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Extracellular amylase production by calcium alginate immobilized bacteria

(Penghasilan amilas ekstraselular oleh bakteria penyekat-gerak kalsium alginat)

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Key words: enzymes, amylase, immobilization, alginate

Abstrak

Bacillus sp. telah disekat gerak di dalam alginat dengan kaedah penggelan dalam dan luar. Kaedah penggelan luar menghasilkan partikel gel yang kukuh dari segi fizikal dibandingkan dengan kaedah penggelan dalam. Kepekatan alginat sehingga 2% (w/v) didapati sesuai untuk penghasilan amilas ekstraselular yang maksimum. Agen pengerasan seperti polietilenaimin dan glutaraldehid dapat menghalang kebocoran sel tetapi boleh mengakibatkan penurunan aktiviti amilas. Berbanding dengan polietilenaimin, glutaraldehid didapati menurunkan aktiviti amilas.

Abstract

Entrapment of *Bacillus* sp. in alginate by both internal and external gelation methods was investigated. Externally gelled *Bacillus* sp. particles were physically superior to the internally immobilized particles. A 2% (w/v) alginate concentration was desirable for maximum extracellular amylase production. Hardening agents such as polyethyleneimine and glutaraldehyde prevented cell leakage but caused reduction in amylase activity. Glutaraldehyde was also found to be more detrimental.

Introduction

Immobilization can be defined as the attachment or entrapment of biocatalysts in or on an inert carrier or matrix. Many different methods can be used to immobilize whole cells, each with its own merits viz. adsorption, aggregation of cells by flocculation, micro-encapsulation, covalent coupling and entrapment into various polymer materials (Adnan 1986). Entrapment methods are the most common for the preparation of immobilized cells (Fukui and Tanaka 1982). Among various natural and synthetic polymers, alginate is one of the most widely used carriers for the immobilization of whole microbial cells (Vorlop and Klein 1983). Alginic acid is an unbranched chain made up of 1:4 D mannuronic acid and 1:4 L guluronic acid residue (McDowell 1975). Alkali and magnesia alginates are soluble in water, whereas alginic acids and the salts of polyvalent metal cations are insoluble. By simple dropping of a sodium alginate solution into $CaCl_2$ solution, rigid spherical gels are formed. This paper describes the investigations on the production of the extracellular amylase by immobilized *Bacillus* sp., a soil isolate and utilizing calcium ions for the ionotropic gelation of

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the alginate. Both the internal (Johansen and Flink 1986) and the external gelation methods (Kierstan and Bucke 1977) were investigated. Preliminary studies on the stabilization of the alginate beads by treating with hardening agents were also explored.

Materials and methods

Organism and growth conditions

The principal bacterium was a soil isolate identified as a Bacillus species and designated as *Bacillus* sp. The strain was maintained on non-supplemented nutrient agar at 4 °C. The medium consisted of 1% (w/v) peptone and 0.2% (w/v) soluble starch, pH 7.0. Cultures were incubated at 25 °C on an orbital shaker set at 75 rpm for 3 days. In all the experiments involved, unless stated otherwise, cultures were grown in peptone and starch medium. The media were sterilized at 121 °C for 15 min.

Amylase activity

Amylase activity was determined with soluble starch as substrate and the release of reducing sugars was measured by the methods of Bernfeld (1955), using dinitrosalicyclic (DNS) reagent. Controls were prepared using heated enzyme while maltose was used as a standard. One unit of amylase activity is defined as the enzyme quantity which produces 1 μmole of maltose in the reaction mixture per 10 min under the assay conditions.

Immobilization of Bacillus sp. by external gelation of alginate

Bacterial cells were aseptically harvested from 3-day cultures by centrifugation at 20 000 x g for 20 min and rinsed in sterile distilled water. After recentrifugation, the cell pellet was resuspended in 50 mL of sterile distilled water and mixed with 50 mL of autoclaved 4% (w/v) alginate solution until homogenous. A peristaltic pump was used to extrude the alginate bacteria mixture through a plastic nozzle. Drops of mixture were allowed to drop onto a solution of 0.1 M $CaCl_2$. Calcium alginate beads formed immediately in the $CaCl_2$ and were allowed to harden for 30 min at room temperature.

