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Production of Thermostable and Acidophilic Amylase from Thermophilic Bacillus licheniformis JAR-26

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Abstract

The present study has been conducted on 120 bacterial isolates collected from spoiled starchy materials like wheat flour and bread dough, soil and spoiled sour materials like tomatoes, citrus fruits and pears etc. These isolates were screened for the production of extracellular acidophilic and thermostable amylases. Five isolates exhibited good amylase activity. Preliminary identification of these isolates showed that they all belong to the genus Bacillus, so they were designated as Bacillus sp. JAR-4, JAR-26, JAR-29, JAR-53 and JAR-76. Isolate JAR-26 showing maximum starch hydrolysis was further subjected to biochemical testing and on the basis of results it was designated as Bacillus licheniformis JAR-26. It was able to grow up to 55 ^{0}C at pH 5.5 and showed remarkable liquefaction as well as saccharification activity. The optimum period for maximum cell growth and amylase production in case of Bacillus sp. JAR-26 was found to be 36 h at 45 ^{0}C. The optimum temperature for enzyme assay was 85 ^{0}C at pH 5.5. The enzyme was also thermostable as 100% activity was observed at 85 ^{0}C and 55% activity retained at 100 ^{0}C and 48% activity at 104 ^{0}C when heated for 30 min.

Key words: Acidophilic amylase; Bacillus sp.; Liquefaction; Thermostable; Saccharification

INTRODUCTION

Amylases are extracellular enzymes that hydrolyze starch into different products like dextrin's, small polymers of glucose (maltooligosaccharides), maltose and glucose. A number of commercial uses have been established for amylase. Gelatinization and liquefaction of the starch are the key processes involved in the manufacture of dextrose and syrups from native starch [1]. Liquefaction of the starch at high temperature using heat stable amylases has definite advantages over the other methods of thinning starch. Use of heat stable amylase can reduce the retro gradation of starch. It also prevents the formation of undesirable byproducts inherent to acid thinning process.

The manufacture of sugars from starch is a multistage process, which involves different microbial enzymes in successive steps. The variation of various parameters in starch conversion process causes many handicaps in the starch industry. Due to pH variation large amounts of salts have to be removed by ion-exchangers. If the operating pH of the amylase used at liquefaction step can be lowered, then chemical addition and cost for ion exchangers are significantly reduced [2]. Therefore, discovery of an enzyme that is active at high temperature and low pH values (4.5-5.0) will significantly lower the cost of sugar syrup production.

The thermostable amylases find wide applications in a number of industrial processes e.g. in starch- glucose, textile, pharmaceutical and brewing industries [3, 4]. Amylases used commercially for starch processing are thermostable but recently the interest has been focused on thermophilic amylase capable of working at low pH range of 4.5-5.5 [5]. Antrim et al. [6] has reported a thermostable α-amylase with activity at pH 5.5. Uguru et al. [7] have isolated a strain of Thermoactinomyces thalpophilus which produced an extracellular amylase with optimum temperature of 90 ^{0}C and pH of 5.0. Kathiresan and Manivannan [8] studied the strain of Penicillium fellutanum that exhibited amylase activity of 94 U/mL, at pH 6.5 and 30°C but none of them has been exploited on industrial scale so far. The present communication deals with the production of thermostable amylase capable of working at low pH range. Relationship between growth and amylase production, amylase production and thermostable characteristics of extracellular amylase has been studied and nature of amylase has also been briefly discussed.

MATERIALS AND METHODS

Isolation of amylase producing bacteria

A total of 120 starch hydrolyzing bacteria were isolated from soil and other spoiled materials like potatoes, wheat flour, bread dough and sour materials such as spoiled tomatoes, citrus fruits, pears etc. and screened for extracellular acidophilic amylase production. The starch medium [9] used for the isolation of bacteria contained (g/L): Starch, 10.0; yeast extract, 5.0; peptone, 2.0; MgSO4.7H2O, 0.5; KH_2PO_4, 0.5; NaCl, 1.5; CaCl2, 0.1; Agar, 20.0. Initial pH was adjusted to 5.5. One gram of each sample was suspended in 9.0 mL of sterile water and 0.1 mL of suitably diluted suspension was spread on the agar plates. The plates were incubated at 45^{0}C, 50^{0}C, 55^{0}C and 60^{0}C for 24 to 48 h. The isolated colonies were flooded with iodine solution. Colonies with good colourless halos around them were picked and maintained on agar slants at 4^{0}C and further assessed for

enzyme production in liquid medium.

