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Scientific Note

Continuous Production of Halophilic α-Amylase Through Whole Cell Immobilization of *Halobacterium salinarium*

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ABSTRACT

The cells of *Halobacterium salinarium* were immobilized in calcium alginate beads and a polyvinyl alcohol film with an aim to improve the production of halophilic α-amylase. The cells of *Halobacterium salinarium* were stabilized by cross-linking with 0.5% glutaraldehyde. Such cells were found to be osmotically stable and showed continuous production of the enzyme for several days (45 d). The stabilized cells could be permeabilized by treatment with chloroform without leakage of intracellular components. This technique can serve as a tool for studying *in situ* regulatory characteristics of intracellular functions in halobacteria and can also help in their reuse under more stabilized conditions for biotechnological applications.

Index Entries: α-Amylase; *Halobacterium salinarium*; halophilic; starch; salt tolerance; immobilization.

INTRODUCTION

As the use of bacterial α-amylases increases, it becomes important to isolate new high-producing strains *(1)* and enzymes with characteristics more amenable to industrial use. Through these studies it became apparent that the characteristics of α-amylase varies as widely as the organism which produces them *(2)*.

The present paper delineates a simple and novel technique for the continuous production of α-amylase from immobilized whole cells of *Halobacterium salinarium*. Enzymes from extremely halophilic bacteria

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constitute a fascinating example of biochemical adaptation. Thus, these enzymes which, both in vivo and in vitro, perform their catalytic functions at 4–5 *M* NaCl and KCl, lose their activity rapidly when exposed to low salt concentrations *(3)*. They constitute therefore a unique system for inactivation-reactivation studies, which can shed light on the type of interactions involved in the maintenance of the integrity of native structures *(4)*. The application of an immobilization approach in these studies was thought to be useful since such an approach had previously been successful in an investigation of the thermostability of immobilized enzymes *(5)* The α-amylase enzyme produced by *H. salinarium* resembles the enzyme from moderate halophiles in its ionic requirement of 0.05–1.0% NaCl for optimal activity. However, this amylase enzyme was more tolerant than most of the enzymes from moderate halophiles to high NaCl levels maintaining 33% of maximum activity at a NaCl concentration of 4.0 *M (6)*. Moreover, the risk of microbial contamination especially in a continuous bioreactor would be reduced if halophilic bacteria are employed since the use of high (4*M*) salt concentrations would effectively curtail microbial growth *(5)*.

MATERIALS AND METHODS

Organism and Growth

Halobacterium salinarium (*Halobacterium halobium* S9) strain was obtained as a gift from Dr. Th. Shreckenbach of the Institut für Biochemie der Universitat, 8700 Wurzburg, Germany. It was maintained at 40°C on a medium containing (g/L) 250, NaCl; 20, $MgSO_4 \cdot 7H_2O$; 2, KCl; 3, trisodium citrate. $2H_2O$; 10, peptone; pH 7.2 *(7)* and subcultured once in every two months.

Enzyme Production

The medium composition used for α-amylase production was the same as above but contained in addition 1% (w/v) soluble starch and 0.1 m*M* $ZnSO_4$, which increased the production of α-amylase *(8)*.

Medium (100 mL) was inoculated with 2 mL of a 1-w-old culture of *H. salinarium*. The broth was centrifuged after a period of 5 d. The cells were obtained in the form of a pellet which were then immobilized in sodium alginate and polyvinyl alcohol.

Amylase Assay

One unit of enzyme activity is expressed as mg of reducing sugar liberated per mL of eluent sample of the reactor at 53°C in 30 min. The reducing sugar was estimated by the dinitrosalicylic acid method *(9)*.

Crosslinking with Glutaraldehyde

Cells (1g wet mass) were suspended in 5 mL of 20% (w/v) NaCl; glutaraldehyde was added to the cell suspension to the required final concentration. The mixture was incubated under gentle shaking for 10 min at room temperature *(10)*.

Permeabilization of Cells

The crosslinked cells (1 g wet mass) were treated with 150 µL of chloroform *(10)*. The mixture was vortexed for 15 s.

