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Parametric Optimization of Extracellular α-Amylase Production by Thermophilic *Bacillus coagulans*

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ABSTRACT. The culture parameters required for optimum production of extracellular α-amylase by the thermophilic *Bacillus coagulans* are described. The optimum pH, temperature and incubation period for amylase production were 7, 50 °C and 48 h, respectively. Age of inoculum (48 h) and its level (2 %) were critical for maximum amylase yield. The enzyme secretion was high in rice starch and beef extract as compared to other carbon and nitrogen sources tested. The addition of mustard oil cake (1 %) and agitation at 1.7 Hz resulted in an enhancement of α-amylase secretion.

Extracellular enzymes of bacteria of the genus *Bacillus* have several applications in paper, textile, food, starch, adhesive and sugar industries (Fogarty 1979). Among the various extracellular enzymes, α-amylase ranks first in terms of commercial exploitation and the extent to which it has been studied. In view of the extensive industrial uses of thermostable α-amylase, the present investigation was planned to select a suitable, α-amylase-producing thermophilic *Bacillus* species and to maximize its production by the optimization of culture conditions.

One-hundred-forty amylolytic bacterial strains were isolated from soil, compost and cereal straw samples on Emerson YpSs agar medium (Emerson 1941) at 50 °C and screened for their ability to hydrolyze starch. Among these, bacterial strain B 49 was found to be an efficient hydrolyzer of starch and it was identified as *Bacillus coagulans* in accordance with Claus and Berkeley (1986) and Gordon (1989). This strain is a moderate thermophile, having a temperature minimum of 25 °C and maximum of 60 °C, with an optimum at 50 °C and is maintained on Emerson YpSs agar slants at 4 °C.

The inoculum was prepared in soluble starch-peptone broth (in %: soluble starch 2.0, peptone 0.5, Yeast extract 0.25, K_2HPO_4 0.2, $MgSO_4 \cdot 7H_2O$ 0.1; pH 7.0) by transferring a loopful of the organism from a fresh culture and incubating at 50 °C in a shaker at 3.3 Hz for 2 d. The amylase production was carried out in a starch-beef extract liquid medium (SB) (in %: soluble starch 2, beef extract 1, Yeast extract 0.2, $CaCl_2$ 0.02, $MgSO_4 \cdot 7H_2O$ 0.01; pH 7.0). Fifty mL of sterile SB medium in a 250-mL Erlenmeyer flask was inoculated with 2 % inoculum and agitated at 3.3 Hz for 2 d in a controlled environment incubator shaker (New Brunswick, USA) at 50 °C. The culture broth was centrifuged at 250 Hz for 20 min at 4 °C. The clear cell-free supernatant was used for amylase assay.

The effect of temperature and pH on enzyme production was studied by growing *B. coagulans* in an SB medium at different temperatures and various pH values. The influence of inoculum age and level was studied (*i*) by using inoculum grown for 8–48 h, and (*ii*) by employing 0.5–8.0 % inoculum, respectively.

In order to formulate a medium for optimum enzyme production, *B. coagulans* was grown in several variants of SB medium prepared by (*i*) substituting soluble starch with different carbon sources (2 %), (*ii*) replacing beef extract with organic and inorganic nitrogen sources (1 %), (*iii*) supplementing with different concentrations of beef extract (0.25–2.0 %) and yeast extract (0.05–4.0 %), and (*iv*) supplementing with different oil seed cakes and surfactants. The effect of agitation on amylase synthesis was studied in modified SB medium (soluble starch was replaced with rice starch, and supplemented with 1 % mustard oil cake) shaken at 1.7, 3.3 and 5.0 Hz.

The products of amylase action on starch were analyzed by thin-layer chromatography. The TLC plates were developed in 1-butanol–ethanol–water (50:30:20) and the spots were revealed by spraying with acetone–silver nitrate solution (0.1 mL of saturated solution of $AgNO_3$ in 20 mL of acetone).

The reaction mixture containing 0.5 mL soluble starch solution (1 %) prepared in phosphate buffer (0.1 mol/L, pH 7.0) and 0.5 mL of a suitably diluted enzyme sample was incubated in a water bath maintained at 60 °C (optimum for amylase activity of this strain) for 1 h and the reducing sugars liberated were determined with the 3,5-dinitrosalicylic acid reagent (Miller 1959). One unit of amylase is defined as the amount of enzyme that liberates one micromole of reducing sugar per minute at 60 °C.

