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PRODUCTION OF ALKALINE AMYLASES FROM ALKALIPHILIC BACILLUS STRAINS

By

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ABSTRACT

For alkaliphilic and haloalkaliphilic Bacillus strains were recorded to produce alkaline amylases in alkaline medium. Bacillus No. N 25 produced maximum alkaline amylase when the medium was supplemented with 2.0 % Na_2CO_3 at pH 10.6 and 37 C. On the other hand, Bacillus strains No. N 28, T 3 & T 6 produced maximum alkaline amylases when the medium was supplemented with 1.5 % Na_2CO_3 and 2 % NaCl at pH 10.4 and 50, 55 & 60 C respectively. Amylases from Bacillus strains No. N 28, T 3 and T 6 were fairly heat stable and about 60 - 85 % of activity was remained after heating at 65 C for 20 min.

INTRODUCTION

Microbial growth under extreme conditions has stimulated the efforts of many investigators (Stetter, 1986 and Krulwich and Guffanti, 1989). High temperatures, extreme alkaline or acidic environments, hypersaline niches

are important factors that determine extremophilicity. Alkaliphilic and haloalkaliphilic bacteria are members of the extremophilic community. They possess certain bioenergetic properties that enable them to produce some industrially important products under extreme environmental conditions (Clejan et al., 1986).

There are numerous applications for enzymes or pharmacologically active chemicals that are stable at high pH (Krulwich and Guffanti, 1989). Horikoshi and Akiba (1982, 1985 & 1988) have reviewed the industrial applications of alkaliphiles and the development of neutrophilic excretion system for alkaliphile enzymes. Amylases have industrial and pharmaceutical uses, particularly as detergent additives at high pH. Extracellular alkaline amylases were not thoroughly investigated (Hayashi et al., 1988 a & b).

The present investigation is therefore concerned with the production of alkaline amylases by four alkaliphilic and haloalkaliphilic *Bacillus* strains previously isolated and identified by Ghanem et al., (1993).

MATERIALS AND METHODS

Organisms :

Four *Bacillus* strains previously isolated and identified to the generic level were used. They were numbered as *Bacillus* N 25, N 28, T 3 & T 6 (Ghanem et al., 1993).

Media:

Medium N0. 3 (Ghanem et al., 1993) was used for the growth and maintenance of the strains under investigation. It contained g/l: $NaNO_3$, 3.0; K_2HPO_4, 1.0; $MgSO_4$. 7 H_2O, 0.2; Glucose, 10.0; yeast extract, 3.0. Sodium carbonate 1.5 % was added to the basal medium after sterilization. NaCl 7.5% was added to the growth medium of strains N 28, T 3 & T 6. For production of alkaline amylases 2 % soluble starch was added instead of glucose.

Preparation of crude enzyme solution:

Bacillus strains under investigation were grown aerobically with shaking (New Brunswick shaker incubator) for 20 h. at 37 C for strain N 25 and 55 C for the rest of strains. Cells were removed by centrifugation at 6000 x g for 10 min and the supernatant fluid was examined as a crude enzyme solution.

Assay of enzyme activity:

A modified blue value method was applied to determine enzyme activity (Horikoshi, 1971). An enzyme solution (10 ul) suitably diluted with 0.02 M Tris - HCl buffer pH 8.0 was mixed with 0.2 ml of 0.2 % potato strach solution (pH 10.0, made up with 0.1 M glycine - NaOH - NaCl buffer) at 40 C. After 30 min incubation the reaction was stopped by adding 0.2 ml of 0.2 N HCl, and to the mixture were added 2 ml of 0 005 % iodine solution. The absorbance of the sample was measured at 700 nm. One unit of enzyme activity is defined as the amount of enzyme to reduce optical density of 0.32, which is equivalent to 100 ug of potato starch under the above conditions.

Effect of various pH's on amylase activity in the crude extract:

The following buffer systems were used: pH 2.5 - 6, 0.2 M acetate buffer; pH 5 - 8, 0.2 M Tris - maleate buffer; pH 7 - 9, 0.2 M Tris - HCl buffer; pH 8.5 - 13, 0.2 M glycine - NaCl - NaOH buffer.