Immobilization of Bacillus sp. by internal gelation of alginate

The method described by Pelaez and Karel (1981), and developed by Flink and Johansen (1985) was adopted. The alginate/bacteria suspension was thoroughly mixed before gelation. To initiate gelation in the system, 1.4 g tri-calcium citrate (suspended in 10 mL water) was first added, after which 1 g glucono-δ-lactone (dissolved in 10 mL of water) was added. The mixture was transferred into a mould (6.5 mm dialysis tubing) for gelation at room temperature. After about 18 h, the gel was pressed out and cut into a 5 mm long cylinder and held in a 0.1 M $CaCl_2$ solution to prevent drying and to ensure saturation of the alginate molecules with Ca^{2+} ions.

Preparation of samples for scanning electron microscopy

The preparation involved the processes of fixing, washing, dehydrating and finally coating the samples with the relevant metals for scanning. Either the whole calcium alginate beads or half beads, obtained by sectioning the whole beads, were fixed in 4% (w/v) glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.3 for 4 h. After fixation, the samples were rinsed in 0.1 M sodium cacodylate, pH 7.3 for 30 min. The samples were then dehydrated by rinsing for 5 min in a series of methanol/water mixture containing 50–100% methanol. This was followed by a second dehydration in a series of methanol/freon. The dehydrated samples were subjected to critical point drying, coated with gold palladium and examined using a JEOL JSM 35 scanning electron microscope.

Leakage of bacterial cells into medium

The leakage of bacteria was determined by measuring the optical density of the medium

in which the immobilized bacteria was incubated. The optical density was measured at 660 nm using the appropriate blank.

Effects of alginate concentration on amylase production

Calcium alginate beads were prepared using the following concentrations of alginate: 0.75, 1.5, 2.0 and 3.0% (w/v). In all cases, the alginate beads were stocked with 2% (w/v) of bacterial cells. About 50 g wet weight of the respective bead was transferred into 100 mL 1% (w/v) peptone plus 0.2% (w/v) soluble starch medium. The flasks were incubated at 25 °C on an orbital shaker set at 75 rpm. After 24 h, samples were taken out for amylase activity determinations and turbidity measurements.

Comparison of amylase production by free and immobilized bacterial cells

Bacterial cells were immobilized by the external gelation method using 2.0% (w/v) alginate. A control was set up using free bacterial cells and amylase activities were compared between the two systems.

Effects of surface area/volume of immobilized particles on amylase production (external gelation method)

Long cylinders of immobilized *Bacillus* sp. were prepared by extruding the alginate cells mixture through a plastic nozzle into a solution of 0.1 M $CaCl_2$. The gels were aseptically cut into cylinders of various lengths (1, 5 or 10 mm), with surface area to volume ratios of 3.3, 1.7 and 1.5 respectively. About 25 g wet weight of the respective cylinder was transferred into 50 mL medium and incubated at 25 °C. Amylase activities were determined after 24 h.

Effects of hardening agents on cell leakage and extracellular amylase production

Alginate beads containing bacteria were stabilized by treating with hardening agents, polyethyleneimine and glutaraldehyde. In the first method, beads were suspended in 0.5% (w/v) polyethyleneimine in 0.05 M $CaCl_2$ for 24 h at 25 °C on a shaker at 75 rpm. The beads were rinsed in sterile distilled water prior to incubation in peptone and soluble starch medium. In the second method, the alginate beads were treated as before but after the polyethyleneimine treatment, they were further resuspended in 1% (w/v) glutaraldehyde for 1 min. After cross-linking with glutaraldehyde, the stabilized beads were thoroughly rinsed with distilled water and then transferred to flasks containing peptone and soluble starch medium. A control flask was also set up containing untreated alginate beads. The three flasks were incubated at 25 °C on a shaker. At fixed intervals, aliquots were withdrawn from the flasks and their amylase activities and turbidity measurements determined.

