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Culture Conditions for Production of Thermostable Amylase by *Bacillus stearothermophilus*

R. A. K. SRIVASTAVA[1]†* AND J. N. BARUAH[2]

Biochemical Research Laboratory, Botany Department, University of Gorakhpur, Gorakhpur 273001,[1] and Regional Research Laboratory, Jorhat, Jorhat 785006, Assam,[2] India

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Bacillus stearothermophilus **grew better on complex and semisynthetic medium than on synthetic medium supplemented with amino acids. Amylase production on the complex medium containing beef extract or corn steep liquor was higher than on semisynthetic medium containing peptone (0.4%). The synthetic medium, however, did not provide a good yield of extracellular amylase. Among the carbohydrates which favored the production of amylase are, in order starch > dextrin > glycogen > cellobiose > maltohexaose-maltopeptaose > maltotetraose and maltotriose. The monosaccharides repressed the enzyme production, whereas inositol and D-sorbitol favored amylase production. Organic and inorganic salts increased amylase production in the order of KCl > sodium malate > potassium succinate, while the yield was comparatively lower with other organic salts of Na and K. Amino acids, in particular isoleucine, cysteine, phenylalanine, and aspartic acids, were found to be vital for amylase synthesis. Medium containing $CaCl_2 \cdot 2H_2O$ enhanced amylase production over that on Ca^{2+}-deficient medium. The detergents Tween-80 and Triton X-100 increased biomass but significantly suppressed amylase synthesis. The amylase powder obtained from the culture filtrate by prechilled acetone treatment was stable over a wide pH range and liquefied thick starch slurries at 80°C. The crude amylase, after $(NH_4)_2SO_4$ fractionation, had an activity of 210.6 U mg^{-1}. The optimum temperature and pH of the enzyme were found to be 82°C and 6.9, respectively. Ca^{2+} was required for the thermostability of the enzyme preparation.**

The composition and concentration of media greatly affect the growth and production of extracellular amylase in bacteria (5, 6, 10), yeasts (9, 12), and *Aspergillus* sp. (2). Shinke et al. (14) reported that medium composition affects amylase production as well as sporulation in *Bacillus cereus*. Starch is considered an inducer for amylase production (4, 8, 16, 18), but there are reports indicating that starch may not be required for amylase production (17). The nature and amount of nitrogen source in the culture medium also affect growth and amylase production (5, 9). Besides carbon and nitrogen, sodium and potassium salts (5, 14, 16), metal ions (5, 17), and detergents (12) are known to affect amylase production and the growth of the organism. The growth medium for amylase production should obviously be one that provides a good yield of extracellular amylase.

The *B. stearothermophilus* strain used in the present studies differed from the strain used in previous studies by Welker and Campbell (18). The strain we used has a lower optimal growth temperature (50 versus 55°C). The amylase produced by the strain used by Welker and Campbell is a low-molecular-weight enzyme with an optimal temperature range of 55 to 70°C, whereas the strain we used produces a high-molecular-weight amylase with an optimal temperature for activity of about 82°C (16). It was therefore necessary to determine the optimal culture conditions for growth of and amylase production by the strain we used. The results of these studies are presented here.

MATERIALS AND METHODS

The organism and its cultivation. *B. stearothermophilus* was isolated from soil (15). Unless otherwise stated, the bacterium was grown in stationary flasks at 50 ± 0.5°C on a basal medium of the following composition: peptone, 0.4%; $(NH_4)_2HPO_4$, 0.4%; corn steep liquor, 0.4%; KCl, 0.1%; $MgSO_4 \cdot 7H_2O$, 0.05; and starch (Difco Laboratories), 0.3%.

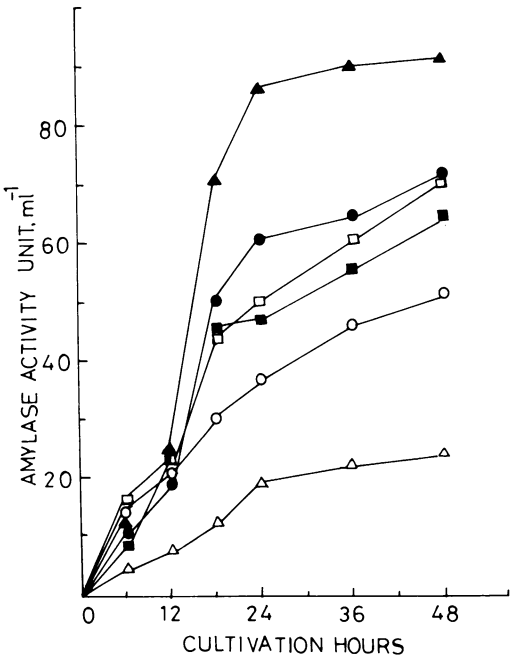

FIG. 1. Effect of various concentrations of starch added to basal medium on the amylase produced by *B. stearothermophilus*. Symbols: △, none; ○, 0.025%; □, 0.050%; ●, 0.10%; ▲, 0.30%; ■, 0.50%.

