immobilized within calcium alginate beads.

MATERIALS AND METHODS

Bacterial Strain and Cultivation Conditions: *Bacillus macerans* was obtained from the Center of Culture Collection of the National Research Centre (NRC), Cairo, Egypt. The culture was maintained on nutrient agar medium at 30^{o}C for 48 h and stored at 4^{o}C. Invertase production was carried out according to the method of Shafiq et al[14]. Twenty-five ml of the

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medium containing (g/l): sucrose, 30.0; peptone, 5.0 and yeast extract, 3.0 at pH 6.0, was transferred to each 250 ml Erlenmeyer flask. Two ml of cell suspension from the 24 h old slant culture was aseptically transferred to the sterilized growth medium. The inoculated flask was incubated at 30ºC and shaked at 160 rpm for 24 h. Two ml of the vegetative inoculum was transferred to 25 ml production medium and the flasks were incubated at the same speed as described previous at 30ºC for 48 h. The results are sum mean of three parallel replicates.

Invertase Activity: The activity was determined by using Nelson's method[10]. 0.2 ml enzyme solution was added to 0.8 ml sucrose solution (3% w/v in 0.2 M acetate buffer pH 4.5) and incubated at 40ºC for 10 min. One ml copper reagent was added to the reaction mixture to terminate the reaction. The tubes were then placed in a boiling water bath for 20 min, cooled and 1 ml arsenomolybdate reagent was added. Finally, 22 ml of distilled water were added to each tube and mixed well by vortexing. After mixing, the absorbance for the blank and experiment were determined at 520 nm with a double beam spectrophotometer (Shimadzu model, UV-1601). One unit of invertase activity was defined as the amount of enzyme required to release 1 µmol glucose equivalent per min under the assay conditions.

Cell Immobilization: Cells were harvested after 48 h by centrifugation at 6000xg for 15 min, 1 mg (dry cell weight) resuspended in 2 ml of sterile distilled water (equivalent to 0.05 g dry cell weight) and added to 2% (w/v) sterile sodium alginate solution to achieve the required cell/alginate ratio. The obtained mixture was then extruded dropwise through a 5 ml syringe into a gently stirred 2% (w/v) $CaCl_2$ solution from about 5 cm height and hardened in this solution for 1 h. The beads (mean diameter of 2 mm) were washed twice with sterile distilled water before being used as inoculum for invertase production. Approximately 100 beads were transferred to 25 ml fermentation medium with an agitation of 160 rpm at 30ºC.

Parameters Investigated for Optimization of Alginate Matrix:Effect of sodium alginate concentration: To determine the optimum concentration of sodium alginate for cell immobilization, various concentrations of sodium alginate (1, 2, 3 and 4% w/v) were used to prepare different types of alginate beads. The beads were prepared as described earlier in 2% $CaCl_2$ solution, transferred into the newly formulated production and incubated at 30°C for 48 h.

Effect of $Cacl_2$ Concentration: The concentration of the cationic solution has a significant effect on the stability and pore size of the bead. Immobilized beads were prepared with the same amount of cells in 3% (w/v) sodium alginate solution using different $CaCl_2$ concentrations (1, 1.5, 2, 2.5, 3 and 3.5% w/v). The beads were transferred into the production medium and incubated at 30°C for 48h.

Effect of Cell/alginate Ratio: To determine the effect of varying amount of cell mass entrapped matrix on invertase production five different cell/alginate ratios (2:1, 1:1, 1:2, 1:3 and 1:4 v/v) were compared. The immobilized beads were prepared using 3% (w/v) $CaCl_2$ solution.

Effect of Beads Number: To optimize the bead number for a particular fixed concentration of substrate, experiments were carried out. In which the number of beads were varied from 50 to 400 beads /flask using 3% sodium alginate and 3% $CaCl_2$ solution.

Effect of Curing Time: The effect of curing time on calcium alginate beads stability and production of invertase enzyme were tested with varying curing time (1, 2, 3, 4, 5 and 6 h).

Effect of Bead Size: To study the effect of bead size on invertase production, three different sizes of alginate beads (2, 4 and 6 mm) were prepared. Equal cell mass containing beads were transferred into flasks and fermentations were conducted.

Reusability of Gel Matrix (Repeated-batch): One of the advantages of using immobilized biocatalysts is that they can be used repeatedly. Therefore, the reusability of *B. macerans* immobilized cells was examined. After attaining the maximum production of invertase (48 h), the beads were washed with distilled water for the next use and added to a fresh production medium (25 ml). The process was repeated for several batches until the enzyme production decreased.

RESULTS AND DISCUSSION

Cell immobilization is one of the common techniques for increasing the overall cell concentration and productivity. The separation of products from immobilized cells is easier compared with suspended cell systems. Immobilization is a strategy for protecting cells from shear forces[3].

