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Amylase produced by *Bacillus sp.* SI-136 isolated from sodic-alkaline soil for efficient starch desizing

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Abstract

Bacillus sp. SI-136, isolated from sodic-alkaline soil, showed 94% similarity to *B. cereus* group based on 16S rDNA sequence. It produced α-amylase of 26 kDa with maximum activity at pH 10.0, stable up to pH 12.0 and 80^{o}C. Mn^{2+} enhanced its activity as also 10% NaCl in medium. Agricultural waste substrates supported growth and enzyme activity was enhanced by 30% with sugarcane bagasse. The partially purified enzyme showed efficient desizing of cotton fabric at 50^{o}C (40-60 min) or 70^{o}C (60 min) with Tegewa rating 7-8, and at 95^{o}C (20 min) with Tegewa rating 9, properties enabling utility in textile industries.

Keywords: *Bacillus sp.* SI-136, Sodic-Alkaline Soil, Alkaliphile, Amylase, Starch Desizing

Introduction

Amylases constitute around 30% of the industrial enzyme market. They break-down starch-based substrates and have largely replaced chemical methods in the food industry. They also find utility as components of detergents, in baking, brewing, production of fruit juice and also in paper and textile processing (Horikoshi 1999; Sivaramakrishnan et al. 2006). These diverse applications require certain unique properties such as thermostability, ability to function efficiently at high pH, stability in presence of chelators and other compounds etc. While amylases are known to be secreted by plants and fungi too, the bacterial sources have best met industrial requirements in terms of cost of production. *Bacillus licheniformis* and *B. amyloliquefaciens* are the principal bacteria used by industry for amylase production.

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Sizing agents like starch are applied to yarn before fabric production to ensure a fast and secure weaving process. Starch is a strengthening (sizing) agent to prevent breaking of the warp thread during the weaving process. It is a very attractive size, because of its cost-effectiveness, availability and ease of removal from fabric. Desizing involves the removal of starch from fabric using large amounts of hot water and chemicals. Enzyme-mediated desizing is an environment-friendly process (Feitkenhauer et al. 2003). The α-amylases function here by removing selectively the size and do not attack the fibres.

Sodic and sodic-alkaline soils are widespread throughout the world and in India, they are found in the northern parts (Garg 2002). Such soils present a severely constrained ecosystem and remain barren. Management of these soils requires information on biodiversity and biological processes occurring in it (Siddikee et al. 2011). There are few studies on microbial diversity from saline and sodic-alkaline environments. Bacterial diversity and population is known to be much higher in non-saline soil than soil with high salinity and sodicity. Barren soil, having maximum sodicity and alkalinity, showed least total bacterial count (Siddikee et al. 2011). In this context, a screening programme was initiated to characterize bacteria from sodic-alkaline soil resulting in more than 500 isolates. We furnish the description of *Bacillus sp.* SI-136, a potentially novel species isolated from barren highly sodic-alkaline soil and production of pH- and temperature-stable α-amylase from this isolate, capable of efficient desizing at lower temperatures (50^{o}C) indicating its usefulness for industrial applications, which are generally carried out at 95^{o}C.

Materials and Methods

Top soil (from 0-5 cm depth) of pH 9.0 was collected from Banthra Village, Lucknow, India and stored at 4^{o}C. Dilutions of 10^{-1} to 10^{-6} were made and alkaliphilic bacteria were selectively isolated on Horikoshi-II medium (in grams litre^{-1}: Starch–10.0, yeast extract–5.0, peptone–5.0, dipotassium hydrogen phosphate–1.0 and magnesium sulfate heptahydrate–0.2) of pH 10.0 (using 10% (w/v) sodium carbonate). Representative colonies were screened for amylase production using starch plate assay (Shanmughapriya et al. 2009). Colonies with clearance zones greater than the positive

control *B. licheniformis* in the plate assay, were quantitatively assayed by dinitrosalicylic acid method and isolate SI-136 was taken up for further studies.