Reusability of immobilized Bacillus sp. for extracellular amylase production

Alginate-bacteria beads were transferred into the culture medium and incubated at 25 °C on a shaker at 75 rpm. After 24 h, amylase activity and turbidity in the supernatant were determined. The beads were removed aseptically, rinsed thoroughly in sterile distilled water and transferred into a fresh medium. Following another 24 h incubation, the amylase activity and turbidity were again determined and the beads were removed, rinsed as before and transferred into another fresh medium. The same cycle was repeated four times to investigate the reusability of the immobilized *Bacillus* sp. beads to produce extracellular amylase. All the data presented are means of triplicate.

Reagents

Nutrient agar and broth were obtained from London Analytical and Bacteriological Media Ltd., Salford, United Kingdom. Peptone was obtained from the British Drug Houses Ltd., Poole, Dorset, U.K. and were of ANALAR grade.

Results

Comparisons between externally and internally gelled immobilized Bacillus sp.

Comparison of the traditional externally gelled beads and the internally immobilized particles, showed that the externally gelled beads were superior. The internally gelled particles produced were soft, crumbly and were difficult to mould into the desired form.

Effects of alginate concentration on extracellular amylase production

Results showed that there was a significant difference between the alginate concentrations (F-test, $p< 0.01$) *(Figure 1)*. Amylase production was highest at 2.0% alginate concentration. There was no significant difference in the leakage of bacteria from beads at various calcium alginate concentrations (F-test, $p> 0.05$) *(Figure 1)*. Alginate concentration of 2.0% was employed in subsequent experiments.

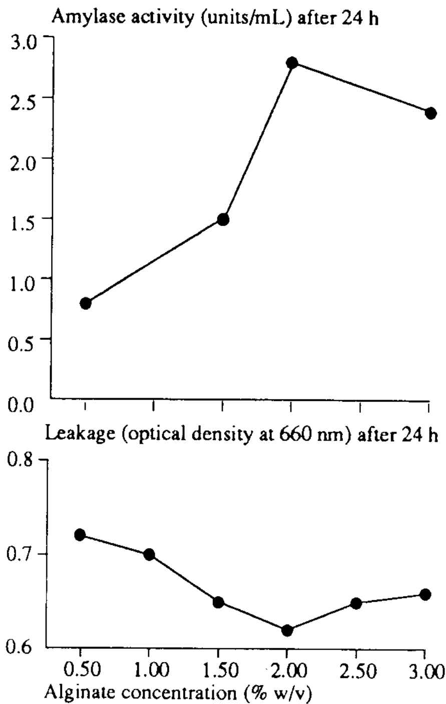

*Figure 1. Effect of calcium alginate concentration on amylase production and cell leakage by beads containing **Bacillus** sp.*

Comparison of amylase production by free and immobilized bacterial cells

The amylase produced by the free bacterial cells was about 4 units/mL after 24 h of incubation. Similar results were observed for the immobilized bacterial cells.

Effects of surface area/volume of immobilized particles on extracellular amylase production (external gelation method)

Figure 2 shows the effects of the surface area to volume ratio of particles containing immobilized *Bacillus* sp. on amylase production. There was a highly significant difference in amylase production betweeen alginate particles with different surface area to volume ratio (F-test, $p< 0.01$). Amylase activity was highest when the ratio of surface area to volume of immobilized particles was greatest.