* Corresponding author.
† Present address: Biochemistry Division, Regional Research Laboratory, Jorhat 785 006, Assam, India.

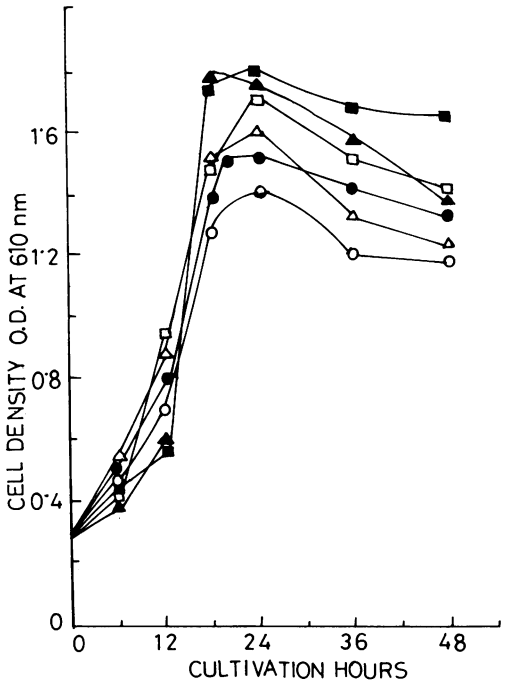

FIG. 2. Effect of various concentrations of starch added to basal medium on the growth of *B. stearothermophilus*. Symbols: ○, none; ●, 0.025; △, 0.050%; □, 0.100%; ▲, 0.30%; ■, 0.50%.

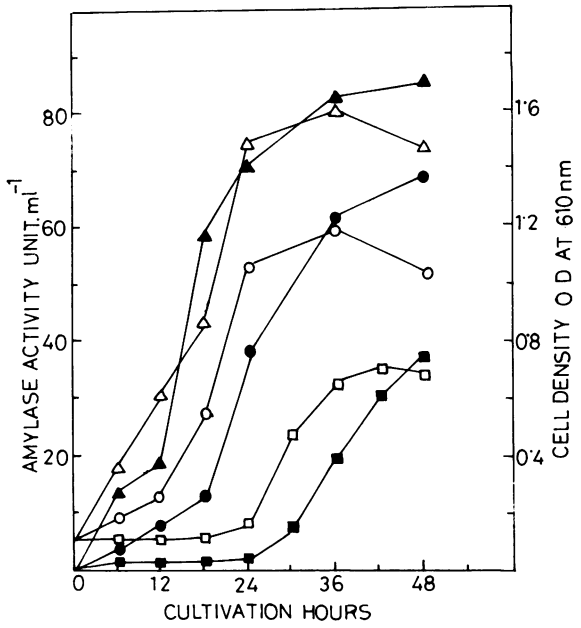

FIG. 3. Effect of complex (△, ▲), semisynthetic (○, ●), and synthetic (□, ■) media on growth and amylase production by *B. stearothermophilus*. Open symbols indicate growth; solid symbols indicate amylase production.

The pH of the medium was adjusted to 7.0. Portions (50 ml) of the basal medium were autoclaved in 250-ml Erlenmeyer flasks and used for bacterial cultivation. The yield of amylase was estimated in the extracellular fluid after removal of the bacterial cells from the culture broth by centrifugation at 10,000 × *g* (0 to 4°C) for 10 min. The cells were thoroughly washed and suspended in distilled water for

TABLE 1. Effect of carbohydrates on amylase formation[a]