Characterization of Bacillus sp. SI-136

Morphological and biochemical tests (Biomerieux Vitek 2 Compact) were done for identification of SI-136. Cell dimensions were studied with Olympus Trinocular microscope (BX-51) using MagnusPro Image Analysis software. 16S rDNA universal primers 8F (5'-AGAGTTTGATCMTGG-3') and 1492R (5'-ACCTTGTTACGACTT-3') were used for amplifying 16S rDNA. The 16S rDNA consensus sequence was compared with those of Gram positive bacteria in GenBank database using BLAST (http:// www. ncbi. nlm.nih.gov) and aligned against corresponding sequences from GenBank/DDBJ/EMBL/RDP databases. Evolutionary distances were computed using Maximum Composite Likelihood (Tamura et al. 2004). The software MEGA 4.0 (Tamura et al. 2007) was used to construct phylogenetic trees; bootstrap consensus tree was inferred from 1000 replicates (Felsenstein 1985). Evolutionary relationship was inferred using neighbor-joining (Saitou and Nei 1987) treeing algorithm. There were a total of 1251 positions in the final dataset.

Media composition and growth conditions

250 mL cultures were assayed for growth (at 600 nm) and amylase activity on Horikoshi II medium pH 10.0. Effect of the following on amylase activity was studied: Starch (0-15%), NaCl (0-15%), alternate sources of carbon (dextrin, glucose, maltose), nitrogen (tryptone, urea), and agricultural waste substrates (1% each of dried and powdered sugarcane bagasse, wheat bran, wheat husk and potato). One unit of amylase activity was defined as the amount of enzyme that released 1 µmol maltose per mL per minute.

Partial purification of amylase

250 mL of cell free supernatant from Horikoshi II medium was fractionated with ammonium sulfate (0-30%, 30-60%, 60-90% and 90-100%) and the pellet collected by centrifugation at 12000 x g for 20 min. The pellet was dissolved in 10 mM Tris-HCl buffer (pH 8.0), dialyzed against the same buffer in dialysis tubing cellulose membrane (Sigma, USA) and amylase activity checked by starch plate assay. The fraction showing clear zones was used as partially purified enzyme for subsequent characterization.

Effect of pH and temperature

Partially purified enzyme was incubated with the following buffers: Phosphate (pH 7.0, 8.0, 11.0 and 12.0), Tris - HCl (pH 9.0), Glycine - NaOH (pH 10.0) and Potassium Chloride (pH 12.5 and pH 13.0). Enzyme activity at different temperatures was determined by incubation at pH 12.0 and varying temperature from 30-100^{o}C. Enzyme stability was assessed by incubating the enzyme at 80^{o}C for 0.5, 1 and 2 hours and determining residual activity.

Effect of metal ions and chelator

2 mM each of metal ions $ZnSO_4.7H_2O$, $CaCl_2$, $MgSO_4.7H_2O$, $CuSO_4.5H_2O$, $FeSO_4.7H_2O$, $CoCl_2.6H_2O$, $MnSO_4.H_2O$ and $HgCl_2$ and chelator EDTA were evaluated for effect on amylase activity.

Native-PAGE and Zymography

Native-PAGE was carried out using partially purified enzyme and proteins stained using Coomassie Blue R-250. Zymography was performed by running the enzyme preparation on SDS-PAGE gel containing 1% soluble starch and amylase bands were visualized using Lugol's iodine solution.

Mode of action

Partially purified enzyme (20 µl) was added to 100 µl 1% soluble starch (w/v) at 60^{o}C and incubated for 0, 30, 60, 120 and 180 min. Analysis was carried out by thin layer chromatography using silica gel as stationary phase and butanol:ethanol:water (5:3:2) as solvent. Products were visualized by spraying with sulphuric acid:methanol (1:1), and drying at 100^{o}C for 10 min. Rf values were compared with glucose and maltose as standards.

Desizing activity

Grey cotton fabric of 1 cm^2 each were dipped in 5% starch solution and allowed to dry till the fabric was stiff (Dalvi et al. 2007). 75 µL of 1:1000 diluted solution of Tween 80 was added as wetting agent. Subsequently, 500 µL of cell-free crude supernatant was directly applied to the grey fabric and the set-up was incubated at 30, 50, 70 and 95^{o}C, each for 0, 20, 40 and 60 minutes. The positive control was pure α-amylase (from *B. amyloliquefaciens*, Sigma Aldrich*)* and negative control was uninoculated Horikoshi-II broth. After each time period, the residual starch content was estimated based on the Tegewa scale method. The cloth was taken out and stained with Tegewa solution for 1 minute. After washing, the immediate colour change in the cloth, if any, was noted and compared to the Tegewa scale. The assay was carried out in triplicates.

Nucleotide Sequence Accession Number

The nearly complete 16S rDNA sequence (1,477 nucleotides) of SI-136 was deposited in GenBank and was assigned the accession number JN314426.