Amylase production in liquid medium

Bacterial isolates selected during preliminary screening were inoculated in production medium [9] containing (g/L). Starch, 10.0; yeast extract, 5.0; peptone, 5.0; KH_2PO_4, 0.12; CaC12.2H20, 0.12; MgSO4.7H20, 0.12; MnSO4.4H20, 0.02. Initial pH of the medium was adjusted at 5.5 and 50 mL of medium in 250 mL of Erlenmeyer flasks were inoculated with a cell suspension of optical density 0.5 (prepared from 24 h old culture). All the flasks were grown for four days on a rotary shaker (Remi) at 170 rpm at 45 ^{0}C. Samples were drawn after a time interval of 12 h and centrifuged at 8000 rpm for 10 minutes. Supernatant was used for assay of enzyme activity.

Assay of amylases

Culture filtrates (supernatant) of different isolates were assayed for both liquefying and saccharifying activity. For assaying liquefying activity the method of Srivastava et al. [10] was followed. One mL of 1% (w/v) starch (Merck) solution was taken in test tube and 0.2 mL of 0.2 M phosphate buffer (pH 5.5) and 0.2 mL of deionized water was added to it. The mixture was equilibrated at 70 ^{0}C for 10 minutes in a water bath. 0.1 mL of crude enzyme (supernatant) was added and then reaction was stopped by adding 1 mL of 1M HC1. Addition of 0.01 mL of iodine solution (0.2% iodine in 2% KI solution) to the reaction mixture developed blue color. The intensity of blue color was measured at 600 nm. One unit of liquefying activity was defined as the amount of amylase that reduces the starch iodine color by 0.05 optical density at 600 nm. For assaying saccharifying activity the reaction procedure was same as above except that the reaction was stopped by adding 1.0 mL of 3, 5-dinitrosalicylic acid (DNS). The mixture was heated for 10 min and the color intensity was measured at 540 nm [11] using a spectrophotometer (Systronics Spectrophotometer 169). One unit of saccharifying amylase activity was defined as the amount of amylase that liberates 1.0 mg of glucose per minute under experimental conditions.

Characterization of the isolates

Five bacterial isolates that showed good thermophilic and acidophilic amylase production were identified based on their characteristics as mentioned in Bergey's Manual of Systematic Bacteriology [12]. The methods of identification were same as given by Collee et al. [13.

Determination of optimum pH, optimum temperature of activity and thermostability of the enzyme

For the determination of the optimum pH of enzyme activity the enzyme- substrate mixture was incubated at different pH values in the range 4.0 to 10.0 for 10 minutes at 70 ^{0}C and then enzyme was assayed for the amylase activity by the same method as described in assay of amylases.

To determine the optimum temperature of activity of amylase the enzyme substrate mixture with appropriate buffer was incubated at different reaction temperatures (40-90 ^{0}C) for 10 minutes and then amylase activity was

TABLE 1. Effect of temperature on growth and starch hydrolysis by different isolates in starch agar medium.

BACTERIAL ISOLATES	CELL GROWTH AND STARCH HYDROLYSIS AT DIFFERENT TEMPERATURE (^{0}C)									
	37^0		45^0		50^0		55^0		60^0	
	G	SH	G	SH	G	SH	G	SH	G	SH
Bacillus sp. JAR-4	+	+ + +	+	+ + +	-	-	-	-	-	-
Bacillus sp. JAR-26	+	+ + +	+	+ + + + +	+	+ + + +	+	+ + +	-	-
Bacillus sp. JAR-29	+	+ + +	+	+ + +	-	-	-	-	-	-
Bacillus sp. JAR-53	+	+ + +	+	+ + +	-	-	-	-	-	-
Bacillus sp. JAR-76	+	+ + +	+	+ + + +	-	-	-	-	-	-

G = Growth, **SH**= Starch hydrolysis

- indicates no growth of bacteria in starch agar medium

+ indicates the growth of bacteria in starch agar medium

Starch hydrolysis in terms of zone size (mm):

+ + + indicates moderate starch hydrolysis (10-12 mm)

+ + + + indicates very good starch hydrolysis (14 mm)

+ + + + + indicates excellent starch hydrolysis (18 mm)

TABLE 2. Amylase production in terms of both Liquefaction and Saccharification activity by five selected bacterial isolates after 36 h of incubation at 45 ^{0}C.

