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Full Length Research Paper

Heat activation and stability of amylases from *Bacillus* species

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Leitch and Collier sporulating Bacillus medium was used to isolate some strains of *Bacillus* species from soil, wastewater and food sources in Ibadan, Oyo State, Nigeria, by heat activation method. Heat treatment at 80oC allowed the growth of sporulating *Bacillus* species, in the culture sample source without other bacteria forms. The amylolytic *Bacillus* species isolated during the study were identified as *Bacillus macerans, Bacillus coagulans Bacillus licheniformis, Bacillus circulans, Bacillus megaterium, Bacillus polymyxa* and *Bacillus subtilis*. Heat treatment at 70oC denatured the β-amylase component of the amylase source while α-amylase retained its potency at this temperature. Calcium cations (Ca2+) enhance the enzyme production than Na+ which was less effective. Physiological studies show that an optimum temperature of 40°C was suitable for the enzyme activity while temperature above 60oC reduced its activity unless positive measures are taken to stabilize it with relevant cations like Ca2+.

Key words: Activation, Bacillus, effect, heat, treatment.

INTRODUCTION

Amylases are starch-degrading enzymes of industrial importance (Reed, 1975). Animal amylase is mainly α-amylase, while β-amylase occurs in plants (Street, 1958). It was established few decades ago that α-amylase occur and can be produced as an extracellular enzyme by microorganisms (Murao et al., 1978; Rose, 1980). First of such discovery was made in 1946 when β-amylase was found to be produced by *Bacillus polymyxa* and later by another *Bacillus* species identified as *Bacillus cereus* var. *mycoides* (Takasaki, 1976). Other amylolytic enzymes can also be obtainable in *Bacillus* strains (Ohdan et al., 2000; Prescott et al., 2002).

It has been demonstrated in past studies that amylases especially the β-amylase is heat labile, thus can be rapidly denatured at temperature above 70°C. Hence this study is aimed at determining appropriate microbial strain and suitable assay temperature for large scale production of enzyme. Starch enzymes, alpha amylase, catalyse the random hydrolysis of α-1,4-glycosidic bounds in starch, while saccharogenic amylase that is, β-amylase is the enzyme that catalyze the sequential hydrolysis of α-1,4-glucans (Matsui et al., 1977). Optimization of cultural condition for maximum production of an enzyme is obligatory for different microbial strains (Bezbaruah et al., 1994). In this regard appropriate media components and suitable conditions must be attained for optimal produc-tion levels of the enzyme required. Effects of heat treat-ment on sporulation (Moran et al., 1990; Amua-Awua and Jakobsen, 1995; Lin et al., 1997), assay and heat stability (Berfeld, 1951; Reed, 1975) have been reported (Lonsane and Ramesh, 1990). Moran et al. (1990) demo-nstrated that heat activation treatments of sporula-ting bacillus species at 80°C for 10 min were suitable for *Bacillus* sporulation than higher temperatures up to 100°C for 10 min. Assay temperature between 40 and 55°C was found optimal for amylolytic activity of *Bacilllus* species (Rose, 1980). Increased temperature up to 70°C rapidly denatures β-amylase, which is heat-labile (Reed, 1975; Bernfeld, 1951) while α-amylase still retains its potency

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at these temperatures. This heat activation patterns in *Bacillus* species is of importance in various industrial and scientific studies. Thus as part of the objective of this study, apart from those earlier enumerated in literatures, the knowledge of the heat stability of *Bacillus* amylase under various cultural conditions is necessary to enhance improved productivity of the enzyme for scale purposes.

MATERIALS AND METHODS

Microbial strains

Bacillus species used for this study were isolated from appropriate sources such as wastewater and selected food sources. The mixed cultures of these samples were subjected to heat activation treatment of 80°C for 10 min using Leitch and Collier sporulating chem.-ically defined medium (Leitch and Collier, 1996; Moran et al., 1990). The *Bacillus* species were identified by standard microbialogical techniques based on their morphological, colour, arrangement of vegetative cell and possession of spores (Robert et al., 1984; Kotzekidou, 1996). Amylolytic bacillus species were screened in this study by using starch hydrolysis procedure (Cowan and Steel, 1985; DIFCO Manual, 1984). Each of the *Bacillus* strain was cultured in nutrient broth medium and nutrient broth constituting 1% soluble starch to estimate their growth nature on incubation overnight.