Results and Discussion

Characterization of Bacillus sp. SI-136

Isolate SI-136 was identified as belonging to *B. cereus* group (*B. cereus*/*B. thuringeinsis*/*B. mycoides*) (Table 1). Cells were Gram positive and rod-shaped. 16S rDNA sequence analysis (Figure 1) showed that SI-136 formed a distinct phyletic line with nearest matches being species belonging to the *B. cereus* group (*B. weihenstephanensis*, *B. mycoides, B. thuringiensis* and *B. cereus*). Maximum sequence similarity (94%) was obtained with B. cereus B-102 (AJ577289) corresponding to 24 nucleotide differences in 1456 sites. The relatively low similarity, along with atypical results in the biochemical tests (utilization of d-mannose, maltotriose, oleandomycin resistance, alanine arylamidase), and growth at high NaCl concentrations (10%) suggest that SI-136 could be a new species, which requires further confirmation. A recent report documents B. cereus isolate to secrete alkaline-tolerant and thermo-stable amylase (Annamalai et al. 2011) though isolated from marine harbor water of non-alkaline pH, SI-136 was selectively isolated in pH 10.0 plates and could grow at pH 12.0. Hence, niche alkaline microhabitats could be present which support the growth of alkaliphilic bacteria (Horikoshi 1999). To our knowledge, our study is one of the very few reports of members of the B. cereus for production of alkaline enzymes (Ghorbel-Frikha et al. 2005; Ravindran et al. 2011). These authors have reported production of alkaline proteases but there is only one other report of alkaline amylase (Annamalai et al. 2011).

Table 1: Morphological and biochemical properties of *Bacillus* sp. SI-136.

Property	Observation
Colony morphology, colour	Round, off–white
Gram reaction	+
Cell shape, length, width	Rod, 1.85 µ, 0.38 µ
Position of endospores	Sub–terminal to terminal
Anaerobic agar	-
Citrate utilization	-
pH (for growth)	7.0-12.0
Motility	-
Growth in NaCl (up to 15%)	+
Lipase	-
Protease	-
Amylase	+
Cellulase	+
Acid from cabohydrates:	
Arabinose	++
Fructose	++
Glucose	++
Maltose	+
Raffinose	+
Sucrose	++
Xylose	-
Growth in *Bacillus* differentiation agar	+
Growth in *Bacillus cereus* medium	-
Gelatin liquefaction	-
L-Pyrrolydonyl Arylamidase	(+)*
Alanine Arylamidase	-
Maltotriose	-
D-Mannose	+
Oleandomycin resistance	+

(+, Positive; -, negative; *Reactions in parentheses are indicative of weak reactions but close to test threshold).

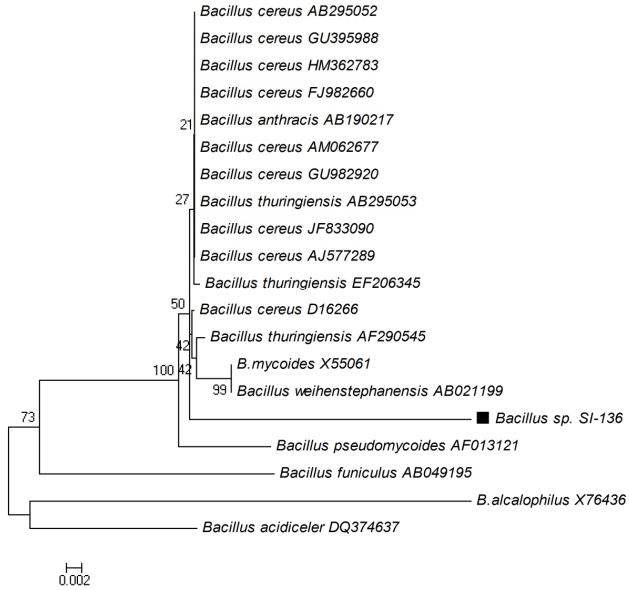

Figure 1: Phylogenetic relationship between *Bacillus* sp. SI-136 and other members of *Bacillus.* Optimal tree with sum of branch length = 0.19390320 is shown.