BACTERIAL ISOLATES	LIQUEFACTION ACTIVITY (U/ml)	SACCHARIFICATION ACTIVITY (U/ml/min)
Bacillus sp. JAR-4	264.2	0.936
Bacillus sp. JAR-26	337.2	2.820
Bacillus sp. JAR-29	232.8	0.884
Bacillus sp. JAR-53	303.8	0.860
Bacillus sp. JAR-76	233.0	1.826

assayed (as described in assay of amylases).

The thermostability of the enzyme produced by Bacillus sp. JAR-26 was studied by the method reported by Srivastava et al. [10]. The supernatant was kept at various temperatures ranging from 70 ^{0}C to 110 ^{0}C in the absence of the substrate and at different time intervals enzyme (0.1 mL) was withdrawn and amylase activity was estimated following the procedure mentioned above.

RESULTS AND DISCUSSION

One hundred twenty microorganisms were isolated from soil, spoiled starchy materials like potato, wheat dough, spoiled and sour material like tomato and pear etc and screened for the production of extracellular acidophilic and thermostable amylases. Preliminary screening of the isolates leads to the selection of five bacterial isolates. These five isolates showed good growth and starch hydrolysis on starch agar medium at 45 ^{0}C (Table 1). Preliminary identification of these isolates showed that all these five isolates belong to the genus Bacillus so they were designated as Bacillus sp. JAR-4, JAR-26, JAR-29, JAR-53 and JAR-76. In comparison to other isolates better growth and starch hydrolysis was observed in case of Bacillus sp. JAR-26 and JAR-76. Bacillus sp. JAR-4, JAR-29 and JAR-76 did not grow at temperature 50 ^{0}C and above whereas Bacillus sp. JAR-26 could also grow at 50 and 55 ^{0}C and caused starch hydrolysis. When these isolates were further assessed for amylase activity in liquid production medium the highest liquefying activities of 337.2 and 303.8 units/mL were observed in culture filtrate of Bacillus sp. JAR-26 followed by Bacillus sp. JAR-53. Culture filtrates of Bacillus sp. JAR-26 also showed highest saccharolytic activity followed by Bacillus sp. JAR-76 (Table 2).

The relationship between growth of different isolates and amylase production by them was studied at different time intervals of incubation period when cultivated in liquid production medium at 45 ^{0}C and pH 5.5. Among all the isolates best growth and amylase production was observed in case of Bacillus sp. JAR-26 at 36 h of growth. Growth in terms of optical density (O.D.) at 600 nm was 2.129 and amylase production was 2.82 units/mL. Further increase in incubation period resulted in decreased enzyme production (2.426 units/mL), however, growth stayed almost constant up to 48 h. Bacillus sp. JAR-76 was the second best isolate for amylase production after 36 hr of growth. In case of Bacillus sp. JAR-4, JAR-29 and JAR-53, maximum enzyme production occurred at 48 h of growth. Amylase yields of 1.962, 1.454 and 1.591 units/mL, respectively, were obtained in these cases (Table 3). Maximum biomass production was also observed at this time interval but yields were lower than those obtained in case of Bacillus sp. JAR-26 and JAR-76. The results of the present study are partially or fully in agreement with those where maximum amylase production has been reported to occur after 24 to 48 h of incubation period. Maximum amylase production was reported in case of Bacillus sp. 64 and Bacillus sp. IMD after 24 h of growth [14, 15]. Similarly in case of Bacillus licheniformis, Bacillus polymyxa and Bacillus subtilis [16-18], maximum amylase production has been found during exponential phase or early stationary phase.