Extraction of amylase from *Bacillus* cultures

The *Bacillus* species were cultured in a medium (50 ml) containing 2% peptone, 0.5% soluble starch, 0.3% K_2HPO_4 and 0.1% $MgSO_4.7H_20$ in Erlenmeyer flask of 200 ml capacity. The cultivation was carried out for about 40 h at 30°C on a rotatory shaker (Model G24, Environmental incubator shaker, N.J., U.S.A.) at 150 rpm. This chemically defined medium aids the synthesis of amylase by the *Bacillus* strains into the culture medium. The cultured cells were removed by centrifugation at 4,000 rpm for 15 min and resultant supernatant was used as enzyme source and assayed for activity according to the dinitrosalicylic acid (DNSA) method (Murao et al., 1978; Bailey, 1988).

Enzyme assay

Amylase activity was assayed by measuring the amount of reducing sugar released according to the DNSA method. The substrate was 1.0% soluble starch dissolved in phosphate buffer (pH 7). α-Amylase was determined by heating the enzyme in a water bath at 70°C for 15 min in order to inactivate the β-amylase (Bernfeld, 1951). The residual α-amylase that is relatively heat stable than β-amylase was similarly assayed for activity as previously described.

Cations considered were that of calcium (Ca^{2+}) and sodium (Na^+). Concentrations of 4 mg/ml of each cation were tested during the study. 4 mg of $CaCl.2H_2O$ was added per unit enzymes solution (swamy et al., 1994; Lonsane and Ramesh, 1990). Sodium cation in form of NaCl was similarly added in the duplicate set of enzyme sample assay.

Effect temperature

Enzyme samples were incubated for one hour at various temperatures between 20 and 70°C in the phosphate substrate buffer (pH 7.0). The samples were then placed at 37°C for 10 min and assayed for activity.

Stability at high temperature

The enzyme samples were subjected to heat treatment in a water bath at 70°C for 15 min and assayed for their enzymatic activity according to the dinitrosalicylic acid (DNSA) method as described by Murao et al. (1979) and Bailey (1988). This process inactivates the β-amylase (Bernfeld, 1951).

RESULTS AND DISCUSSION

Table 1a shows the amylolytic nature of the *Bacillus* strains. *Bacillus subtilis* (WBS), *Bacillus licheniformis* (WBL) and *Bacillus macerans* (MBM) with 6.24, 4.2 and 3.0 unit/ml, respectively, showed more proficiency for amylase than other *Bacillus* strains. Heat treatment at 70°C rapidly denatures the heat-labile β-amylase content of the enzyme recovered. Table 1b clarifies the importance of cation of Ca^{2+} that stabilizes the residual α-amylase in the assay systems. Comparative study of the enzyme activity shows that *B. macerans* (SBMI), *B. licheniformis* (WBL) and *B. subtilis* (WBS) had their activity increased from initial value of 0.12 and 0.06 unit/ml to 2.4 unit/ml. respectively.

Ca^{2+} further enhances amylase production activity than Na^+ in this study (Table 2). For example, *B. licheniformis (*WBL) with original amylolytic value of 0.12 unit/ml increased its amylase production to 11.88 unit/ml on addition of Ca^{2+}. The protective or enhanced activity of Ca^{2+} was also seen on other *Bacillus* strains amylolytic activity. The effects of assay temperature on *Bacillus* strains enzyme samples are shown in Table 3. The optimal assay temperature observed for amylase activity ranged between 40 and 50°C.

Heat treatment at 70°C and above rapidly denatures the β-amylase enzyme samples. Table 4 shows that the enzyme samples had their activities reduced from 13.61 and 18.7 unit/ml to low 0.00 value that cannot be detected and 0.60 unit/ml at pH 7 for *B. licheniformis* (WBL) and *B. subtilis* (WBS). Similarly, at pH 7.4, β-amylase cannot be detected in *B. licheniformis* (WBL) while 0.1 unit/ml was obtained in *B. subtilis* (WBS) after heat treatment (Table 4).