Optimization of Supporting Matrix for Maximum Invertase Production: Among various supporting matrices studied for whole cell immobilization of *B.macerans*, calcium alginate was found to be better

entrapment matrix for invertase production (data not shown). Very little study on the production of invertase enzyme using immobilized bacterial cells. Moreover, study on invertase production by *B. macerans* cells entrapped in calcium alginate beads represents the first report. Immobilization of microorganisms presents many advantages, but it implies strong modifications in the microenvironment of the cells, where possible effects should be investigated. Therefore, in this work, the effects of some parameters such as alginate and $CaCl_2$ concentrations, bead diameter, initial cell loading, beads number and curing time were studied.

Effect of Various Alginate Concentrations on Invertase Production: In order to find out the optimum alginate concentration for *B. macerans* cells immobilization, alginate solution of different concentrations were used. Results shown in Fig. (1) indicate that alginate concentration plays a prominent role in the production of invertase by immobilized cells. It was observed that the enzyme titer was reduced with increased alginate concentration, which may be due to reduced porosity of the beads limiting the nutrient supply and oxygen diffusion. This result is agreement with Elibol and Moreira[5]. Low alginate concentration resulted in leakage of biomass out the beads, which might be due to increase in pore size of the beads[4]. Alginate at 3% was found to be the optimum concentration for formulation of spherical and stable beads with better enzyme production and was used in all other experiments.

Effect of $Cacl_2$ Concentration: The concentration of $CaCl_2$ is important for the stability and pore size of the bead[5]. Immobilized beads were prepared with the same amount of cells in 3% sodium alginate solution using different $CaCl_2$ concentration. The immobilized cells prepared in 3% (w/v) $CaCl_2$ solution was found to be the best as it resulted in the highest invertase activity (Fig. 2). These results are similar to that reported by Elibol and Moreira[5]. on the production of protease by immobilized *Teredinobacter turnirae* cells. The enzyme level did not change significantly with increasing of $CaCl_2$ concentration.

Effect of Cell/alginate Ratio: Increasing or decreasing the amount of cells added to initiate a culture could influence the growth and productivity of the culture. The immobilized beads were prepared using 3% sodium alginate and 3% $CaCl_2$ solution (Fig. 3). Maximum invertase level was obtained in the case of a 1:1 v/v (cell/alginate ratio). Increase cell density did not cause any significant increase in the maximum invertase activity. Culturing a volume fraction of encapsulated cells lower than 1:1 lead to delayed growth as well as invertase production.

Eeffect of Beads Number: By varying the number of beads from 50 to 400 beads/flask, the influence of the initial beads number was tested. It was observed that the maximum invertase level was obtained up to 200 beads (Fig. 4). Enzyme yield decreased at high initial cell loading (400 beads/flask). This could be attributed to the fact that, when the number of beads increases, the nutrient/bead ratio decreases, which may become limiting[4]. Therefore, this number of beads was used for the rest of the study.

Effect of Curing Time: To find the appropriate conditions in calcium alginate were prepared with 3% alginate and 3% $CaCl_2$ solution with varying curing times (1, 2, 3, 4, 5 and 6h). Immobilized beads were used for the production of enzyme and results are shown in Fig. (5). Beads prepared with 2 h curing time were better enzyme producers than beads prepared at higher or lower curing times. Increase of curing time resulted in a hared type of beads with less enzyme production[2]. These results indicate that a curing time of 2 h is optimal for the formulation of stable calcium alginate beads and better enzyme production.

Effect of Alginate Beads Size: Mass transfer is an important consideration for immobilized cell growth. Although the diffusion of small substrate in alginate is the same as in water, the diffusion of larger molecules may be restricted Li *et al*[7]. Since the cell primarily grow near the bead surface, cell growth and production behavior may be influenced by the square of the bead diameter. For this reason, the production behavior of *B. macerans* cells immobilized in 2, 4, 6 mm beads was compared. The results in Fig. (6) showed that the smallest size of beads exhibited better invertase production when compared to large size beads. This might be due to increased surface area of the beads, which enhances the mass transfer.

Reusability of Gel Matrix (Repeated-batch): Batch culture is a common mode for commercial fermentation. In addition to fermentation time, however, the production cycle also includes turnaround time (needed for sterilization, inoculation, turnaround etc.) which leads to a reduction in overall productivity and add to production costs[5]. The semi-continuous fermentation was terminated to investigate the stability of the biocatalyst and its ability to produce invertase under repeated batch cultivation condition using optimized alginate beads[4]. Immobilized cells on alginate beads were reused in seven successive reaction cycles (each cycle 48 h) without any loss of biocatalytic activity and 12 cycles with 72% residual activity (Fig. 7). This result is higher than that obtained by Karandikar et al[6]. on using immobilized cells five cycles for invertase production.