The thin-layer chromatography of starch hydrolyzate revealed the formation of glucose, maltose, maltotriose and other oligosaccharides (Fig. 1), confirming that the enzyme is α-amylase.

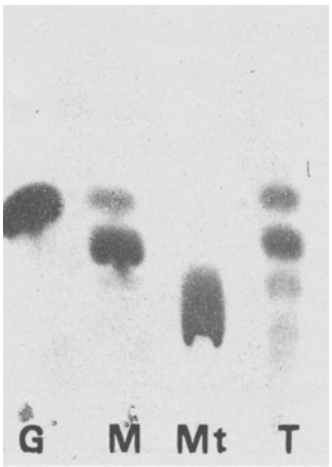

Fig. 1. Thin-layer chromatogram showing the products liberated by the action of amylolytic enzymes of *B. coagulans*; G – glucose, M – maltose, Mt – maltotriose, T – test sample.

The amylase production was very poor at 30 °C and moderate at 37 and 45 °C. At 50 °C, maximum growth as well as enzyme production was recorded. The α-amylase accumulation was slightly less at 55 °C than that at 37 and 45 °C (Fig. 2 *left*). Medda and Chandra (1980) reported the optimum amylase production by *B. coagulans* at 53 °C. *B. coagulans* is known as an acidophilic organism (Belly and Brock 1974). However, this strain failed to grow at pH below 5. The optimum pH for growth as well as enzyme secretion was 7.0 (Fig. 2 *right*).

The accumulation of α-amylase increased linearly with the increase in inoculum age, the maximum being at 48 h. This is not in agreement with the report of Haddad (1974) that the age of the inoculum has no such effect in *Bacillus mesentericus* P.B. A noticeable positive effect of inoculum level (0.5–2 %) was observed on amylase secretion. However, at higher inoculum densities, enzyme production was inhibited. Haddad (1974) has also reported similar observations. The enzyme secretion reached a peak by 48 h of incubation and it did not increase further. Shinmyo *et al.* (1982), Chojecki and Blaschek (1986), Sunna and Hashwa (1990) have reported a similar finding.

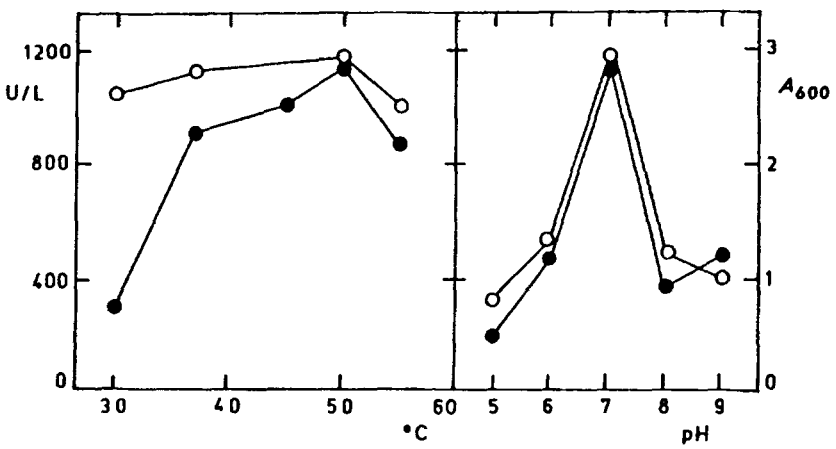

Fig. 2. Effect of temperature (*left*, °C) and pH (*right*) on α-amylase production (U/L, *closed symbols*) and growth (cell density, absorbance A_{600}).

Good cell growth was seen on soluble starch, sucrose, galactose, xylose, glucose, fructose and raffinose. Moderate growth was observed on malt extract and maltose, while poor growth was recorded on dextrin and lactose. The amylase production was high on rice starch as compared to that on other carbon sources (Table I). In xylose, maltose, lactose and glucose there was no detectable amount of amylase. Thus, various sugars showed differential effects on enzyme formation. The fall in amylase synthesis by low-molar-mass metabolizable sugars may be due to the repression of enzyme biosynthesis (Saito and Yamamoto 1975).