Effects of hardening agents on cell leakage and amylase production

Figure 3 shows the effects of polyethyleneimine and glutaraldehyde on cell leakage and amylase production. The data showed that during the early stages of the experimental period, both

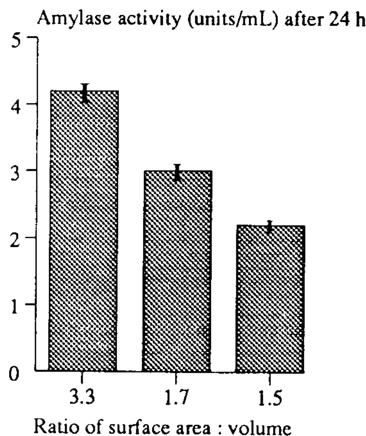

Figure 2. Effects of surface area: volume of particles containing bacteria on extracelluar amylase production

polyethyleneimine treatment and polyethyleneimine plus glutaraldehyde reduced cell leakage from treated beads compared with untreated beads. However, in both cases, the prevention of cell leakage was not sustained. Both treatments reduced amylase activities. In the polyethyleneimine treatment, the amylase activity was only slightly reduced to about 89% of control beads *(Figure 3)*. On the other hand, polyethyleneimine plus glutaraldehyde treatment reduced the amylase activity to almost zero. Towards the end of the experimental period, amylase activities were low in all cases.

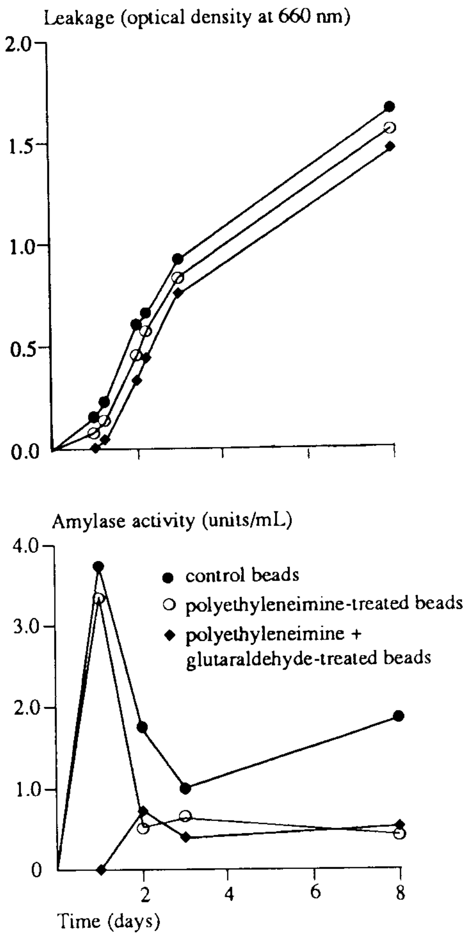

Figure 3. Effects of hardening agents on cell leakage and amylase production

Usability of immobilized Bacillus sp. for amylase production

The reusability of immobilized *Bacillus* sp. for amylase production was investigated and the results are shown in *Figure 4*. The alginate beads containing *Bacillus* sp. could repeatedly be used for at least four cycles for amylase production. Highest amylase activity was obtained in the second cycle. Amylase activity declined slightly to about 68% of the initial activity in the fourth cycle. Cell leakage was also highest in the second cycle.

Discussion and conclusion

The technique of immobilization of *Bacillus* sp., by the internal gelation of alginate, was found to be inferior to the traditional

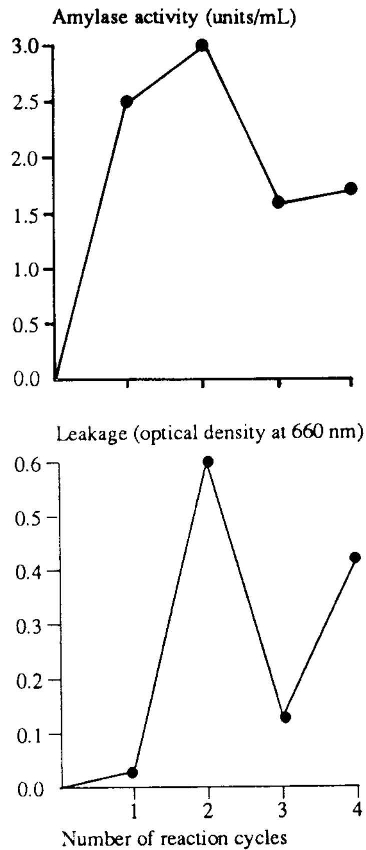

*Figure 4. Reusability of immobilized **Bacillus** sp. for extracellular amylase batch production using calcium alginate beads*