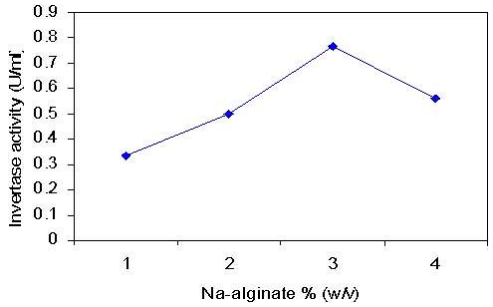

Fig. 1: Effect of sodium alginate concentration on invertase production by immobilized *B. macerans* cells.

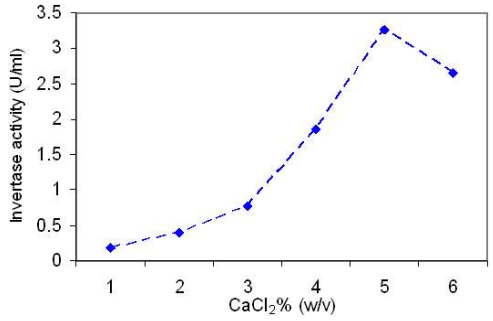

Fig. 2: Effect of $CaCl_2$ concentration on invertase production by immobilized *B. macerans* cells.

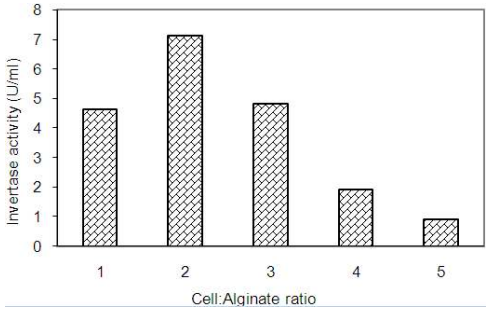

Fig. 3: Effect of cell alginate ratio on invertase production by immobilized *B. macerans* cells.

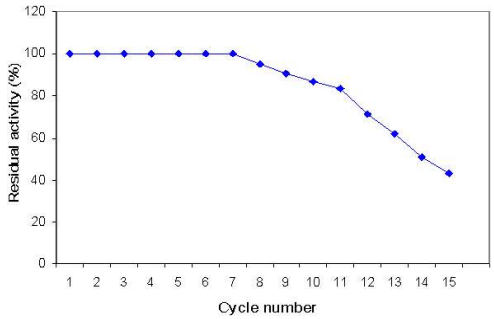

Fig. 4: Effect of initial cell loading (beads number) on invertase Production by immobilized *B. macerans* cells.

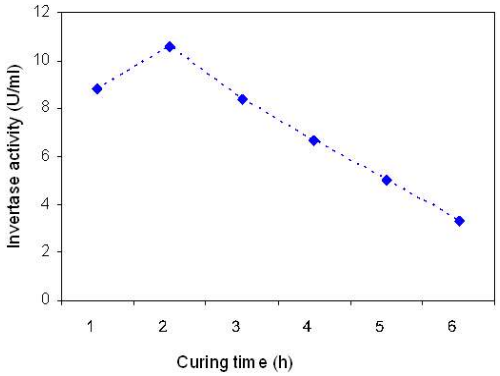

Fig. 5: Effect of curing time on the stability of calcium alginate beads and the production of invertase.

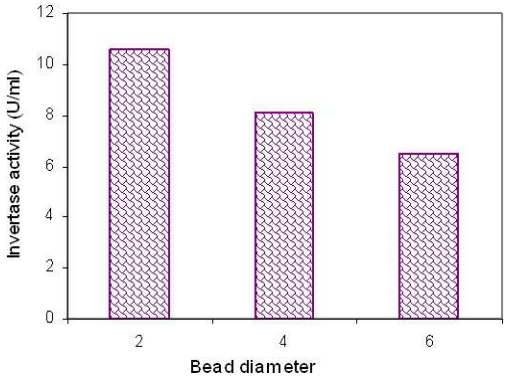

Fig. 6: Effect of alginate bead size on invertase production by immobilized *B. macerans* cells.

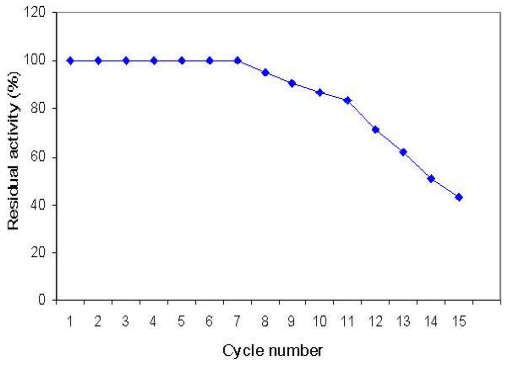

Fig. 7: Reusability of alginate beads (repeated batch) on invertase production.

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