Immobilization of Cells in Sodium Alginate

The cell culture was added to 3.5% sodium alginate slurry prepared in buffer and allowed to stand for 30 min. Beads were formed by dripping the alginate-cell suspension into a solution containing 0.05*M* $CaCl_2$. They were washed and stored in a buffer.

Immobilization of Cells in a Polyvinyl Alcohol Film

An aqueous solution of 10.4% polyvinyl alcohol (PVA) with 0.02% sodium alginate was prepared. Here solution containing PVA was carefully heated to a temperature of 60°C to completely dissolve the PVA. A solution of 0.02% sodium alginate in buffer was prepared by gently stirring for 30 min and added to the PVA solution. The PVA-alginate solution was then cooled to a temperature of 35°C.

The centrifuged cells were mixed with the growth medium. This mixture was then added to the PVA solution and mixed thoroughly. This was then spread uniformly on a glass plate and allowed to harden for 24 h. The PVA coated plate was rinsed with 2% $CaCl_2$. The PVA film was cut into small squares and used in a packed-bed reactor.

The slurry of sodium alginate and PVA was prepared in 0.01*M* sodium β-glycerophosphate buffer as well as 0.01*M* TES [*N*-tris (hydroxymethyl) methyl-2-amino ethane sulfonic acid] buffer (pH 7.2). The cells were then immobilized and comparative data of their activity was obtained.

Bioreactor Using Immobilized Whole Cells

Immobilized whole cells in the form of beads were packed in a tubular glass column with a void volume of 50 mL (30 g gel beads) which was operated in a continuous fashion. The medium was passed continuously to give an upward flow. Similarly, the PVA pieces containing the immobilized whole cells were packed in a similar column. The flow rate was adjusted using a peristaltic pump and the eluent samples were routinely analysed for amylase activity. It was expressed as rate of formation of enzyme (in terms of units) per hour at a particular flow rate. Experiments

were carried out in quadruplicate for each flow rate and the data has been presented with standard deviation.

RESULTS AND DISCUSSION

α-Amylase is an extracellular enzyme produced by *H. salinarium* which possesses unique properties in terms of its salt tolerance and therefore, can be of importance. However, *H. salinarium* is not a good producer of α-amylase. Its production goes down significantly after 5d in submerged process. One of the approaches to improve its production efficiency is by longterm continuous production of amylase under cell immobilization. Scant information is available regarding the production of α-amylase *(5,8)*. Therefore, here we report our observation of continuous production of α-amylase in an upward flow packed-bed reactor using growth medium of *H. salinarium* containing 1% (w/v) soluble starch and 0.1 m*M* $ZnSO_4$.

In order to find out the optimum alginate concentration for whole cell immobilization, alginate solution of different concentrations were used which ranged from 2 to 4.5% (w/v) with an increment of 0.5% between the sets. Amylase production improved with an increasing concentration of alginate reaching a maximum of 3.5% (w/v) (data not given). At low concentration the beads were too soft and showed rapid leakage of the cells from the beads. At very high concentration the amylase production further reduced considerably as probably the beads were too strong making them less permeable for the release of amylase.

It is necessary to find out the optimum cell density which can be taken up by alginate. Therefore, to determine optimum cell loading in alginate different quantities of cell culture (cell pellet) were mixed with sodium alginate solution (3.5%). It was observed that 5 mL of a 5-d-old culture in a 50 mL solution of sodium alginate gave maximum production (data not given).

H. salinarium cells were also characterized in terms of temperature and pH in alginate immobilized conditions for α-amylase production. The pH of 7.2 and temperature of 40°C were found to be optimum for maximum amylase production (data not given). These conditions were the same as for the free cells in submerged fermentation. However, amylase production was not affected significantly with alteration in temperature and pH.

Upflow-packed bed reactor was operated at three different flow rates of 0.5 mL/min, 1 mL/min, and 1.5 mL/min. At a low flow rate that is at a higher retention time the enzyme concentration was higher as the sample expressed high activity. However, the total amount of enzyme per unit time was less. Best results were obtained when the flow rate was maintained at 1 mL/min.