external gelation of alginate beads. The results contrasted with the findings by Johansen and Flink (1986) that the immobilization of yeast cells by internal gelation of alginate yielded more stable particles, compared with the traditional alginate beads. In principle, the internal gelation method involves blending the cell-containing alginate solution with a suspension of calcium citrate and D-glucono-1, 5-lactone (GDL). GDL hydrolyses in solution to form gluconic acid and this lowers the pH of the solution. As the solubility of calcium citrate is pH-dependent, Ca^{2+} is liberated and causes the gelation of the alginate solution. With this method, a uniform distribution of alginate Ca^{2+} in the alginate solution is obtained and gelation occurs simultaneously throughout the solution. In contrast, the external gelation method involves the diffusion of the Ca^{2+} from the surrounding liquid into the alginate solution, causing gradual gelation at the surface and into the particles at the diffusion-limited rate (Johansen and Flink 1986). The data in *Figure 2* show that the smallest alginate gel particles, with the highest surface area to volume ratio, produced the highest amylase activity. In practice, the bead was found to be a convenient configuration for entrapment of whole cells in alginate by the external gelation method. It can be easily manufactured in large quantities without much effort. Besides having a high surface area to volume ratio, the alginate bead size was kept approximately uniform without much variation. This was not possible by any other configurations because other shapes are dependent on manual designing or shaping of the alginate gels. Small spherical biocatalyst beads of uniform size are desirable for several reasons. Large beads reduce the substrate and/or product diffusion rate. On the contrary, too small and irregularly shaped particles with a wide distribution causes problems such as clogging of the packed-bed reactors, abrasion and washout in stirred vessels (Hulst et al. 1985). The alginate beads prepared from 0.75% (w/v) and 1.5% (w/v) alginate were spherical, but those prepared from 2.0% and 3.0% (w/v) alginate were pear-shaped. This is probably due to the high viscosity of the suspension at higher alginate concentration. The lower concentrations of alginate seemed to promote more bacterial leakage and this is probably due to the weak infrastructure of the alginate gel. At the 2% (w/v) alginate concentration, the bacterial leakage was minimal.

Scanning electron micrographs of the 2% (w/v) whole calcium alginate beads with entrapped *Bacillus* sp. and sections of the beads taken immediately and a few days after immobilization are shown in *Figure 5* to *Figure 9*. The beads are pear-shaped (*Figure 5*) with a smooth outer cortex devoid of large pores (*Figure 6* and *Figure 7*). A honey comb-like structure with pores was observed within the beads. The bead matrix was similar in basic appearance to that reported by Laretta et al. (1983), Scherer-Garde et al. (1981) and Musgrave et

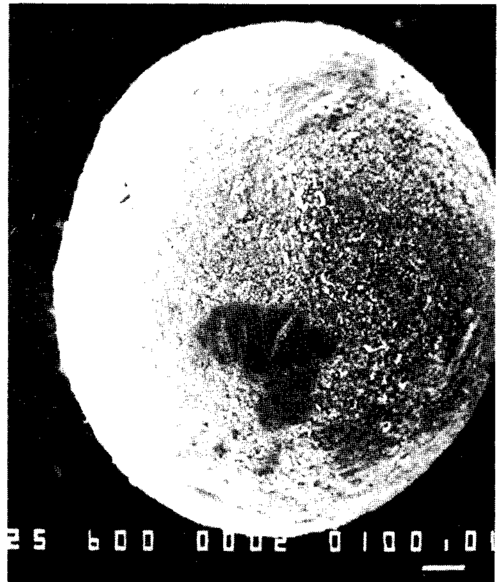

*Figure 5. Scanning electron micrograph of calcium alginate bead containing **Bacillus** sp. immediately after immobilization prior to use in bioreactor (Bar = 100 μm)*