As Bacillus sp. JAR-26 showed the maximum liquefaction activity as well as saccharification activity, so further biochemical and morphological tests of this isolate were performed (Table 4). When all these characteristics were compared to the characteristics of different Bacillus species as given in Bergey's Manual of Systematic Bacteriology [12], it could be concluded that isolate JAR-26 belongs to genus Bacillus and species licheniformis. Hence, the isolate was designated as Bacillus licheniformis JAR-26.

Optimum pH for maximum activity of enzyme was observed 5.5. At pH 6.5 the enzyme activity decreased to about 90 % of the maximum. Likewise, when the pH of the reaction mixture was adjusted at 5.0 and 4.0 the activity observed were 95 and 71 % of the peak activity, respectively (Fig. 1). The amylase produced by Bacillus licheniformis JAR-26 was found to be maximally active

TABLE 3: Effect of incubation period on cell growth and enzyme production by five bacterial isolates at 45 0C and pH 5.5.

BACTERIAL ISOLATES	CELL GROWTH AND AMYLASE PRODUCTION AT DIFFERENT INCUBATION PERIOD														
	24 h			36 h			48 h			72 h			96 h		
	G (OD)	S.A. U/ml	L.A. U/ml	G (OD)	S.A. U/ml	L.A. U/ml	G (OD)	S.A. U/ml	L.A. U/ml	G (OD)	S.A. U/ml	L.A. U/ml	G (OD)	S.A. U/ml	L.A. U/ml
Bacillus sp. JAR-4	1.156	0.812	22.91	1.424	0.936	26.71	1.629	1.962	32.14	1.308	1.248	18.9	0.924	0.801	15.91
Bacillus sp. JAR-26	1.274	1.793	26.22	1.348	2.820	39.84	1.310	2.426	35.19	1.287	1.858	21.42	1.260	0.894	18.73
Bacillus sp. JAR-29	1.241	0.528	19.94	1.312	0.884	27.92	1.523	1.454	24.12	1.312	1.203	16.61	0.918	0.420	11.24
Bacillus sp. JAR-53	0.927	0.720	18.11	1.146	0.860	20.43	1.208	1.591	24.42	1.104	0.817	16.70	0.978	0.603	11.12
Bacillus sp. JAR-76	1.242	0.942	20.64	1.357	1.826	28.17	1.421	1.428	24.71	1.301	0.862	18.41	0.876	0.712	15.62

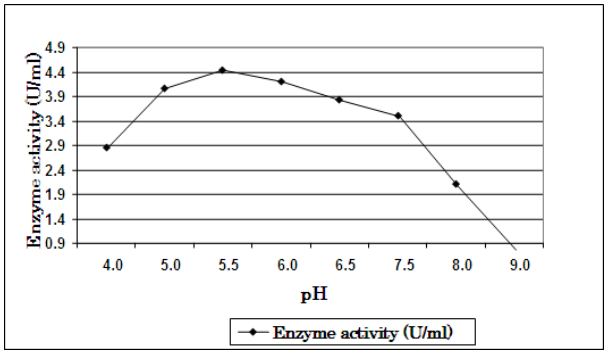
G = growth at 600 nm, OD= optical density, S.A.= saccharification activity, L.A.= liquefaction activity

Table 4: Morphological and biochemical characteristics of isolate JAR-26.

S.No.	CHARACTERISTICS	RESULTS
A	**Morphological Characteristics**	
1.	Gram's staining	Gram positive rods measuring 0.5-0.8 x 3-5 µm in size
2.	Spore	Spore oval, central and not bulging the mother cell
3.	Motility	Negative
B	**Biochemical characteristics**	
4.	Gas from glucose	Negative
5.	Acid from	
(a)	Glucose	Positive
(b)	Maltose	Positive
(c)	Sucrose	Positive
(d)	Lactose	Negative
(e)	Mannitol	Positive
(f)	Xylose	Negative
(g)	Arabinose	Negative
6.	Hydrolysis of	
(a)	Starch	Positive
(b)	Gelatin	Positive
7.	Catalase	Positive
8.	Voges Proskauer	Positive
9.	pH in V.P. broth	6.8
10.	Growth (in nutrient broth) at pH	
	6.8	Positive
	5.7	Positive
11.	Growth in NaCl	
	2%	Positive
	5%	Positive
	7%	Positive
	10%	Negative
12.	Growth at	
	30 ^{0}C	Positive
	40 ^{0}C	Positive
	50 ^{0}C	Positive
	55 ^{0}C	Positive
	60 ^{0}C	Negative