Effect of different culture conditions on alkaline amylase production:

The following tests were made: 1- Effect of different salts at different pH's: Different concentrations of NaCl, KCl. Na_2CO_3 and $NaHCO_3$ were added separately to the basal production medium. The crude extract was assayed for amylolytic activity after each treatment, 2- Effect of different incubation temperatures, and 3- Effect of different incubation periods.

Determination of thermal stability:

The culture broth was mixed with 0.1 M Borax - NaOH buffer (pH 10.0) in the presence or absence of 10m M $CaCl_2$, incubated at 55 C, 60 C and 65 C for 20 min, and the residual activity was measured at pH 10.0.

Effect of pH on the stability of the crude enzyme:

The same method used for thermal stability was performed with the exception that the enzyme mixture was incubated at 50 C.

RESULTS AND DISCUSSION

Four alkaliphilic and haloalkaliphilic *Bacillus* strains were recorded to produce alkaline amylases in alkaline media (Ghanem et al., 1993). *Bacillus* N 25 was reported to be obligate alkaliphile where *Bacillus* strains N 28, T 3 and T 6 were reported to be facultative haloalkaliphiles that grow well under both alkaline and neutral conditions with the presence or absence of NaCl. The latter three strains were also reported to be thermophilic.

Culture conditions for the production of alkaline amylases:

Growth of the four extremophilic *Bacillus* strains and production of amylases were examined in the media containing sodium carbonate, or various carbonate sources. The effect of adition of sodium chloride or potassium chloride was also tested. Growth was measured as optical density units at 600 nm after 20 h. Alkaline amylase activity was also measured at the same time in the crude extract. Results recorded in Table (1) showed that addition of carbonate of bicarbonate salts is an important factor for the production of alkaline amylase for all strains under investigation. Interestingly, the addition of NaCl increased the productivity of alkaline amylases for the haloalkaliphilic strains N 28, T 3 and T 6. Maximum alkaline amylase activity of 3200 units/ml culture was measured at pH 10.6 in the presence of 2.0 % Na_2CO_3 for the obligate alkaliphilic strain N 25. On the other hand, maximum productivities of the facultative strains were recorded at pH 10.4 in the presence of 1.5 % Na_2CO_3 and 3 % NaCl. Strain T 6 was the most potent alkaline amylase producing organism in the present investigation.

Effect of incubation temperature on the production of alkaline amylases:

Crude enzyme extracts were assayed for alkaline amylase activities after different incubation temperatures. Results recorded in Fig. (1) showed that strain N 25 achived maximum productivity at 37 C, strain N 28 at 40 C and strain T 3 & T 6 at 55 & 60 C respectively.

Effect of incubation period on alkaline amylases production:

Results represented in Fig. (2) showed that maximum enzyme production was attained after 20 - 25 h. of incubation for all strains under investigation.

Detection of pH stability:

Different buffers were used to cover pH ranges from 2.5 to 13. Results were calculated in terms of relative activities (%) remained after treating the crude extract with $CaCl_2$ and heating at 50 C for 15 min. Fig. (3) showed that alkaline amylase residual activity of strain N 25 was above 60 % at pH 7 - 10. Alkaline amylases of strains N 28 and T 3 showed also pH stability in the range of 7 - 9.8 and the residual activities recorded were above 60 %. Interestingly, alkaline amylases produced by strain T 6 showed stabilities in the acidic as well as alkaline ranges where two pH peaks of residual activities were recorded. The first was obtained at a pH range of 4 - 6 with residual activity around 60 %, and the second was recorded at a pH range of 8 - 11 with residual activity above 60 %.

Detection of thermal stability:

Residual alkaline amylase activities were measured after heating the crude extract to 55, 60 and 65 C respectively in the presence or absence of $CaCl_2$. As shown in Table (2) all the amylases tested were stabilized by Ca^{2+}. Amylases of strains N 28, T 3 and T 6 are fairly heat stable, and about 60 - 85 % of activity was remained after heating at 65 C. On the other hand, amylase of strain N 25 showed less heat stability where 10 % activity was recorded after heating at 65 C, 18 % activity after heating at 60 C and 20 % after heating at 55 C.