Carbohydrate	% Concn (wt/vol)	Final pH	Cell growth (OD_{610})	Sp act (unit/unit cell mass)	
				Liquefying	Saccharifying
Monosaccharides and polyols					
D-Arabinose	0.20	7.9	0.90	11.7	2.4
	0.40	8.0	1.05	11.4	2.3
D-Xylose	0.20	7.4	0.75	10.7	ND
	0.40	7.4	0.85	11.2	ND
D-Fructose	0.2	7.2	0.75	ND[c]	ND
	0.4	7.1	0.80	ND	ND
D-Galactose	0.2	6.9	0.85	ND	ND
	0.4	6.8	1.00	ND	ND
D-Glucose	0.2	6.0	1.00	ND	ND
	0.4	5.3	1.35	ND	ND
Inositol	0.2	7.4	0.75	46.0	15.3
	0.4	7.2	0.90	36.7	15.3
D-Sorbitol	0.2	7.3	0.60	42.5	18.3
	0.4	7.1	0.95	30.5	13.6
Maltooligosaccharides					
Maltose (purified)	0.2	6.8	0.60	ND	ND
	0.4	6.6	0.70	ND	ND
Maltotriose	0.2	6.9	0.70	27.8	12.1
	0.4	6.7	0.80	28.6	11.3
Maltotetraose	0.2	7.0	0.70	31.4	11.7
	0.4	6.7	0.85	30.0	10.5
Maltopentaose	0.2	6.7	0.65	44.6	14.6
	0.4	6.3	0.85	39.4	12.1
Maltohexaose	0.2	6.8	0.90	35.0	12.4
	0.4	6.5	1.00	34.0	12.4

[a] The basal medium contained (% wt/vol) peptone, 0.4; $(NH_4)_2HPO_4$, 0.4; $NH_4H_2PO_4$, 0.2; $MgSO_4 \cdot 7H_2O$, 0.05; and KCl, 0.1.
[b] OD_{610}, Optical density at 610 nm.
[c] ND, Not detectable.

TABLE 2. Effect of carbohydrates on amylase formation[a]

Carbohydrate	% Concn (wt/vol)	Final pH	Growth (OD_{610})[b]	Sp act (unit/unit cell mass)	
				Liquefying	Saccharifying
Polysaccharides					
Dextrin	0.05	6.60	0.85	69.0	18.24
	0.10	6.60	0.95	65.3	17.79
	0.20	6.40	1.05	61.9	17.14
Glycogen	0.10	6.40	0.75	62.7	13.46
	0.20	6.20	0.90	57.2	12.44
	0.40	5.90	1.00	61.0	12.00
Starch (soluble)	0.10	6.90	0.85	78.8	20.59
	0.20	6.80	1.05	66.2	20.86
	0.30	6.80	1.15	67.8	21.30
Trisaccharides					
Raffinose	0.10	7.10	0.55	53.64	7.09
	0.20	6.90	0.75	44.67	14.00
	0.40	6.85	0.95	35.79	11.58
Disaccharides					
Cellobiose	0.10	6.85	0.65	46.62	9.08
	0.20	6.70	0.70	44.29	19.00
	0.40	6.60	0.80	41.88	18.12
Lactose	0.10	6.70	0.70	21.43	ND[c]
	0.20	6.60	2.90	20.56	ND
	0.40	6.60	1.00	20.00	ND
Maltose	0.10	6.80	0.60	17.50	ND
	0.20	6.70	0.65	18.30	ND
	0.40	6.60	0.75	17.33	ND
Sucrose	0.10	6.90	0.65	19.23	ND
	0.20	6.70	0.75	17.33	ND
	0.40	6.60	0.80	18.12	ND

[a] Basal medium (see Table 1 footnote) containing 0.3% starch was used as control for the above studies.
[b] OD_{610}, Optical density at 610 nm.
[c] ND, Not detectable.

measurement of growth in cuvettes (light path, 1 cm) at 610 nm in a Beckman DU-2 spectrophotometer.

Amylase assay. The amylase in the culture filtrate was assayed by the methods of Bernfeld (3). The enzyme was precipitated from the culture filtrate with prechilled acetone to study the pH stability, heat stability, and pH and temperature optima of the enzyme. The amylase assay in each case was done in triplicate. One unit of dextrinizing activity was expressed as the amount of enzyme required to decrease the starch-iodine color by 0.05 optical density unit at 610 nm per minute, and 1 unit of saccharifying activity was expressed as the amount of enzyme required to liberate 1 mg of maltose per min.

