ISSN: 2278-778X
Research Article
www.ijbio.com

STUDIES ON THE EFFECTS OF CERTAIN NUTRITIONAL AND ENVIRONMENTAL FACTORS ON THE PRODUCTION OF AMYLASE BY *BACILLUS SUBTILIS* BS5

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Received for publication: September 13, 2012; **Accepted**: October 24, 2012.

Abstract: The effect of different starch and nitrogen sources on the production of amylase by the highly amylolytic isolates *Bacillus subtilis* BS5 from the gut of *Amitermes evuncifer* Silvestri was studied. Cocoyam starch proved to be the best carbon source for the enzyme production. Highest yield of amylase was achieved at pH 6.0. Enzyme synthesis occurred at 20 to 55°C with an optimum at 50°C. Amylase production was stimulated by Ca^{2+}, Na^{+} and Mg^{2+} but inhibited by EDTA and $HgCl_2$.

Keywords: Starch, Termite, Inhibition, Amylase

INTRODUCTION

Alpha amylase (1, 4-α-D- glucanohydrolase; EC 3.2.1.1) which hydrolyses α 1, 4- glucosidic linkage in starch related molecules is one of the several enzyme involved in the degradation of starch. This enzyme is widespread among aerobe and anaerobes. Starch tubers and cereals occur abundantly in most developing in most developing countries of the tropics. They form important staple foods in the diet of the people in most developing countries of the tropics[1]. Several microorganisms have been reported to produce raw starch digesting amylase, however most of them were effective for cereal starches but root and or tuber starches were more resistant to the enzyme reaction. A lot of them are lost due to inadequate and ineffective storage facilities[1,7]. However they can be converted to reducing sugars by acid or enzymatic saccharification[9]. Various factors like carbon and nitrogen sources, pH, and temperature and incubation periods are known to affect production of several enzymes. Although the use of microbial amylase for the hydrolysis of raw starches has long been advocated and practiced to a limited extent, yet there is a dearth of information on the hydrolysis raw starches of locally available tubers in the tropics. The present work describes the effects of some physiological factors on amylase production by a strain of *Bacillus subtilis* isolated from the hindgut of wood-eating termites *Amitermes evuncifer* Silvestri.

MATERIALS AND METHODS

Raw materials and cultures:

Yam (*Discorea* spp), cocoyam (*Colocasia esculenta*), cassava (*Mannihot utilissima*), plantain (*Ipomea battatas*), corn, rice and millet, soy bean, cow meat and fish were purchased at King's market, Ado-Ekiti, Nigeria.

Preparation of native starches:

Starches from the tubers were prepared in the laboratory according to standard procedure as described by Obi and Odibo (1984). The tubers were washed thoroughly with water, peeled and individually reduced to pulps using a hand grater. Thereafter, each pulp was homogenized in a blender. From each homogenate in a bag of fine white cloth, starch was leached into a bowl by churning with excess water. The crude starch suspension was allowed to settle overnight, decanted and dried at 50°C for 48h. The resultant starch flakes were ground to fine powder.

Forty grams each of corn, rice and millet was dried and steeped in 200mL of 0.45% sodium metabisulphite solution and placed in a water bath at 50°C for 24h. Thereafter the steep liquor was drained off and the grains homogenized in a warring blender with 70mL of distilled water. The slurry was screened through silk cloth. The starch suspension was allowed to settle overnight and the supernatant decanted. The resultant starch was dried at 50°C in an oven and ground to fine powder.

Preparation of organic nitrogen sources:

Cow blood meal: Freshly collected cow blood was steamed for 30mins, oven dried at 50°C for 24h and homogenate was dried at 60°C for 24h and then ground to powder.

Cow meat and fish meal: Fifty grams each of cow meat and fish were cut into smaller pieces, dried in the oven at 50°C for 5h after which it was homogenized in a warring blender. The homogenate was further dried at the same temperature for 24h. The dried meat and fish were ground separately and passed through a sieve to obtain a fine powder.

Bean and Soy bean meal: Fifty grams of each sample was ground in a blender and screened the grits through a sieve to obtain a fine powder.

Organism and culture condition:

Bacillus subtilis BS5 used was previously isolated from the hindgut of wood-eating termites *Amitermes evuncifer* Silvestri. The organism was found to produce amylase on starch agar after flooding the plate with iodine solution. The organism was propagated at 35°C for 36h in a basal medium containing the following in g/L; K_2HPO_4, 1.5; KH_2PO_4, 0.5; $MgSO_4$, 0.05; NaCl, 1.5; $(NH_4)_2SO_4$, 1.0; $CaCl_2.2H_2O$, 0.02; $FeSO_4.7H_20$, 0.02; Yeast extract, 0.5 and 1% (w/v) soluble starch. Inocula for experiments were prepared by growing the organism in 0.2% peptone water at 35°C on a rotary shaker for 12h. Sterilized medium (100mL) was inoculated with 1mL of inocula (8.6×10^6 cells/mL). Inoculated flasks were incubated at 35°C for 36h on a rotary shaker (120rpm) and then centrifuged at 5000 rpm for 20mins he supernatant obtained was used for enzyme assay.