Results of the present investigation showed that alkaline amylases produced by certain extremophlic *Bacillus* strains possess pH and heat stability properties. Table (3) summarizes such properties. The enzymes produced look quit different than other bacterial amylases in being heat and pH stable enzymes since they resist pH ranges up to 11 and temperature up to 65 C. Moreover, amylases produced by strain T 6 showed tow pH ranges of activity i.e. acidic (4 - 6) and alkaline (8 - 11).

Interestingly, alkaline amylases produced from alkaliphilic *Bacillus* strains were recently reported by few authors (Hayashi et al., 1988 a & b; Ikura and Horikoshi, 1987; Boyer et al., 1973; Yamamato et al., 1972; and Horikoshi, 1971). Horikoshi (1971), reported that *Bacillus* No. 40 - 2 isolated from Japanese soil produced alkaline amylase of the saccharifying a - amylase type. It was produced at pH 10.5 and stable at pH 8.5 and at 55 C. Yamamato et al., (1972) also reported that many *Bacillus* strains from Japanese soils were alkaline amylases producers. They stated that four types

of amylases were investigated that showed stability under specific pH ranges i.e. 7 - 9.5; 7 - 9; 5 - 10.5; and 6.5 - 11, beside their thermal stability at 55 C. More recently Hayashi et al., (1988) reported that *Bacillus* strain H-167 which is able to grow at pH 7 - 12 produced three alkaline amylases with different molecular weights but with optimal activity at pH 10.5 and stable at 55 C.

As far as we know, no other report concerning alkaline amylase production from alkaliphilic *Bacillus* strains was published. Therefore, the importance of the present investigation lies in the ability of the produced alkaline amylases to act under extreme coditions of high pH and temperature.

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Table 1: Amylase activities of Bacillus strains N25, N28, T3 and T6 under different culture conditions

Salts added	Initial pH	Growth (at 600 nm)				Amylase activity			
		N25	N28	T3	T6	N25	N28	T3	T6
None	7	nd	0.1	0.1	0.2	nd	nd	nd	nd
None*	8	nd	0.3	0.4	0.3	nd	nd	nd	nd
None*	10	nd	0.9	0.7	0.6	nd	nd	nd	nd
NaCl 1.0%	7	nd	0.3	0.2	0.4	nd	nd	nd	nd
NaCl 2.0%	7	nd	0.6	0.6	0.5	nd	nd	nd	nd
NaCl* 1.0%	10.5	0.1	1	1	0.9	nd	nd	nd	150
NaCl* 2.0%	10.5	0.1	1.2	1.4	1.4	nd	nd	nd	150
KCl 1.0%	7	0.1	0.1	1	1.1	nd	nd	nd	nd
KCl* 1.0%	10.5	0.1	0.2	0.5	0.7	nd	bd	nd	100
Na2CO3 0.5%	9.7	0.5	1.2	1	0.9	1500	2500	3000	3500
Na2CO3 1.0%	10.2	1.2	1.7	1.5	1.4	2000	2600	3200	3600
Na2CO3 1.5%	10.4	1.8	1.7	1.8	1.7	3000	3000	3400	4000
Na2CO3 2.0%	10.6	1.6	1.5	1.2	1.7	3200	3000	3400	3800
NaHCO3 1.0%	9	0.4	0.6	1.1	1.1	1000	1500	1350	2000
NaHCO3 1.5%	8.2	0.4	0.6	1.4	1.3	1000	1450	1300	2000
NaHCO3 2.0%	9.3	0.3	0.7	1.4	1.4	1100	1500	1340	2100
K2CO3 1.0%	10.2	0.7	1	0.9	1.1	1400	1650	1800	2000
Na2CO3 1% + NaCl 1%	10.2	0.2	1.3	1.9	1.9	600	2500	3100	4000
Na2CO3 1.5%+NaCl 2%	10.4	0.2	1.7	2	2	250	3500	4400	5000
Na2CO3 1.5%+NaCl 3%	10.4	0.1	1.9	2	2	180	3500	4500	5100

