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Characterization of Alkaliphilic, Surfactant Stable and Raw Starch Digesting A-Amylase from *Bacillus subtilis* Strain JS-16

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Abstract

An alkali-thermophilic and surfactant stable α-amylase was obtained from *Bacillus subtilis* strain JS-16, isolated from sludge samples of a soda ash industry, located on west coast of Gujarat. It was active over a wide temperature range (30°C-80°C) exhibiting optimum activity at 50°C and pH 9.0. Unique feature of this amylase was two-fold enhancement of activity in SDS. Three-step purification yielded 15.16-fold purified enzyme with specific activity of 13.5 U/mg proteins. K_m and V_{max} were 10 mg/ml and 0.2 µmol/min/ml (11.56 µmol/ min/mg protein) respectively. Enzyme activity enhanced with Fe^{3+} but was strongly inhibited by Hg^{2+} ions. The amylase hydrolyzed 12% raw wheat starch and 5% corn starch granules after 12 h incubation. Surfactant stability, alkaliphilic nature, activity under wide temperature range and hydrolyses of raw starch makes this amylase a promising candidate for liquid detergent and starch industry.

Keywords: Alkaline amylase; Alkaliphilic; *Bacillus subtilis*

Introduction

α-Amylases (E.C. 3.2.1.1) produced by plants, animals and microbes, hydrolyze α-1-4 glycosidic linkages in starch to dextrin, maltotriose, maltose and glucose. Amylases have been extensively used in food, fermentation, textile and paper industries with 30% production worldwide and 25% share in the enzyme market. Microbial amylases are preferred for industrial production considering the economics, faster production, wide range of operation parameters and minimum space requirements [1-4]. Demand for extremozymes with a blend of unique catalytic properties for specific biochemical processes is ever increasing. Extremozymes from alkaliphiles have found their applications in tanneries, paper and pulp, laundry, detergents and waste water treatment *Bacillus* sp. (*B. subtilis*, *B. stearothermophilus*, *B. licheniformis* and *B. amyloliquefaciens*), a dominant and omnipresent representative of Phylum Firmicutes has been widely used source of microbial amylase [5,6]. Alkaline amylases are best suited as detergent additives and for starch saccharification in food and textile industries [7].

Present study deals with production of alkaline amylase by an alkaliphilic *Bacillus subtilis* strain JS-16 isolated from a soda ash industry located in west coast of Gujarat, India. Further, it was purified and characterized for its unique properties that make it a potential biochemical catalyst for various industries.

Materials and Methods

Isolation and screening of amylolytic bacteria

The bacterium, *Bacillus subtilis* JS-16, reported in current study was isolated from sodic sludge (pH 9.24) sampled from west coast of Gujarat (21.8619 N 72.2775 E). Modified Horikoshi I medium (in g/l; glucose 10.0, peptone 5.0, yeast extract 5.0, K_2HPO_4 1.0, $MgSO_4.7H_2O$ 0.2, agar 20.0, pH adjusted to 9.0 with sterilized saturated NaOH solution) was used as an alkaline basal medium for isolation and storage. Purified colonies were streaked on M9 medium containing soluble starch (in g/l; soluble starch 5.0, Yeast extract 2.0, NaCl 2.5, NH_4Cl 5.0, KH_2PO_4 15.0, Na_2HPO_4 30.0, $MgSO_4$ 0.25, agar 20.0, final pH adjusted to 9.0). After incubation at 37°C for 96 h, plates were flooded with Lugol's Iodine. Based on zone of clearance, strain JS-16 was selected for further study.