Amylase assay:

Enzyme assay was estimated by the dinitrosalicyclic acid (DNSA) method of Miller (1959). The reaction mixtures consist of 0.5ml of substrate solution (1% soluble starch in 0.05M phosphate buffer, pH 6.9) and 0.5mL of the enzyme solution. The reaction mixture was incubated for 3 minutes at 30°C. The reaction was terminated by the addition of 1ml of dinitrosalicyclic (DNSA) reagent. The mixture was heated at 100°C for 5 minutes and cooled. The optical density was read at 540nm in a spectrophotometer (Jenway, 6305). One unit of alpha amylase activity (U) was defined as the amount of enzyme that liberated reducing sugar equivalent to one micromole of D-glucose from starch under the assay condition.

Effect of starch sources on enzyme production:

To determine the effect of starch sources, the soluble starch in complete medium was replaced with cassava, yam, cocoyam, plantain, millet, rice and corn starches at a concentration of 1% (w/v).

Effect of concentration of cocoyam starch sources on enzyme production:

The effect of different concentration of cocoyam starch on amylase production by the organism was determined using values of 0.5 to 4.0%.

Effect of nitrogen sources on enzyme production:

The influence of the different nitrogen sources (fish meal, blood meal, cow meat, soy beans and beans) on amylase production was determined by incorporating each of them into basal medium at a final concentration of 1.1% (w/v) and the initial pH adjusted to 7.0.

Effect of concentration of fish meal on enzyme production:

The effect of fish meal at various concentration of 0.5 to 1.2%, on amylase production was determined.

Effect of initial pH of basal medium on enzyme production:

The effect of initial pH on enzyme production was determined by measuring activity in basal medium having pH ranging from 3.0 to 9.0. Final pH was also determined after incubating inoculated medium at 35°C on a rotary shaker at 120 rpm for 36h.

Effect of metal ion on enzyme production:

Effects of $CuSO_4$, $MnSO_4$, $FeSO_4$, $MgSO_4$, $ZnSO_4$ and $CaCO_3$ on enzyme production were examined with the addition of these chemicals to growth medium. Enzyme activity was determined after 36h incubation.

Effect of heavy metals on enzyme production:

Effects of ethylene diaminetetra acetic acid (EDTA) and HgCl on amylase production was determined by adding these chemicals to growth medium. Enzyme activity was determined after 36h incubation.

RESULTS AND DISCUSSION

Effect of starch sources on alpha amylase production: The ability of *Bacillus subtilis* BS5 to grow and produce amylases on different starch sources was studied. Significant growth of the organism was observed in all, while cocoyam starch gave the highest amylase yield at a concentration of 4% (w/v) (Table.1, 2).

Table.1: Effect of starch sources on alpha amylase production

Starch sources (1%w/v)	Final Ph	α-amylase activity (U/ml)
Cassava	5.0	0.153
Yam	5.7	0.144
Cocoyam	6.1	0.261
Plantain	5.4	0.201
Soluble starch	5.0	0.186
Millet	6.0	0.255
Rice	6.6	0.216
Corn	5.4	0.231

Table.2: Effect of concentration of cocoyam on amylase production

Concentration (w/v)	α-amylase activity (U/ml)
0.5	0.145
1.0	0.254
1.5	0.268
2.0	0.246
2.5	0.203
3.0	0.138
3.5	0.065
4.0	0.058

Effect of nitrogen sources on alpha amylase production: As shown in Table.3, fish meal was the most suitable nitrogen source at a concentration of 0.5% (w/v) (Table.3, 4) for amylase production by *B.*

subtilis BS5. Obineme *et* al., [6] (2003) reported that cocoyam starch and fish meal supported maximum amylase production by *Aspergillus oryzae*.

Table 3: Effect of nitrogen sources on alpha amylase production

Nitrogen (1% w/v)	Final pH	α-amylase activity (U /ml)
Fish meal	8.8	0.75
Blood meal	8.5	0.53
Cow meat	8.3	0.48
Soy beans	7.4	0.72
Beans	8.1	0.65

Table.4: Effect of concentration of fish meal on amylase production

Concentration (w/v)	α-amylase activity (U /ml)
0.5	0.17
0.6	0.18
0.7	0.19
0.8	0.20
0.9	0.22
1.0	0.23
1.1	0.23
1.2	0.18

Effect of initial pH on alpha amylase production: Enzyme synthesis was observed at pH 4.5 to 10.0. Maximum enzyme productions were obtained at pH 6.0 to 8.0. The maximum production was 90% at pH 6 to 7. Enzyme production was decreased at pH 8.5 to 10 (Figure.1). *Bacillus licheniformis* TCRDC-B13 was reported to continue enzyme synthesis until pH 10^2, while *B. subtilis* K-12 synthesized amylase within the pH range 4.5 to 10.5[4].

Table.5: Effect of initial pH on alpha amylase production

pH	α-amylase activity (U/ml)
3.0	0.58
4.0	0.65
5.0	0.48
6.0	1.33
7.0	0.53
8.0	0.58

Effect of metal ions on alpha amylase production: Results in Table.5 showed that metal ions Ca^{2+}, Na^{+} and Mn^{2+} were found to stimulate enzyme production in this organism. Kadrekar and Ramasarma[3] (1990) and Kiran *et al.*,[4] (2005) reported that Mn^{2+} favored the synthesis of amylase. Shatta *et al.*,[8] (1990) however reported that amylase production decreased in medium containing Mn^{2+}.

Table.6: Effect of metal ions on alpha amylase production

Metal ions	Relative activity (%)
None	100
Ca^{2+}	113.4
Cu^{2+}	80.8
Fe^{3+}	88.8
Mg^{2+}	57.9
Mn^{2+}	146.3
Zn^{2+}	77.1
Na^{+}	105.0

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Source of support: Nil
Conflict of interest: None Declared