* pH was adjusted with NaOH

Table 2: Thermal stabilities of alkaline amylases under investigation

Strain No.	Residual activity (%)					
	55 C		60 C		65 C	
	CaCl2 10 mM		CaCl2 10 mM		CaCl2 10 mM	
	-	+	-	+	-	+
N25	5.2	20	0	18	0	10
N28	40	75	40	77	30	60
T3	45	73	44	76	40	70
T6	87	94	87	100	70	85

Table 3: Properties of alkaline amylases of the four Bacillus strains

Strain No.	Opt. pH	max. act./ act. 10	pH stability**	protection by Ca+2
N25	10.8	1	7.5 - 10	+
N28	10.4	1	7 - 10	+
T3	10.4	1	8.7 - 9.8	+
T6	10.4	1	4 - 6 and 8 - 11	+

* maximum activity /activity at pH 10
** 50% of the activity was remained

Fig 1: Effect of incubation temperature on alkaline amylases

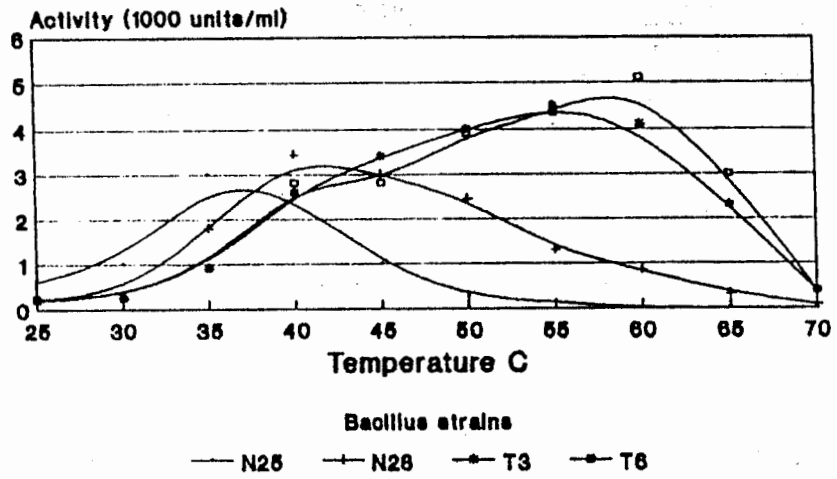

Fig 2: Effect of incubation period on alkaline amylases production

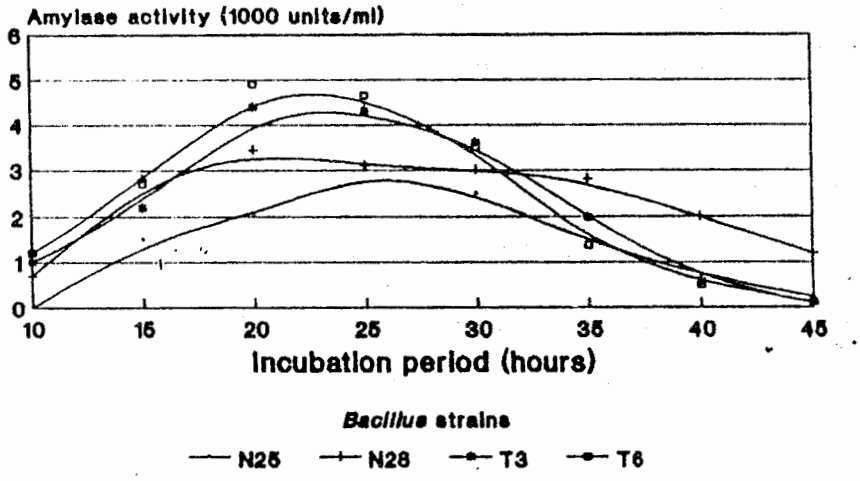

Fig 3: Effect of different pH's on the activity of crude enzyme extract