Table I. α-Amylase production by *B. coagulans* in different carbon (2 %) and nitrogen (1 %) sources

Carbon source	Cell density[a]	Amylase U/L	Nitrogen source	Cell density[a]	Amylase U/L
Rice starch	-	2 007	Beef extract	2.294	1 350
Wheat flour	-	1 266	Peptone	3.000	1 267
Soluble starch	2.494	1 138	Casein hydrolyzate	1.550	933
Cross-linked starch	-	695	Asparagine	0.860	0
Malt extract	1.170	665	NH_4NO_3	-	228
Dextrin	0.656	483	$(NH_4)_2HPO_4$	1.536	218
Corn starch	-	365	KNO_3	0.558	0
Fructose	2.000	265	$(NH_4)_2SO_4$	0.572	0
Raffinose	2.194	202			
Sucrose	2.444	171			
Galactose	0.450	0			
Maltose	1.772	0			
Glucose	2.194	0			
Xylose	2.230	0			

[a]Absorbance at 600 nm.

Organic nitrogen sources such as peptone and beef extract supported fine cell growth. The enzyme production was maximum on beef extract followed by peptone and casein hydrolyzate. Asparagine was not suitable for growth or amylase production (Table I). Chandra *et al.* (1980) found greater production of α-amylase in peptone than in other nitrogen sources. With inorganic nitrogen sources (ammonium nitrate, ammonium phosphate) amylase production was very low. Ammonium sulfate and potassium nitrate supported very poor growth and enzyme formation that could not be measured. The optimum levels of beef extract and yeast extract for high amylase production were 1 and 0.2 %, respectively (Fig. 3).

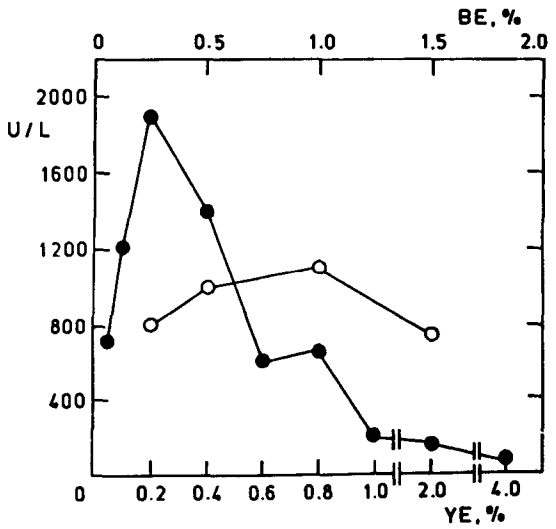

Fig. 3. Amylase production (U/L) at different concentrations of yeast extract (YE, *closed symbols*) and beef extract (BE, *open symbols*).

Table II. Production of α-amylase in modified SB medium with different oil seed cakes (concentration 1.5 %)

Oil seed cake	Enzyme production U/L
Control	567
Mustard	2 500
Sesame	1 100
Coconut	733
Linseed	633
Castor	700
Ground nut	433

The addition of surfactants such as Tween-80 (1 ppm), Triton X100 (100 ppm) and SDS (20 ppm) to the medium did not result in any significant improvement in α-amylase production. Contrary to our observation, Chandra *et al.* (1980) reported a stimulatory effect of surfactants on amylase secretion by *Bacillus licheniformis*. Among the oil cakes tested, mustard cake was found to be the most suitable additive since it showed a 4-fold increase in amylase production while sesame gave a 2-fold increase and coconut and castor cakes improved the enzyme formation to a small extent. However, the addition of groundnut cake was found to be inhibitory (Table II). One percent concentration of mustard cake was found to be adequate for enhanced amylase production. Krishnan and Chandra (1982) also reported a similar finding, observing that mustard oil cake contains a lower level of proteinaceous

matter and a higher level of saccharide than any other oil seed cake used. The speed of the agitation had a marked effect on amylase formation. Maximum enzyme production was attained at 1.7 Hz (2 582 U/L) as compared to 3.3 (894 U/L) and 5 (840 U/L). Amylase secretion was negligible when the fermentation was carried out in static conditions (214 U/L).

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