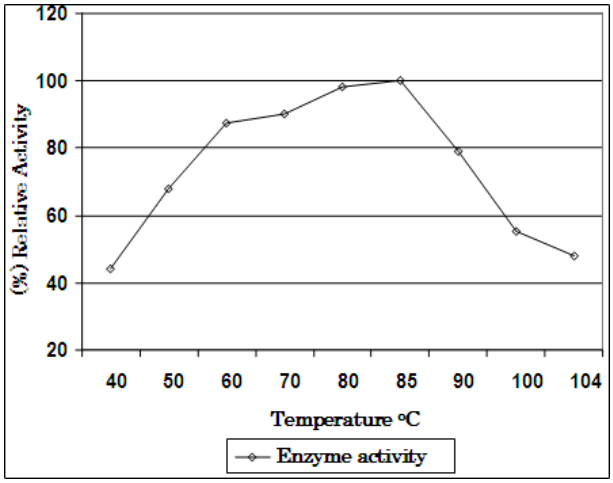
at 85 ^{0}C where the enzyme was found to be 100 % active. However, at 80 ^{0}C the enzyme showed good activity of 98 % which was decreased to about 79 % at 90 ^{0}C. The activity further decreased to about 55 % at 100 ^{0}C (Fig. 2). Out of the two α-amylases used commercially on industrial scale from Bacillus sp., the α-amylase from Bacillus amyloliquefaciens has an optimum temperatures of activity around 65-70^{0}C and other enzyme from Bacillus licheniformis was optimally active at temperature 90 ^{0}C. But both enzymes had optimum pH of activity in the range of 6.5-7.0 [19, 20]. The α-amylase secreted by Bacillus licheniformis JAR-26 the organism of the present study was optimally active at 85 ^{0}C and it had appreciable good activity at 90 ^{0}C (79 %). But the characteristic feature of this amylase is that it is active at low pH of 5.5 and even at 5.0 (Fig. 1).

Figure 1: Effect of different pH on amylase activity by Bacillus licheniformis JAR-26 at temperature 45 ^{0}C with incubation period of 36 h.



When the thermostability was checked, it was found that the amylase synthesized by Bacillus licheniformis JAR-26 could retain 100 % activity when heated at 85 0C for 30 minutes (Fig. 3). However, at 90 0C the residual amylase activity detected was about 88%. The activity further decreased to about 55 % and 48 %, respectively, when enzyme solution was heated at 100 and 104 0C. Earlier different strains of Bacillus licheniformis have been reported to produce α-amylases. A thermostable α-amylase was reported by Madsen et al. [21] from Bacillus licheniformis with optimum activity at 90 0C and optimum pH in the range of 6.0-7.0. It required 5 ppm Ca2+ ions for its activity and is currently used for starch liquefaction on industrial scale [2]. A similar type of α-amylase has been reported by Morgan and Priest [19] from Bacillus licheniformis NCIB 6346 with optimum pH for activity in the range 7.0-9.0. Another α-amylase from Bacillus licheniformis M27 with optimum temperature of activity 90 0C has been reported by Padmanabhan et al. [22]. This enzyme shows peak for pH optima at 6.5-7.0 and 8.5-9.0. As in starch industry large amount of salts have to be removed by ion exchangers in second step because of unavailability of thermostable amylases capable of working at low pH. This study indicates that Bacillus licheniformis JAR-26 could grow at elevated temperature of 55 ^{0}C and it produces thermostable and acidophilic amylase, which can find use in starch saccharification. Hence, this isolate was selected for further study on optimization of parameters, characterization and purification of enzyme.

Figure 2: Effect of temperature on activity of amylase from Bacillus licheniformis JAR-26 at pH 5.5 with incubation period of 36 h.



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