The effect or various heat activation treatments on sporulation of the amylolytic *Bacillus* species and assay temperature conditions for good enzymatic production of amylase from suitable strains of bacillus species was determined during the study. The *Bacillus* species encountered through the use of heat activation method at 80°C for 10 min from soil, food and wastewater sources include *B. macerans, Bacillus coagulans, B. licheniformis, Bacillus circulans, Bacillus megaterium, B. polymyxa* and *B. subtilis* (Table 1).

Heat labile nature of β-amylase was shown in this study, because the heat treatment of 70°C which denatures it still keep the α-amylase content of the *Bacillus* species (Tables 1 and 4). Previous studies showed that heating amylase enzyme in water bath at 70°C for 15 min

Table 1a. Heat treatment of amylases produced by *Bacillus* species at 70°C for 15 min.

Strain code	*Bacillus* species	Total amylase (unit/ml)	Denatured β-amylase (unit/ml)
MBM	*B. macerans*	3.0	3.0
SBM1	*B. macerans*	1.56	1.44
SBM2	*B. macerans*	1.80	-
MBC	*B. coagulans*	0.84	0.36
WBL	*B. licheniformis*	4.2	4.2
WBCI	*B. circulans*	0.72	0.72
SBG	*B. megaterium*	0.12	0.12
WBP	*B. polymyxa*	0.48	0.48
WBS	*B. subtilis*	6.24	5.64

Table 1b. Effect of Ca^{2+} on α-amylase at high temperature (70°C).

Strain code	*Bacillus* species	Amylase (unit/ml)
MBM	*B. macerans*	1.68
SBM1	*B. macerans*	2.4
SBM2	*B. macerans*	1.80
WBC	*B. coagulans*	0.6
MBC	*B. coagulans*	0.60
SBL	*B. licheniformis*	2.4
WBL	*B. licheniformis*	1.68
WBC1	*B. circulans*	0.48
SBG	*B. megaterium*	0.12
WBP	*B. polymyxa*	0.36
WBS	*B. subtilis*	2.4

Table 2. Effect of Ca^{2+} and Na^{+} cations on total amylase activity.

Strain code	*Bacillus* species	Amylase (unit/ml)	Ca^{2+}	Na^{+}
MBM	*B. macerans*	3.0	1.32	2.28
SBM1	*B. macerans*	1.56	-	-
SBM2	*B. macerans*	1.80	0.80	-
MBC	*B. coagulans*	0.84	1.64	0.72
WBL	*B. licheniformis*	4.2	-	-
WBCI	*B. circulans*	0.72	1.32	-
SBG	*B. megaterium*	0.12	11.88	-
WBP	*B. polymyxa*	0.48	0.72	-
WBS	*B. subtilis*	6.24	7.20	-

inactivate β-amylase content (Bernfeld, 1951). It also shown in this study that Ca^{2+} cation improves enzymatic activity of the *Bacillus* amylase than Na^{+} (Tables 1b and 2). Swamy et al. (1994) in their study showed that Ca^{2+} has stabilizing effect on amylase activity when the temperature is raised. This shows the protective nature of Ca^{2+} cation on amylase activity. The range of assay temperature observed for good production activity of amylase from *Bacillus* species was 40 to 50°C. This is consistent with the studies of previous investigators (Rose, 1980; Reed, 1975; Saito, 1973).

Table 3. Effect of temperature on *Bacillus* amylase.

Temperature (°C)	Amylase (unit/ml))
20	0.72
30	3.84
40	6.12
50	5.28
60	1.20

In conclusion, this study helps to formulate suitable the-

Table 4. Enzyme stability at high temperature (70°C).

pH values of assayed samples	*B. licheniformis* (WBL) amylase (Unit/ml)		*B. subtilis* (WBS) amylase (Unit/ml)	
	Initial enzyme activity	Heat treated samples	Initial enzyme activity	Heat treated samples
5.8	3.26	1.44	7.26	2.97
7.0	13.61	-	18.7	0.60
7.4	8.52	-	11.7	0.1

rmal condition using appropriate culture medium for the isolation of various strains of amylolytic *Bacillus* species and subsequently adapt on optimal assay temperature to improve the amylase production from various strains of the genus *Bacillus.* Furthermore the protective property of calcium (Ca^{2+}) cation on amylase activity coupled with the cautionary measures to be taken in maintaining specified temperatures for effective amylase production will also be useful as guide for research, clinical and industrial amylase productions purposes.

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