Amylase production in shaken flasks. Amylase production was measured in shaking flasks at various pHs and temperatures and the optima were the same as in stationary flasks, namely 50 ± 0.5°C and pH 6.9, respectively. Each autoclaved Erlenmeyer flask, containing 50 ml of the basal medium, was fitted in a reciprocal shaker at 50 ± 0.5°C, and at 6-h intervals the amylase activity in the filtrate and the growth of the bacterium were recorded.

Amylase production in various media. (i) Complex media. Various complex media containing different concentrations of beef extract, yeast extract, corn steep liquor, meat extract, peptone, and starch were used. The other constituents were as follows: $(NH_4)_2HPO_4$, 0.4%; KCl, 0.1%; $MgSO_4 \cdot 7H_2O$, 0.05%; and starch, 0.3%.

(ii) Semisynthetic and synthetic media. A semisynthetic medium containing 0.4% peptone, 0.4% $(NH_4)_2HPO_4$ 0.4% $NH_4H_2PO_4$, 0.1% KCl, 0.05% $MgSO_4 \cdot 7H_2O$, and 0.3% starch was found to be the best and was used in the present study. Growth and amylase production were lower in the synthetic than in the semisynthetic medium, but synthetic medium of the following composition gave highest activity of amylase: $(NH_4)_2HPO_4$, 0.4%; $NH_4H_2PO_4$, 0.4%; starch, 0.3%, $MgSO_4 \cdot 7H_2O$, 0.05%; KCl, 0.1%; isoleucine, 0.001%; cysteine, 0.001%; phenylalanine, 0.001; aspartic acid, 0.05%; and $CaCl_2 \cdot 2H_2O$, 0.001%.

Effect of carbon and nitrogen source on amylase production. The effect of different carbon sources was studied in the basal medium. Three concentrations (1.0, 2.0, and 4.0%) each of monosaccharides, disaccharides, trisaccharides, and polysaccharides were used. Except for polysaccharides, solutions of saccharides were sterilized by membrane filtration (pore size, 0.45 μm; diameter, 25 mm; Millipore Corp.), whereas polysaccharides were autoclaved. All these saccharides were added aseptically to the culture medium to give the desired concentration.

The effects of different inorganic nitrogen sources and concentrations were studied in the basal medium. The sources of inorganic nitrogen were $(NH_4)_2SO_4$, $(NH_4)_2HPO_4$, $NH_4H_2PO_4$, and NH_4NO_3. The organic nitrogen sources were amino acids and nucleotides.

Effect of sodium and potassium salts, detergents, and metal ions. Various organic salts of sodium and potassium were studied for their effects on the synthesis and secretion of extracellular amylases. The effect of a number of divalent cations such as Ca^{2+}, Sr^{2+}, Mg^{2+}, Mn^{2+}, Ba^{2+}, Zn^{2+}, and Cu^{2+} and trivalent cations such as Fe^{3+}, Sb^{3+}, Au^{3+} were studied. A control without the addition of extraneous metal ions was maintained for comparison.

RESULTS AND DISCUSSION

Amylase production in various media. A complex medium for maximal production of extracellular amylase by a thermophilic *B. stearothermophilus* strain was formulated. Among various complex media tried for good amylase yield, corn steep liquor was found to be the best, followed by meat extract and beef extract. Growth on yeast extract was comparable to growth on meat extract or beef extract, but amylase production was lower. Of the various concentrations of corn steep liquor and peptone tried, 0.4% of each was found to be optimal for amylase production, but in the absence of starch very little amylase was produced (Fig. 1). However, the growth of the bacterium in the presence or absence of starch was good (Fig. 2). When various complex media were used, the pH of the culture broth varied between 6.5 and 7.2 during the course of cultivation. Since corn steep liquor contains many chemical ingredients, it was difficult to ascertain which of them induced amylase production. It therefore became necessary to replace corn steep liquor with synthetic material of known chemical composition. In the semisynthetic medium, a prolonged lag phase was observed and growth as well as amylase production was comparatively lower (Fig. 3). In the present study, maximal production of amylase was noticed during the postexponential and stationary growth phase. In *B. subtilis* var. *amyloliquefaciens* 3053 (1), it has been reported that the composition and concentration of the media greatly affected the growth and synthesis of amylases. A number of carbon and nitrogen sources have been examined for amylase production in *B. subtilis* (6). As in the present study (Fig. 1), increasing the concentration of starch stimulated amylase formation in *Bacillus* sp. (16) and in *Aspergillus niger* (2). Diammonium hydrogen phosphate served as a good nitrogen and phosphate source in the present study. Shaking had little effect on growth and amylase production.