Bacterial identification and phylogenetic analysis

Morphological, physiological and biochemical characteristics of JS-16 was studied according to Bergey's Manual of Determinative Bacteriology [8]. Fatty Acid Methyl Ester (FAME) analysis was performed (MIDI Sherlock Microbial Identification System). For molecular identification genomic DNA was extracted by standard chloroform-isoamyl alcohol method [9]. PCR amplification of 16S rRNA was performed using forward and reverse primers as 8f (fD1) 5′-AGA GTT TGA TCC TGG CTC AG-3′ and 1495r (rP2) 5′-ACG GCT ACC TTG TTA CGA CTT -3′ respectively [10]. Reaction mixture for PCR amplification contained 10X PCR buffer 5 µl, 200 mM dNTPs 5 µl, 2.5 U Taq DNA polymerase, 20 pM of each primers (Sigma, India) and 50 ng of bulk DNA. Amplification was performed in a thermal cycler (Bio-Rad MyCycler, Thermal cycler, California, USA) for an initial denaturation at 94°C for 4 min followed by 35 cycles of 94°C for 1 min, 58°C for 1 min and 72°C for 2 min and a final extension at 72°C for 5 min. Purified PCR product was sequenced for BLAST analysis [11]. 16S rRNA gene sequence was deposited in GenBank with accession number GQ280086. Phylogenetic analysis was done by MEGA 4.1 [12] software and tree was constructed using neighbor-joining method [13].

Amylase production

Culture was inoculated to 500 ml broth of M9 medium containing soluble starch and incubated at 37°C for 96 h. Culture supernatant after centrifugation (8000X g for 10 min) was used as crude enzyme.

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Effect of carbon sources

Amylase production was carried out using soluble starch, maltose, dextrin and sugar cane bagasse. M9 medium supplemented with 0.5% of the above mentioned carbon source was inoculated with the culture and incubated at 37°C for 96 h.

Amylase assay

Amylase activity was determined at 50°C for 15 min in 20 mM Tris-HCl buffer (pH-9.0) by measuring the release of reducing sugar from starch using Dinitrosalicylic acid (DNS) method [14]. In blank, enzyme was added after addition of DNS reagent. The absorbance was taken at 540 nm. One unit of amylase activity was defined as the amount of enzyme that produced 1 µmol of maltose equivalent per minute under specified conditions.

Protein estimation

Protein concentration was determined by Folin phenol method [15] with bovine serum albumin as standard. Protein content of the chromatographic fractions was measured at 280 nm.

Purification of Amylase

Ammonium sulfate precipitation and dialysis: Crude amylase was precipitated with 70% saturation of ammonium sulfate and kept overnight at 4°C. Precipitate was centrifuged and dissolved in minimum volume of 20 mM Tris-HCl buffer (pH 9.0). Dialysis was done using dialysis tubing (Sigma, D-0655).

Anion exchange chromatography: Prior to equilibration, the DEAE-cellulose (SRL, Mumbai, India) was activated by suspending in 0.5 M HCl, degassed for 20 min, and washed with distilled water till it was acid free followed by treatment with 0.5 M NaOH and finally washed with distilled water. This process was repeated thrice to activate the support. The support is loaded on the column (2 cm×18 cm) and was equilibrated with 20 mM Tris- HCl buffer of pH 9.0. The ammonium sulfate precipitated and dialyzed enzyme solution was loaded on the column and eluted with a stepwise concentration gradient of sodium chloride (0, 0.25, 0.5, 0.75 and 1 M NaCl) in the same buffer. The fractions, each 3 ml, were collected at a flow rate of 0.6 ml/min. Protein concentration and amylase activity of each fraction was determined. Active fractions were pooled.

Size exclusion chromatography: Pooled fraction (5 ml) from ion exchange chromatography was loaded to a Sephadex-G-100 (GE Healthcare, Uppsala, Sweden) column (1.25 cm×25 cm) pre-equilibrated with 20 mM of Tris-HCl buffer (pH 9.0) and then eluted with the same buffer. Fractions of 2 ml each were collected at a flow rate of 19 ml/h. Protein concentration and amylase activity of each fraction was determined. Active fractions were pooled, concentrated by lyophilization and checked for purity by SDS-PAGE.

SDS-PAGE and zymogram analysis

SDS-PAGE (10%) [16] was run for determining molecular mass of the purified enzyme. Standard molecular mass markers (Genei, Bangalore, India) used were, phosphorylase b (97.4 kDa), bovine serum albumin (66 kDa), ovalbumin (43 kDa), carbonic anhydrase (29 kDa) and lactoglobulin (18.4 kDa). Silver staining was done to view the protein bands [9].