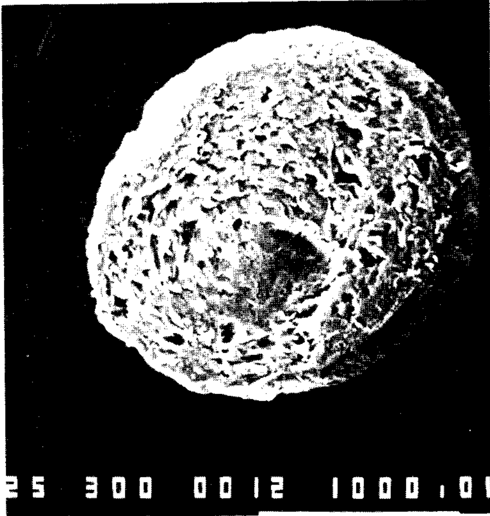

Figure 6. Scanning electron micrograph of a cross section of a freshly formed bead showing honey-comb matrix (Bar = 100 μm)

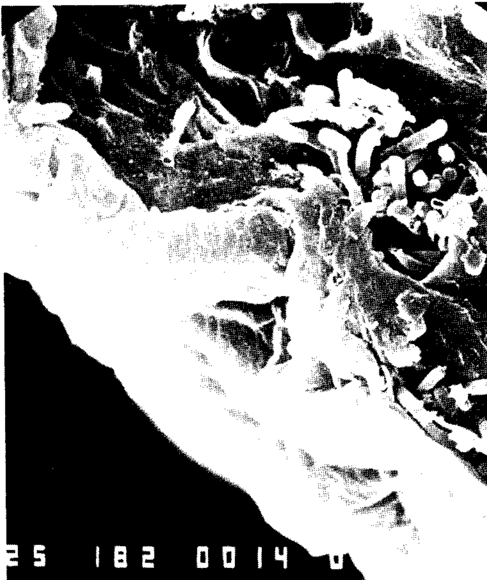

Figure 7. Scanning electron micrograph of a section through a freshly formed bead showing outer cortex (Bar = 10 μm)

al. (1981). The *Bacillus* sp. cells were contained singly or in chains within the bead matrix *(Figure 8)*. The structure of alginate beads was found to be unstable and susceptible to rupture *(Figure 9)*, resulting in the leakage of bacteria cells from immobilized beads.

Figure 8. Scanning electron micrograph of a section through a 4-day-old bead showing entrapped Bacillus sp. (Bar = 10 μm)

Figure 9. Surface of a 4-day-old bead showing rupture of cortex, permitting escape of cells (Bar = 10 μm)

Attempts to stabilize beads by treating with hardening agent, polyethyleneimine (PEI) shows that the PEI treatment reduced cell leakage for about 1 day, while

permitting the release of a reasonable amount of amylase. However, by the second day of the experiment, cell leakage began to increase to a value similar to that of the control beads. On the other hand, PEI plus glutaraldehyde treatment of beads was capable of minimizing cell leakage for the first day. Nevertheless, it appeared to cause a lower level of amylase production. This may have been due to the toxic effect of glutaraldehyde on the organism, similar to the results of Birnbaum et al. (1981), who found that glutaraldehyde caused a lower fermentation activity in immobilized yeast cells. Repeated use of *Bacillus* sp. for the production of extracellular amylase was possible for at least four cycles as shown in *Figure 6*. Amylase activity was high in the first two cycles but declined slightly in the third and fourth cycle. Cell leakage was inconsistent, being the lowest in the first cycle. Clearly, further studies on the physical properties of the alginate beads are necessary to reduce cell leakage, but the findings have demonstrated the feasibility of using beads of *Bacillus* sp. for the production of extracellular amylase. It is hoped that an understanding of the immobilization technique will help in future research, in areas of production of substances of economical importance (e.g. acetic acid, lactic acid, citric acid, enzymes and alcohol).

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