TABLE 3. Effect of organic nitrogen on amylase production[a]

Organic nitrogen source	Concn (mg/100 ml)	Final pH	Growth (OD_{610})[b]	Sp act (unit/unit cell mass)[c]
Amino acids				
Glycine	10	6.9	0.70	2.57
Glutamic acid	10	6.9	0.80	8.25
Methionine	10	6.8	0.80	2.62
Isoleucine	10	6.8	0.85	10.00
Cysteine	10	6.7	0.70	8.86
Phenylalanine	10	6.9	0.90	9.90
Aspartic acid	10	6.9	0.90	8.67
Nucleotides				
ATP	100	6.6	0.60	3.67
ADP	100	6.7	0.70	4.57
GTP	100	6.7	0.75	2.40
Guanine	100	6.9	0.55	2.18
Cytosine	100	6.9	0.65	ND[d]
Adenine	100	6.8	0.70	ND
Uracil	100	6.8	0.60	ND
Xanthine	100	6.7	0.70	2.57
NADP	100	6.9	0.75	4.27

[a] The basal medium contained $(NH_4)_2HPO_4$, 0.4%; $NH_4H_2PO_4$, 0.4%; starch, 0.3%; $MgSO_4 \cdot 7H_2O$, 0.05%; KCl, 0.1%. All other amino acids tried did not result amylase production. The above concentration is given after optimization.

[b] OD_{610}, Optical density at 610 nm.

[c] Saccharifying activity.

[d] ND, Not detectable.

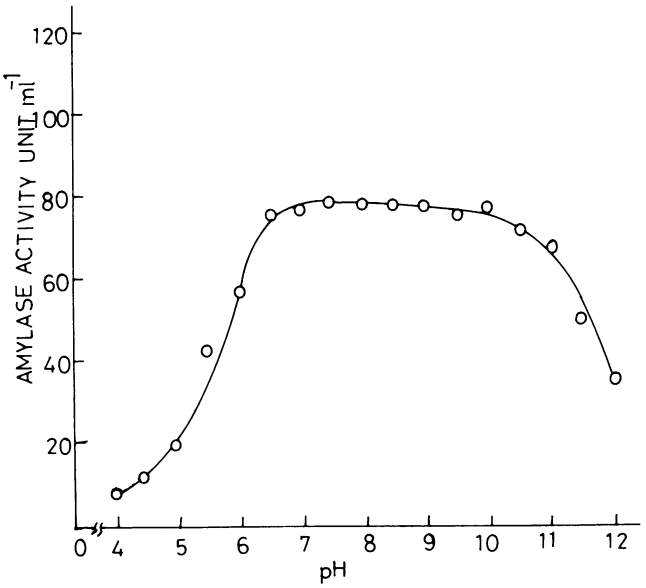

FIG. 4. pH stability of amylase at 4°C. The amylase solution was kept in buffers of different pHs for 24 h at 4°C, and then activity was measured under optimum conditions.

Effect of KCl and organic salts of sodium and potassium. Because growth and amylase production in the synthetic medium were comparatively lower, the semisynthetic medium was used to study the effects of various salts and detergents on amylase production. Addition of KCl (0.1%) to the semisynthetic medium increased production. The role of K^+ and Na^+ on the release of amylase was reported in *B. cereus* (14), yeasts (12), and *A. oryzae* (11). Of the various organic salts of sodium and potassium, potassium citrate, potassium succinate, potassium fumarate, sodium malate, sodium glutamate, and sodium aspartate were found to be better than the others.

The effect of detergents on the release of amylase into the extracellular fluid was studied in the semisynthetic medium in a similar way. In yeasts, addition of detergents increased extracellular amylase production owing to release of cell wall-bound amylase into the extracellular fluid (12). However, in the present studies, with semisynthetic medium containing Tween 80 and Triton X-100, growth was increased but amylase production was suppressed.