Media composition, growth conditions and amylase production

Log phase was reached by 20 hours but maximum amylase production was towards late log-stationary phase (Figure 2a), as also reported earlier (Annamalai et al. 2011). Specific activity of amylase from SI-136 is higher (Table 2) than that of some alkaliphiles (Annamalai et al. 2011; Burhan et al. 2003; Kim et al. 1996; Murakami et al. 2007). 1% starch was best for inducing amylase production (Figure 2b). SI-136 could grow with 15% NaCl but maximum amylase activity (Figure 2c) was in 10% NaCl (130% increase over control). Growth in 10% NaCl has not been reported for members of the *B. cereus* group. The original soil sample has been documented to have exchangeable sodium percentage of 73%, pH of 9.6 (0-20 cm depth), and electrical conductivity of 2.18 dS m^{-1} (Garg 2002), pointing to its sodicity, alkalinity and mildly saline nature, which accounts for the isolate's ability to grow in high NaCl concentrations.

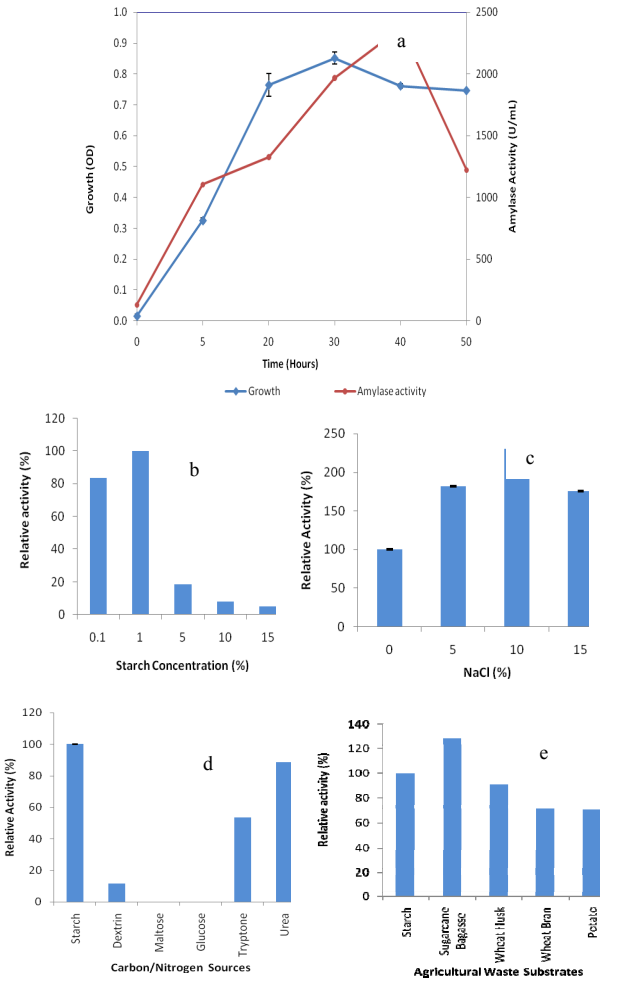

Figure 2: Amylase production in *Bacillus* sp. SI-136. (a) Growth and amylase activity on Horikoshi II medium. Effect of (b) starch (c) NaCl (d) alternate carbon and nitrogen sources and (e) agricultural waste substrates on amylase activity. Absolute activity was 1193.07 U mg^{-1}.

Starch and maltose elicit good amylase production as also seen with SI-136 (Fig. 2d). Overall, amylase activity was best with the original nitrogen components in Horikoshi-II media. Conflicting results have been reported on the best carbon or nitrogen source for amylase production. While starch and peptone were reported optimal for *S. gulbargensis* (Dastager et al. 2009), sucrose and beef extract were found better for *Halobacterium salinarum* MMD047 (Shanmughapriya et al. 2009) and glucose for *B. cereus* MTCC1305 (Anto et al. 2006). Sugarcane bagasse was best (30% increase over soluble starch) amongst agricultural waste substrates (Fig. 2e).

Partial purification of amylase

Amylase activity was detected in the 60-90% ammonium sulfate fraction with a 2.9-fold purification (Table 3). Zymography showed

a single clear band of size 26 kDa (Figure 3b) corresponding to similar sized band in Native-PAGE (Figure 3a). The additional 30 kDa band does not have amylolytic activity. The partially purified enzyme had specific activity of 1193.07 U mg^{-1}. Members of

Table 2: Comparison of amylase produced by *Bacillus* sp. SI-136 with that of other bacterial strains producing high temperature/pH tolerant amylases.