Maximum stabilization was observed when the cells were treated with 0.5% gluteraldehyde for 10 min. Treatment with glutaraldehyde improved the srength of the beads and could show enzyme production for

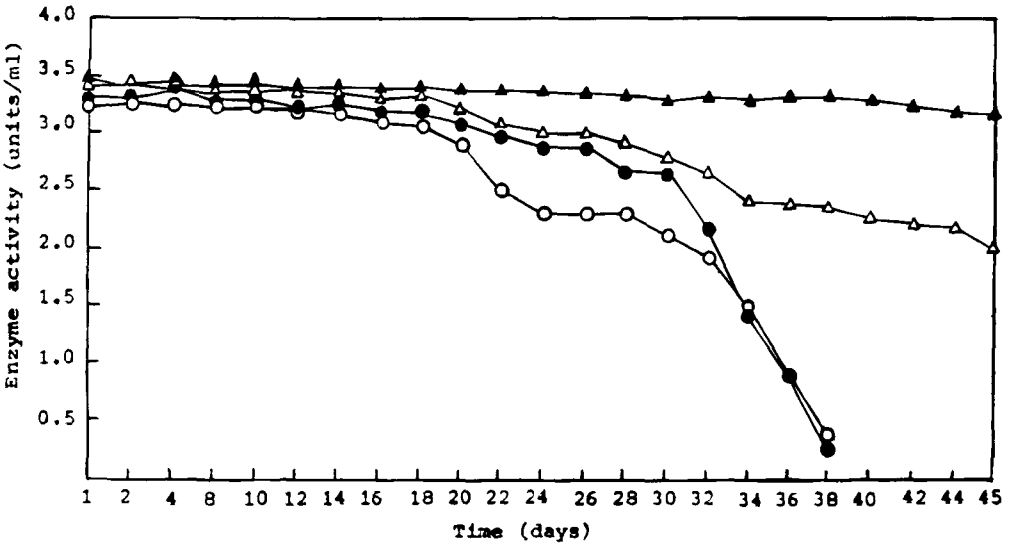

Fig. 1 Comparative data of α-amylase production by *Halobacterium salinarium* cells immobilized after treatment with glutaraldehyde and chloroform, buffered with Sodium β-glycerophosphate and TES (0.01 *M*). Conditions: Temperature, 40°C; flow rate, 1 mL/min and void volume of the reactor is 50mL.

Symbols: Sodium alginate slurry prepared in Sodium β-glycerophosphate buffer, (○); PVA slurry prepared in Sodium β-glycerophosphate buffer, (●); Sodium alginate slurry prepared in TES buffer, (△); PVA slurry prepared in TES buffer, (▲).

a longer period. Intact cells are known to be impermeable to many substrates, effectors and products. These limitations have been obliviated by permeabilizing the bacterial cells while retaining intact, the macromolecular machinery. Treatment of the stabilized cells with chloroform was found to result in the permeabilization of the cells *(10)*.

One of the major inherent problems of alginate beads is that with time the beads become soft which gradually leads to a loss of cells *(11)*. Recently, the use of polyvinyl alcohol (PVA) for cell immobilization has been investigated *(12)*. The film thus produced proved to be very durable, outlasting beads produced by other cell immobilization techniques such as polyacrylamide and calcium alginate *(12)*. It has been observed that cells immobilized in the slurry of sodium alginate and PVA prepared in TES buffer give higher activity compared to sodium-β-glycerophosphate buffer. Due to the presence of phosphate in sodium β glycerophosphate buffer the beads become soft which results in the leakage of cells (Fig. 1).

In the free state there is a remarkable reduction in the production of α-amylase within a few days which is evident by a drop in the rate of conversion of starch to reducing sugar. In contrast to this, calcium alginate and PVA immobilized *H. salinarium* cells showed the same activity as the free cells for the initial 4 h. However, after this, the production was reduced by

about 20% and reached a steady state. This steady state remains constant for the next 45 d.

Even though potentials of halobacteria to catalyse reactions under extreme conditions of salt are known hitherto no reports are available on their application in an immobilized form. This is mainly because these cells are mechanically very fragile towards shear forces and lyse with a decrease in salt concentration in the external medium. Thus, the technique developed in this study could obviate these problems and may also open up new avenues for utilization of halobacterial cells for various biotechnological applications *(10)*.

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