Effect of carbon and nitrogen sources. The synthesis of amylase was greatly suppressed when the bacterium was grown either on glucose, maltose, or sucrose (Table 1), but amylase production was good when the bacterium was grown on starch or other polysaccharides (Table 2). Cell

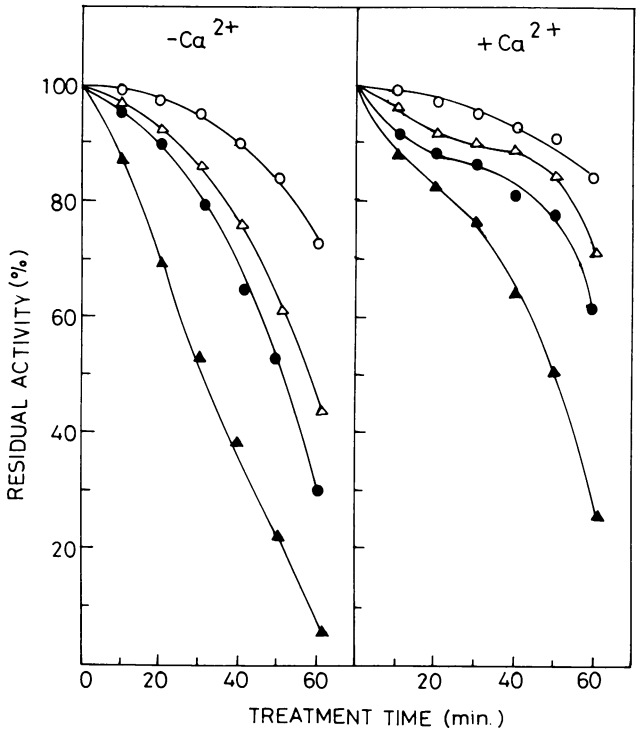

FIG. 5. Heat stability of amylase. The amylase solution was given heat treatment at different temperatures for various periods ranging from 0 to 60 min, and then the remaining activity was estimated by bringing the assay conditions to optimum. Symbols: ○, 60°C; △, 70°C; ●, 80°C; ▲, 90°C.

growth was not affected in the presence of monosaccharides. Ensley (7) reported that the slowest growth rate and amylase production occurred when fructose was the carbon source for *Clostridium acetobutylicum*. The level of growth resulting from the utilization of various carbohydrates did not affect formation of the amylase. The level of growth was comparable to that caused by glucose and maltose. However, amylase production was greatly reduced when these carbon sources were used. In the present study maltose repressed amylase production, but in *Pseudomonas burtonii* (7) maltose induced amylase production. Afunasyeva et al. (1) observed repression of amylase synthesis by glucose, maltose, sucrose, and α-methyl glucose in *Endomycopsis fibuligera;* this repression was reversed by cyclic AMP. Contrary to most of studies on amylase synthesis by bacteria, Tobey and Yosten (17) observed in *B. thuringiensis* that starch was not required for amylase production and that glucose or glycerol served as a good source. Other polysaccharides such as glycogen and dextrin also induced amylase production (Table 2). None of the oligosaccharides except maltotriose, maltotetraose, maltopentaose, and maltohexaose were found to induce amylase synthesis. Similar results have been obtained with *B. licheniformis* (13).

Of the four inorganic nitrogenous compounds used, diammonium hydrogen phosphate and ammonium dihydrogen phosphate were found to be better than ammonium sulfate and ammonium nitrate. For *B. cereus* BQ 10-S1 Spo (14), it was reported that the medium containing only ammonium sulfate or ammonium acetate did not produce amylase. Of the various amino acids examined, only seven (Table 3) had some effect on amylase production. Cysteine, phenylalanine, isoleucine, and aspartic acid were found to be indispensable for amylase production. Similar results have been obtained by other workers (5). Of the nucleotides, only ATP had a slight stimulatory effect on amylase production.

Of the divalent and trivalent cations tested for their effect on the synthesis or secretion, or both, of amylase, Ca^{2+}, Sr^{2+}, Mg^{2+}, and Ba^{2+} slightly induced amylase production, but Ag^{+}, Cu^{2+}, Fe^{3+}, Sb^{3+}, As^{3+}, and Au^{3+} inhibited amylase production. However, Zn^{2+} and Mn^{2+} had no effect on growth or amylase production (data not shown).

From the culture filtrate, amylase was precipitated out with prechilled acetone, and further studies on pH and temperature stability were done with this enzyme powder after ammonium sulfate fractionation (0.45 to 0.60 saturation). It was found that the amylase was fairly active at 80°C. The pH stability was studied at 4°C to avoid contamination by any airborne microorganisms. It was found that the amylase enzyme was stable over a wide range of pH (6 to 11) (Fig. 4).

Ca^{2+} (20 mM) was found to protect the enzyme from inactivation when exposed to various high temperatures (Fig. 5). Thus the present enzyme could be of better use in liquefying thick starch slurries at 80°C over a wide pH range.

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