Zymogram analysis was done by running native PAGE (10%). A 3% agarose plate was prepared into which 0.5% soluble starch was incorporated. After electrophoresis, the native PAGE gel was laid over the agarose gel and incubated at 40°C for 3 h. The agarose gel after incubation was overlaid with Lugol's Iodine solution to visualize the band.

Effect of pH and temperature on amylase activity

Optimum pH for amylase activity was determined at 50°C for 15 min using different buffers viz. citrate buffer (6.0), Mc Levine buffer (7.0), Tris- HCl buffer (8.0-9.0) and glycine-NaOH buffer (10.0-11.0). Stability of the purified enzyme with respect to varying pH (6.0-10.0) was determined by incubating in respective buffer for 30 min.

The optimum temperature for amylase activity was determined by incubating the assay system from 30°C to 80°C. Stability of the purified enzyme with respect to temperature was also determined by incubating the enzyme from 30°C to 80°C at pH 9.0 for 30 min.

Effect of additives and surfactants

Amylase activity was determined in presence of different cations (Ca^{2+}, Co^{2+}, Hg^{2+}, Mn^{2+}, Fe^{2+}, Mg^{2+}, Cu^{2+}, Cd^{2+}, Zn^{2+} and K^{+}) at 5 mM concentration. Effect of additives like β Mercaptoethanol, EDTA and PMSF (5 mM), SDS, Triton X-100, Tween-80, commercial detergents (Surf Excel and Tide) {1% (w/v)} was checked by pre-incubating the enzyme for 30 min.

Kinetic studies

The kinetic properties of amylase were determined using varying concentration of soluble starch. K_m and V_{max} values were calculated by Lineweaver Burk double reciprocal plot.

Raw starch hydrolysis by crude amylase

Raw corn, wheat and potato starch granules (10 mg) were separately mixed with 5 ml of the crude enzyme and final volume made up to 10 ml with 20 mM Tris-HCl buffer (pH 9.0). The reactions were incubated at 50°C in a shaking (100 rpm) water bath. Sample aliquots were collected after 4, 8, 12, 16, 20 and 24 h for the estimation of reducing sugars [14]. Degree of hydrolysis of raw starch (Rh) was defined by the formula: Rh (%)=(A1/A0)×100, where A1 was - amount of sugar in the supernatant after hydrolysis and A0 was the initial amount of raw starch [17].

Results and Discussion

Bacterial identification

Strain JS-16 was found to be Gram-positive, motile, rod shaped, exopolysaccharide producer (Figure 1A). The biochemical characteristics of JS-16 are given in Table 1. Isolate JS-16 produced six alkaliphilic extracellular enzymes namely, amylase (Figure 1B), caseinase, pectinase, lipase, xylanase and gelatinase. It was strongly inhibited by Chloramphenicol, Ciprofloxacin and Erythromycin. JS-

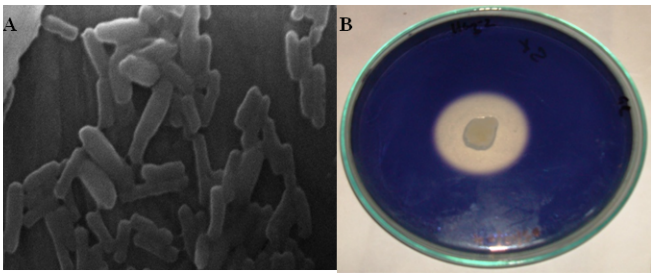

Figure 1: (A) SEM image of JS-16 (B) Plate assay showing a zone of clearance.

Tests	Results
Colour, size	Creamy round medium sized
Gram reaction	Gram positive
Motility	+
Catalase	-
Oxidase	+
Indole	-
MR	-
VP	-
Simmons citrate	-
Amylase	+
Pectinase	+
Gelatinase	+
Lipase	+
Caesinase	+
Phenylalanine	-
Urease	-
H2S production	-
Nitrate reduction	-
Arginine	+
Lysine	-
Ornithine	+
Dextrose	-
Fructose	+/-
Galactose	+/-
Lactose	-
Maltose	+/-
Mannose	+
Sucrose	+
Xylose	+

Table 1: Morphological and biochemical characteristics of *Bacillus subtilis* JS-16.