Organism	Activity (U mg^{-1})	Optimum Temperature (^oC)/pH	Molecular Weight (kDa)	Reference
Bacillus sp. SI-136	1193.07	70-80/10.0	26	This study
Bacillus sp. GM8901	157.5	60/11.0–12.0, stable from pH 6.0–13.0	97	Kim et al. 1996
Bacillus sp. KSM-1378	5,000	55/ 8.0–8.5	53	Igarashi et al. 1998
Bacillus sp. ANT-6	195	80/10.5		Burhan et al. 2003
Bacillus halodurans 38C-2-1	130, 18	50–60, 10.0–11.0	105 (α-amylase I) 75 (II)	Murakami et al. 2007
Streptomyces gulbargensis	1,341.3	45/8.5-11.0	55	Dastager et al. 2009
Bacillus sp. BKL20		70/6.0-11.0		Kubrak et al. 2010
Bacillus cereus	43.9	65/8.0-11.0	42	Annamalai et al. 2011

Bacillus, obtained from diverse sources and environments, can produce different types of amylases with varying molecular weights (Table 2). Amylase of 26 kDa has not been reported till date. A thermostable amylase stable at pH 9.0 from *B. cohnii* US147 (Ghorbel et al. 2009) had an apparent molecular weight of 30 kDa. Two starch-binding protein bands of 26 kDa and 105 kDa were reported in *Arthrobacter psychrolactophilus* (Smith and Zahnley 2005) but amylolytic activity of the 26 kDa band could not be confirmed.

Table 3: Summary of partial purification of amylase of *Bacillus* sp. SI-136.

Step	Total Activity (U)	Protein concentration (µg mL^{-1})	Specific activity (U mg^{-1})	Yield (%)
Crude culture filtrate (250 mL)	1064	86	407.79	100 (1.00)*
Lyophilized filtrate (50 mL)	1038	82	421.82	97.4 (1.03)*
$(NH_4)_2SO_4$ precipitation (60–90%) and dialysis (10 mL)	898	19	1193.07	84.3 (2.93)*

*Purification fold

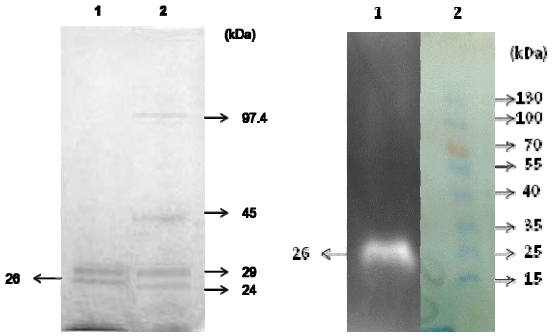

Figure 3a-b: (a) Native PAGE (b) Zymogram of dialyzed 60-90% fraction from *Bacillus* sp. SI-136. Lane 1: Dialyzed fraction (60-90%); Lane 2: Molecular weight marker.

Properties of amylase

SI-136 amylase was stable from pH 9.0 to 12.0 (Figure 4a), retaining more than 80% activity at pH 12.5. It was also stable from 35°C to 90°C (Figure 4b), retaining 90% activity at 100°C and after incubation at 80°C for 2 hours (Figure 4c). Earlier reports have documented that amylases can be active at a wide range of pH and temperatures (Table 2), depending mainly on the environmental source from they have isolated. Amylase activity was promoted (Table 4) by Mn^{2+} (104%) which could be an essential co-factor (Annamalai et al. 2011). The amylase was inhibited to varying extents (10-83%) by Co^{2+}, Cu^{2+}, Ca^{2+}, Fe^{2+}, Mg^{2+}, Hg^{2+} and EDTA. Zn^{2+} was completely inhibitory. Analyses of hydrolytic products by TLC showed that the spots corresponded to glucose and maltose, confirming the enzyme to be an α-amylase.

