June 2010 ISSN : 0974 - 7435 Volume 4 Issue 2

Trade Science Inc.

BioTechnology

An Indian Journal

FULL PAPER

BTAIJ, 4(2), 2010 [107-110]

Production of cell free α-amylase by immobilized *Bacillus subtilis* (ATCC 6633) under the substrate stress

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Received: 21st April, 2010 ; Accepted: 1st May, 2010

ABSTRACT

Living cells of *Bacillus subtilis* were entrapped in calcium alginate beads for the α-amylase production. There was maximum production after 120 Hrs at pH 7 when 8×10^8 cell/ml immobilized in 2% sodium alginate was inoculated in the production medium containing 5% starch and calcium chloride as inducer. Amylase extracted by acetone precipitation has specific activity 29.341 IU/mg of protein, optimum pH 7 and temperature 60°C, K_m 333μgm/ml and V_{max} 1.5IU/ml/minute.

KEYWORDS

α-amylase;
Bacillus subtilis;
Immobilization;
Starch and substrate stress.

INTRODUCTION

Starch-degrading amylolytic enzymes are of great significance in biotechnological applications ranging from food, fermentation, textile to paper industries[4,11]. The amylases can be derived from several sources such as plants, animals and microbes. The microbial amylases meet industrial demands a large number of them are available commercially; and, they have almost completely replaced chemical hydrolysis of starch in starch processing industry[11]. The major advantage of using microorganisms for production of amylases is in economical bulk production capacity and microbes are also easy to manipulate to obtain enzymes of desired characteristics[5]. Amylase has been derived from several fungi, yeasts, bacteria and actinomycetes, however, enzymes from fungal and bacterial sources have dominated applications in industrial sectors[11]. Immobilized growing cells show many advantages over the free cells, such as increase in enzyme productivity and long term stability. Because of this, during the past few decades enzyme production by immobilized bacteria is an active research area despite the fact that industrial applications are still limited. Under the substrate stress conditions the cells produce only a limited number of proteins. The purpose of the present work was to decrease the purification cost and enhance the cell free α-amylase production by providing substrate stress conditions to immobilized living cells of *B. subtilis*.

MATERIALS AND METHODS

Microorganism

The bacterial strain *B. subtilis* (*ATCC 6633*) was used for the production of α-amylase in this study. Culture was maintained on nutrient agar at 4-6°C and sub

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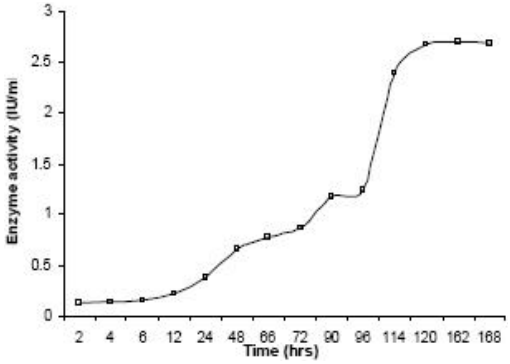

Figure 1 : Optimization of time intervals for the α-amylase production from immobilized *Bacillus subtilis*

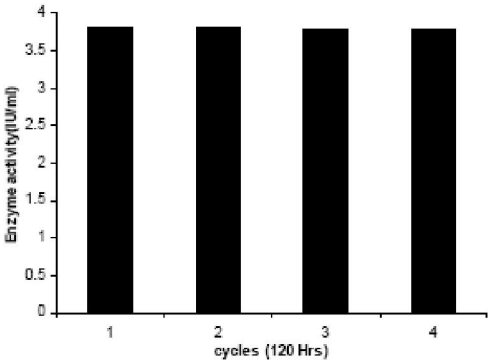

Figure 2 : Optimization of Number of Cycles for α-amylase production from immobilized *B. subtilis* cells

cultured after every 20 days.

Chemicals

All the chemicals and reagents of analytical grade were procured from Glaxo India Limited, Himedia, SD Fine Chemicals, SRL and Ranbaxy.