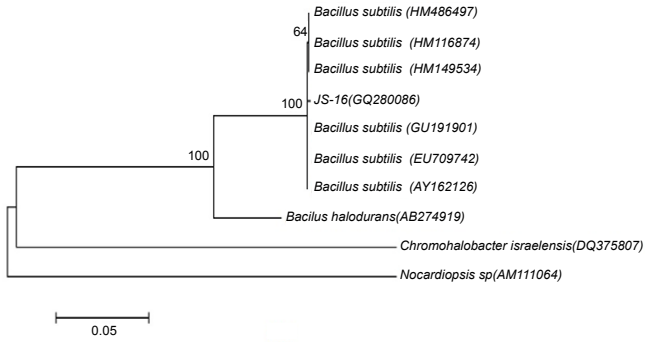

Figure 2: Phylogenetic tree showing taxonomic position of JS-16. Figures within brackets represent GenBank accession numbers.

16 could tolerate broad pH range (7.0-12.0) with optimum growth at pH 9.0 and 5% salt concentration. FAME analysis of JS-16 showed a Similarity Index (SI) of 0.75 with *Bacillus subtilis* accounted from -RTSBA library (Sherlock software). BLAST search of 16S rRNA gene sequence (1424 bp) showed 99% homology with that of *Bacillus subtilis* strain W1. The phylogenetic tree (Figure 2) shows taxonomic position of isolate JS-16.

Amylase production and purification

Soluble starch was the best carbon source for amylase production followed by dextrin and maltose (Figure 3). Sugar cane bagasse was not a good inducer. *Bacillus* sp. strain TSCVKK produced optimal amylase with 1% dextrin [18]. The three-step purification yielded 15.16 fold purification of amylase with 4.13% yield and specific activity of 13.5 Units/mg of protein (Table 2). 1.7 fold purification and 74% yield was reported from *Bacillus subtilis* WB600 recombinant amylase [19].

SDS-PAGE and zymogram analysis

The purification homogeneity assayed from native page revealed two bands of equal intensities corresponding to molecular mass of about 99 and 87 kDa. Two zones of clearance in zymogram supported the trends obtained in native PAGE. Correspondingly, four bands of molecular mass of about 35, 42, 48 and 58 kDa were obtained in the SDS-PAGE (Figure 4). Two bands with amylase activity in zymogram might be a possible explanation for the hetero-dimeric forms. *Bacillus* sp. A 3-15 amylase also reported two forms with molecular mass of 86 and 60.5 kDa after partial purification [20]. Similar results were

Enzyme	Total vol (ml)	Total Protein (mg)	Total activity (units)	Specific activity Units/ mg protein	Fold purification	%Yield
Crude	500	620	540	0.89	1	100
Ammonium sulfate precipitation	45	61.2	81	1.33	1.5	15.05
DEAE column chromatography	27	9.45	36	3.85	4.33	6.75
Gel permeation	10	1.73	23.3	13.5	15.16	4.31

Table 2: Purification of amylase isolated from *Bacillus subtilis* JS 16.

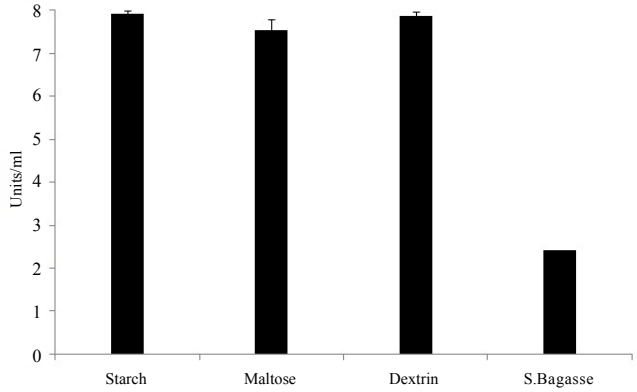

Figure 3: Effect of different carbon sources on amylase production where S Bagasse is Sugarcane Bagasse.