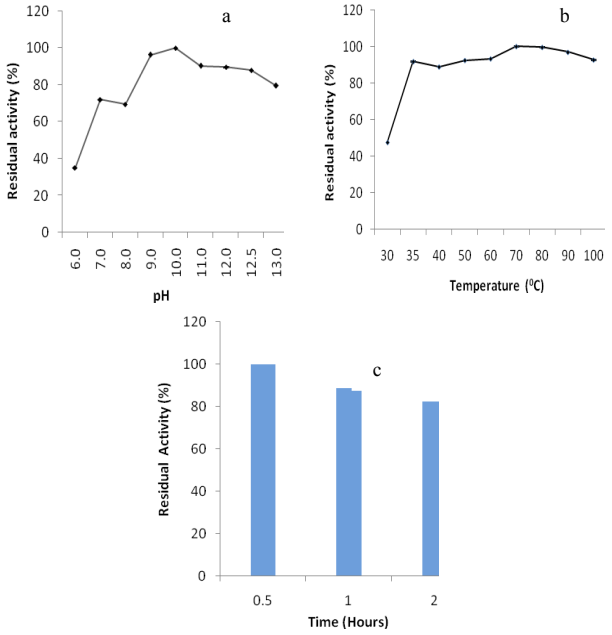

Figure 4: Properties of amylase. Effect of (a) pH, (b) temperature (c) stability (at 80°C) of amylase from *Bacillus sp.* SI-136. Absolute activity was 1193.07 U mg^{-1}.

Table 4: Effect of metal ions and chelator on amylase activity from *Bacillus sp.* SI-136.

Metal ion/chelator (2 mM)	Relative Activity (%)
None	100
Ca	68.03±0.02
Fe	57.23±0.01
Mg	16.73±0.03
Hg	24.73±0.03
Mn	109.43±0.00
Zn	0
Co	89.63±0.00
Cu	88.53±0.00
EDTA	85.53±0.00

Absolute activity was 1193.07 U mg^{-1}

Desizing activity

Results of desizing activity (Figure 5, Table 5) show that at 50°C (40 min), the amylase showed Tegewa rating of 7, which improved to 8 on incubation for 60 min. Incubation at 70°C for 60 min,

resulted in rating of 8. At 95°C, for even a minimum period of 20 min, desired Tegewa rating of 9 was observed.

Industrial processes involving amylase for textile desizing are carried out at 95°C for 90 min (Au and Holme 1999; Dalvi et al. 2007). Fabric showing rating of 7-8 is considered to be free from size and these ratings conform to approximately 0-15% starch on warp threads and 0.0725% starch on fabric, while rating of 9 implies 0.08% and 0.04% respectively (Au and Holme 1999). Hence SI-136 amylase could be potentially useful for desizing at lower temperatures (50°C) leading to energy savings as well as being environment-friendly. Being pH stable, its potential increases for industrial application.

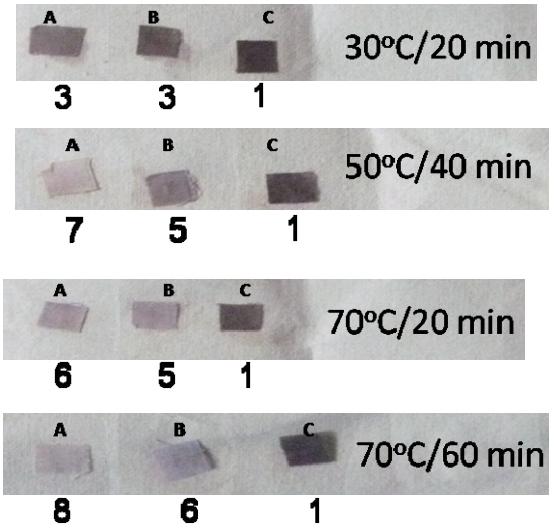

Figure 5: Removal of size from grey fabric after enzymatic desizing. A: Partially purified SI-136 amylase; B: Positive control; C: Negative control. Numbers below figures represent Tegewa rating.

Table 5: Tegewa rating of grey fabric subjected to enzymatic desizing at different temperatures and for different time periods.

Temp. (°C)	30			50			70			95		
Time (min)	20	40	60	20	40	60	20	40	60	20	40	60
Partially purified amylase (*Bacillus* sp. SI-136)	3	3	3	5	7	8	6	7	8	9	9	9
α-amylase (*B. amyloliquefaciens)*	3	3	3	3	5	6	5	5	6	5	6	6
Uninoculated Horikoshi-II broth	1	1	1	1	1	1	1	1	1	1	1	1

Conclusions

The present report shows that sodic-alkaline habitats harbor microbial flora with interesting potential. The partially purified amylase from *Bacillus* sp. SI-136 is pH- and thermo-stable as well as stable in high salt concentrations. It shows excellent properties for textile desizing and moreover, shows efficient desizing at lower temperatures and hence could facilitate in bringing down energy costs during industrial processes. *Bacillus* sp. SI-136 is also able to grow and produce amylase on waste substrates which is an added advantage.

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