Production of biomass

50ml of the medium containing (g/l): starch 20, $(NH_4)_2N0_3$ 5.6, $MgSO_4$ 0.5; $CaCl_2$ 0.1; Trisodium citrate 5, Peptone 10 and pH 7.0 was inoculated in 250ml flask with *B. subtilis* and incubated in orbital shaker (250rpm) at 37°C. After 24 h biomass was centrifuged (4°C and 7000 rpm) and washing was given with saline solution. Cell pellets was suspended in the saline and at 600nm absorbance was adjusted for the desired cell concentration.

Immobilization of cells

Sterilized sodium alginate was mixed with cell suspension (2% w/v) and dissolved properly by gently shaking the mixture. 5ml of the above solution was then added to chilled $CaCl_2$ solution (0.2 M), drop-wise, with the help of a disposable syringe to produce spherical beads of about 3 mm diameter[10].

Optimization of culture conditions for the enzyme production from the immobilized cells of *B. subtilis*

For the optimization of amylase production from the immobilized cells, the experiments were conducted in 250ml Erlenmeyer flask containing (g/L); starch 40, $MgSO_4$ 0.5; $CaCl_2$ 0.1; trisodium citrate 5, and pH 7.0 was inoculated with immobilized cells and incubated in orbital shaker (250 rpm) at 37°C. The factors such as time, sodium alginate, pH, $CaCl_2$, starch concentration, cells concentration and stability of sodium alginate beads, inducer and inhibitors were studied.

Partial extraction and kinetic characterizations of amylase

α-amylase produced by immobilized cells was extracted by acetone precipitation. After extraction kinetic characterization and optimum pH and temperature for the maximum activity was determined.

Enzyme assay and protein estimation

Amylase assay was made by using a reaction mixture (4ml) consisted of 1ml of enzyme solution and 2ml of soluble starch in phosphate buffer, pH 6.5[13]. The mixture was incubated for 10 min at 60°C. Level of reducing sugars was determined by dinitrosalicylate method[7] and is expressed in units (one unit is the amount of enzyme which releases 1μ mole glucose). The protein concentration was determined by Folin-Lowry method[6].

RESULTS AND DISCUSSION

Optimization of time intervals for the maximum production of amylase form immobilized *B. subtilis*

Microorganism produces different enzymes at different times of their life cycle. To check the optimum time for the maximum production of amylase from immobilized cells of *Bacillus subtillus*, samples were collected after different intervals and enzyme activity was observed. From the results (Figure 1) it was observed that enzyme production increases with time and there was maximum production after 120 hours. After that

TABLE 1 : Effect of sodium alginate, starch and Calcium Chloride concentration on α-amylase production from immobilized *B. subtilis*

Sodium alginate Conc. (%)[a]	Enzyme activity (U/ml)	Starch conc. (%)[b]	Enzyme activity (U/ml)
1.0	2.41	0	0.09
1.5	2.57	1	0.91
2.0	2.69	2	1.23
2.5	1.86	3	1.96
3.0	1.59	4	2.65
3.5	1.04	5	2.64
4.0	0.67	6	2.60

[a]pH 7, Starch 4 %, Temperature 37°C, pH 7, calcium chloride 0.02%, [b]Sodium alginate 2 %, pH 7, Temperature 37°C, calcium chloride 0.02

TABLE 2 : Effect of pH and cells concentration on α-amylase production from immobilized *B. subtilis**

pH	Enzyme activity (U/ml)	Cell Conc (ml)	Enzyme activity (U/ml)
4	0.07	8×10^{2}	1.11
5	0.11	8×10^{4}	1.21
6	2.26	8×10^{6}	2.41
7	2.61	8×10^{8}	2.66
8	1.16	8×10^{10}	2.65
9	0.13	8×10^{12}	2.68

*Sodium alginate 2 %, starch 4%, Temperature 37°C, calcium chloride 0.02%

TABLE 3 : Effect of calcium chloride concentartions on the α-amylase production from immobilized *B. subtilis*

S.No	Calcium chloride conc.(%)	Enzyme activity (U/ml)
1	0.01	1.05
2	0.02	2.65
3	0.03	3.05
4	0.04	3.57
5	0.05	3.89
6	0.06	3.85

there was almost constant rate of enzyme production up to 168 Hrs. This time is in comparison to the enzyme synthesis from *Bacillus polymyxa* (CBTB-25) and *Bacillus lichaniformis* that was 120 and 144 hrs respectively[2,8].