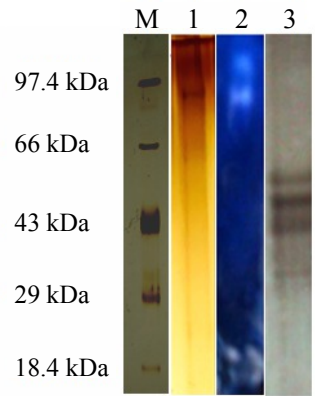

Figure 4: Native and SDS-PAGE of purified amylase where M denotes molecular markers Lane 1 native PAGE of purified amylase 2 Zymogram of the purified amylase 3 SDS-PAGE of purified amylase.

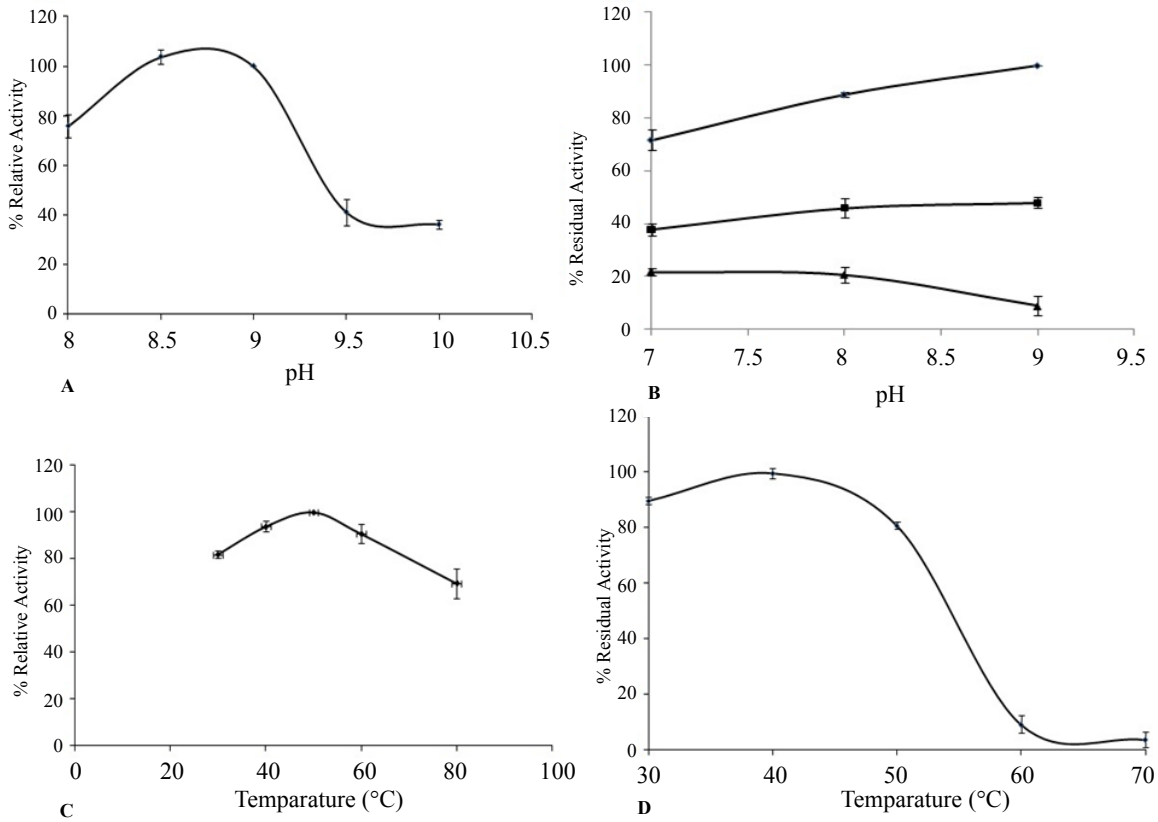

Figure 5: (A) Effect of pH on amylase activity (B) Effect of pH on amylase stability. (Solid diamond) denotes activity at 0 min; (Solid square) denotes activity after 30 min incubation; (Solid triangle) denotes activity after 60 min incubation. (C) Effect of temperature on amylase activity (D) Thermostability of amylase after preincubating for 30 min.

also reported from halophilic bacterial species Chromohalobacter sp. TVSP101 [21].

Effect of pH and temperature on amylase activity

Bacillus subtilis strain JS-16 amylase exhibited good activity from pH 8.0 (76.0% relative activity) to 9.0 with optimum at pH 9.0 (Figure 5A). An optimum pH of 6.0 was reported by amylase from *Bacillus subtilis* AX20 [22]. After 30 min incubation at pH 9.0, approximately 48% residual activity was observed, which was drastically reduced after 60 min incubation (Figure 5B).

JS-16 amylase exhibited good activity from 20°C-80°C with optimum at 50°C. Moreover, 70% relative activity was seen at 80°C (Figure 5C). Thermo stability test showed that about 80% residual activity was observed at 50°C which was drastically decreased at 60°C (Figure 5D). This trend is in accordance to that of *Nesterenkonia* sp. strain F amylase [23].

Effect of additives and surfactants on amylase activity

Fe^{3+} ions enhanced amylase activity while Hg^{2+} strongly inhibited it. 55% residual activity was seen with Ca^{2+} ions (Figure 6A). JS-16 amylase was Ca^{2+} ions independent similar to that from *Bacillus subtilis* AX20 [22] and in contrast to *Bacillus* sp. strain TSCVKK amylase [18]. Considerable activity was observed with PMSF and β-mercaptoethanol (Figure 6B). Enhanced activity in PMSF was contradictory to a thermostable alkaline α-amylase from *Bacillus* sp. A 3-15 that was inhibited by 3 mM PMSF [20]. EDTA did not affect amylase activity indicating it as metal independent. SDS enhanced amylase activity by two-fold. In Triton X-100 and Tween 80, residual activity of 62% and 72% respectively was retained. *Nesterenkonia* sp. strain F amylase retained 90% activity on incubation with 0.1-0.5% SDS [23]. Similarly 82% and 80% activity was exhibited by *Bacillus* sp. A 3-15 and *Bacillus* sp. PN5 respectively, with 1% SDS [20,24]. 90% activity was retained by *Bacillus* sp. strain TSCVKK amylase with 0.1% Triton X-100 and Tween 80 [18]. Present study reports massive enhancement of amylase activity with SDS and greater stability in surfactants (Tween 80 and Triton X-100) making it a potential candidate for detergent market. However, when surf and tide were used as additives, amylase retained a very low residual activity of 27 and 13% respectively. Sensitivity to oxidants present in the detergents should be a plausible explanation for reduction in amylase activity. Increase of α-amylase activity in the presence of SDS might be the first of its kind to be reported.

Kinetic studies

Km value was 10 mg/ml and the Vmax was 0.2 g μmol/min/ml (11. 56 μmol/min/mg protein). Values of kinetic parameters differ with different substrates or assay conditions, and thus, the Km value of amylase from JS-16 was well within the range of other α-amylases (0.35-11.66 mg/ml) [25].

Raw starch hydrolysis by crude amylase

JS-16 amylase hydrolyzed raw wheat and corn starch granules at the rate of 12% and 5% respectively, after 12 h incubation (Figure 7). Significant hydrolysis of potato starch was not obtained. The degradation