Optimization of sodium alginate and Starch concentration for the production of amylase

Sodium alginate was used to immobilize the cells of *B. subtilis*. Pore size depends upon the concentration of sodium alginate so different concentrations of sodium alginate (1.0, 1.5, 2.0, 2.5, 3.0, 3.5, and 4.0%) were used to immobilize the cells. There was increase in amylase production as concentration of sodium alginate was increased from 1-2% but after that there was decrease in the enzyme production (TABLE 1) This concentration value is comparable to concentration of sodium alginate used for the immobilization By Zoe and Maria, 2006 but lower than the 4% sodium alginate concentration used for the immobilization of of *Bacillus polymyxa* (CBTB-25) and *Bacillus lichaniformis* Mohandas and Chandersekaran, 1994; Dobreva et al., 1996). Amylase is an inducible enzyme and is generally induced in the presence of carbon sources such as starch and glucose[1,3,9,12]. Starch was used to give the substrate stress condition to the bacteria for the production of amylase. There was increase in the enzyme production as the concentration was increased from 1-4% (TABLE 1) with respect to the control. After this there was no significant increase in production of enzyme with increase of substrate concentration.

Effect of Biomass concentration and medium pH for the synthesis of amylase

Biomass concentration was determined by checking the absorbance at 600 nm. As the cell concentration was increased there was increase in the enzyme activity (1.11-2.66U/ml). There was maximum enzyme production with biomass having cells concentration 8×10^{8} cells /ml. Enzyme production was observed at pH 4.0-9.0. The maximum enzyme production was obtained at pH 7.0 and above that enzyme production decreased (TABLE 2).

Effect of metals on the enzyme production

Calcium chloride works as inducer for the amylase production[1]. There was 47% increase in the amylase production (1.05-3.89U/ml) as the concentration of calcium chloride was increased from 0.01-0.05% (TABLE 3).

From the results (results not shown) it was observed the Antimony chloride and Bismuth nitrate in the medium increased the 43% enzyme production. Where as cobalt nitrate, mercuric chloride and trisodium arsenate decreased the production by 30, 37 and 37% respectively.

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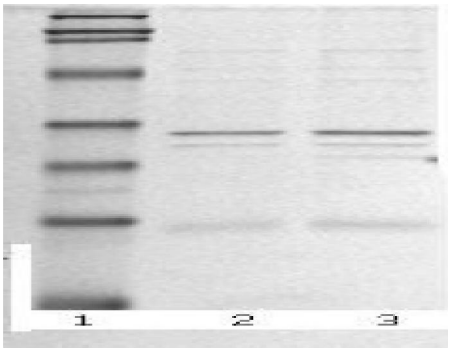

Figure 3 : Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) of protein extracted by acetone precipitation Line 1. Marker 2,3.Protein extracted by acetone precipitation , produced by immobilized cells of *B. subtilis*

The beads containing the immobilized cells were used for number of times for the amylase production and (Figure 2) enzyme production remains almost same for the first four cycles (each cycle 120 h) after that there was disintegration of beads.

Partial extraction and kinetic characterizations of amylase

Enzyme produced by immobilized cells was partially extracted by acetone precipitation methods. Protein extracted by acetone precipitation has specific activity 29.341 IU/mg of protein, optimum pH 7 and temperature 60°C, K_m 333µgm/ml and V_{max} 1.5IU/ml/minute.SDS-PAGE was performed to check the purity of amylase produced by immobilized *B. subtilis* (Figure 3) this shows that only limited numbers of proteins are produced by microorganism under substrate stress conditions.

CONCLUSION

There was maximum production of amylase after 120 h at pH 7 when 8×10^8 cell/ml immobilized in 2% sodium alginate was inoculated in the production medium containing 5% starch and 0.05% calcium chloride which works as inducer. Amylase extracted by acetone precipitation has specific activity 29.341 IU/mg of protein, optimum pH 7 and temperature 60°C, K_m 333µgm/ml and V_{max} 1.5IU/ml/minute. So under the substrate stress conditions the cells produce only a limited number of proteins which decrease the purification cost and enhance the cell free α-amylase production.

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