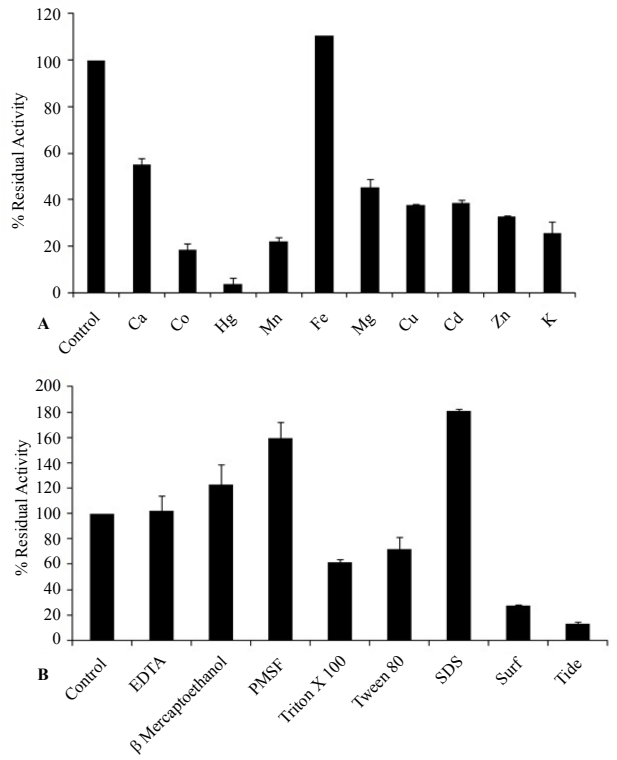

Figure 6: (A) Effect of metal ions on amylase activity. (B) Effect of organic additives, surfactants and commercial detergents on amylase activity.

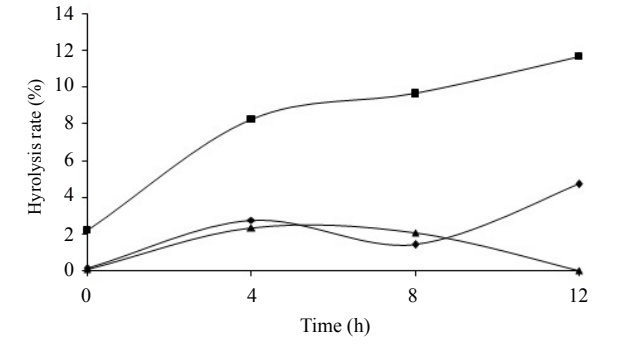

Figure 7: Hydrolysis at 50°C of raw corn (Solid diamond), wheat (Solid square) and potato (Solid triangle) starch granules by crude amylase.

rates of corn, wheat and potato starch granules at 1% concentration were 63.2%, 56.4%, 48.6% at 12 h, respectively from *Bacillus* sp. YX-1 amylase [17]. A Ca^{2+} dependent thermostable α-amylase from *Bacillus* sp. I-3 hydrolyzed raw potato starch granules in a temperature range of 60-90°C at a concentration of 12.5% in 12 h [26]. Similarly *Bacillus* sp. GRE1 amylase hydrolyzed starch granules from wheat, corn and tapioca at 40-70°C, with 50% degradation of starch granules after 12 h at 70°C without Ca^{2+} supplementation [27,28]. Halophilic α-amylase from *Nesterenkonia* sp. strain F hydrolyzed raw wheat and corn granules at the rate of 38% and 20% after 48 h incubation, respectively [23,29].

Conclusion

The *Bacillus subtilis* strain JS-16 isolated from the sludge samples of a soda ash industry in Gujarat produced α-amylase with promising properties. Activity of amylase at an alkaline pH, over a broad range of temperature and stimulation in the presence of SDS, are the key properties which could make this isolate a potential candidate for its use as a source of amylase suitable especially for the liquid detergent industry where SDS is largely used as surface active agent.

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References

1. Burhan A, Nisa U, Gokhan C, Omer C, Ashabil A, Osman G (2003) Enzymatic properties of a novel thermostable, thermophilic, alkaline and chelator resistant amylase from an alkaliphilic Bacillus sp.isolate ANT-6. Process Biochem 38: 1397-1403.
2. Gupta R, Gigras P, Mohapatra H, Goswami VK, Chauhan B (2003) Microbial a-amylases: a biotechnological perspective. Process Biochem 38: 1599-1616.
3. Rao MB, Tanksale AM, Ghatge MS, Deshpande VV (1998) Molecular and biotechnological aspects of microbial proteases. Microbiol Mol Biol Rev 62: 597-635.
4. Sivaramakrishnan S, Gangadharan D, Nampoothiri KM, Soccol CR, Pandey A (2006) a-Amylases from microbial sources—an overview on recent developments. Food Technol Biotechnol 44: 173-184.
5. Hashim SO, Delgado O, Hatti-Kaul R, Mulaa FJ, Mattiasson B (2004) Starch hydrolysing Bacillus halodurans isolates from a Kenyan soda lake. Biotechnol Lett 26: 823-828.
6. Horikoshi K (1996) Alkalophiles-from an industrial point of view. FEMS Microbiol Rev 18: 259-270.
7. Kim TU, Gu BG, Jeong JY, Byun SM, Shin YC (1995) Purification and Characterization of a Maltotetraose-Forming Alkaline (alpha)-Amylase from an Alkalophilic Bacillus Strain, GM8901. Appl Environ Microbiol 61: 3105-3112.
8. Holt JD (1994) Bergey's Manual of Determinative Bacteriology, 9thed. Williams and Wilkins, Baltimore.
9. Sambrook J, Fritsch EF, Maniatis T (2001) Molecular Cloning: A Laboratory Manual. 3rd ed. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
10. Weisburg WG, Barns SM, Pelletier DA, Lane DJ (1991) 16S ribosomal DNA amplification for phylogenetic study. J Bacteriol 173: 697-703.
11. Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990) Basic local alignment search tool. J Mol Biol 215: 403-410.
12. Tamura K, Dudley J, Nei M, Kumar S (2007) MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Mol Biol Evol 24: 1596-1599.
13. Saitou N, Nei M (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol 4: 406-425.
14. Miller G (1959) Use of dinitrosalicylic acid reagent for determination of reducing sugars. Anal Chem 31: 426-428.
15. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the Folin phenol reagent. J Biol Chem 193: 265-275.
16. Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227: 680-685.
17. Liu XD, Xu Y (2008) A novel raw starch digesting alpha-amylase from a newly isolated Bacillus sp. YX-1: purification and characterization. Bioresour Technol 99: 4315-4320.
18. Kiran KK, Chandra TS (2008) Production of surfactant and detergent-stable, halophilic, and alkalitolerant alpha-amylase by a moderately halophilic Bacillus sp. Strain TSCVKK. Appl Microbiol Biotechnol 77: 1023-1031.
19. Liu Y, Lu F, Chen G, Snyder CL, Sun J, et al. (2010) High-level expression, purification and characterization of a recombinant medium-temperature alpha-amylase from Bacillus subtilis. Biotechnol Lett 32: 119-124.
20. Arikan B (2008) Highly thermostable, thermophilic, alkaline, SDS and chelator resistant amylase from a thermophilic Bacillus sp. isolate A3-15. Bioresour Technol 99: 3071-3076.

21. Prakash B, Vidyasagar M, Madhukumar MS, Muralikrishna G, Sreeramulu K (2009) Production, purification, and characterization of two extremely halotolerant, thermostable, and alkali-stable a-amylases from Chromohalobacter sp. TVSP101. Process Biochem 44: 210-215.

22. Najafi MF, Deobagkar D, Deobagkar D (2005) Purification and characterization of an extracellular alpha-amylase from *Bacillus subtilis* AX20. Protein Expr Purif 41: 349-354.

23. Shafiei M, Ziaee A, Amoozegar MA (2010) Purification and biochemical characterization of a novel SDS and surfactant stable, raw starch digesting, and halophilic a-amylase from a moderately halophilic bacterium, Nesterenkonia sp. strain F. Process Biochem 45: 694-699.

24. Saxena RK, Dutt K, Agarwal L, Nayyar P (2007) A highly thermostable and alkaline amylase from a Bacillus sp. PN5. Bioresour Technol 98: 260-265.

25. Najafi MF, Kembhavi A (2005) One step purification and characterization of an extracellular a-amylase from marine Vibrio sp. Enzyme Microb Technol 36: 535-539.

26. Goyal N, Gupta JK, Soni SK (2005) A novel raw starch digesting thermostable a-amylase from Bacillus sp. I-3 and its use in the direct hydrolysis of raw potato starch. Enzyme Microb Technol 37: 723-734.

27. Haki GD, Anceno AJ, Rakshit SK (2008) Atypical Ca2+-independent, raw-starch hydrolysing a-amylase from Bacillus sp. GRE1: characterization and gene isolation. World J Microbiol Biotechnol 24: 2517-2524.

28. Pathak A, Rathod MG (2013) Bioprospecting an alkaline ecosystem of Lonar Lake for novel alkaline protease producer. Innovative Research in Chemistry 1: 22-26.

29. Antony CP, Kumaresan D, Hunger S, Drake HL, Murrell JC, et al. (2013) Microbiology of Lonar Lake and other soda lakes. ISME J 7: 468-476.

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