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Cold-active detergent-stable extracellular α-amylase from *Bacillus cereus* GA6: Biochemical characteristics and its perspectives in laundry detergent formulation

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Abstract

A psychro-halo-tolerant *Bacillus cereus* GA6 was isolated from soil of Gangotri glacier, Western Himalaya that produced maximum cold-active α-amylase at 20±1 °C after 96 h of incubation in alkaline medium by using glycerol and ammonium acetate as a substrate. The enzyme was purified to homogeneity (55 kDa) with 53.33% yield and 175.92 U/mg specific activity; and was stable at low temperature (4-37 °C) and in alkaline medium (pH 7-11). The purified enzyme showed maximum activity at 22±1 °C (pH 9) along with K_m and V_{max} value of 0.27 mg/ml and 2600 U/ml, respectively. The activity was strongly inhibited by Fe^{2+}, Zn^{2+}, H_2O_2 and $CuSO_4$ while enhanced considerably by Ca^{2+}. The hydrolysates of soluble starch by the enzyme were mainly glucose, maltose and maltotriose. The enzyme showed exceptional resistance ability against chemical denaturants (Urea, SDS and EDTA) and significant compatibility with commercial laundry detergents thus advocating its suitability for detergent formulation.

Keywords: Cold-active enzyme; α-amylase; *Bacillus cereus*; biocatalyst; biodetergent.

Introduction

Amylases (EC 3.2.1.1) are of ubiquitous occurrence and are produced by plants, animals, and microbes but microbial sources are the most preferred one for large scale production to meet industrial demands (Pandey et al. 2000). Out of different factors affecting microbial environment, temperature is one of the most important as it influences most biochemical reactions. Thereby psychro-tolerantmicrobes have developed various adaptive strategies to live in harsh environmental conditions (Margesin et al. 2002). Psychrotrophs are known to synthesize enzymes with low activation energies and high activities at low temperatures (Morita et al. 1997) conferring considerable progress toward energy savings. These enzymes are of commercial interest because they might be used in applications at low temperature and there is scientific interest in the relationship between protein structure and thermal stability of enzymes.

Amylases constitute one of the most important groups of industrial enzymes and account for nearly 25% of the total sale of enzymes (Burhan et al. 2003). Alpha amylases (EC 3.2.1.1) belongs to the enzyme class of hydrolases which randomly cleaves the 1,4-α-D-glucosidic linkages between the adjacent glucose units in linear amylose chain of starch. For commercial purpose of enzymes, detergent industries are the primary consumers in terms of both volume and value. Detergent enzymes account for about 30% of the total worldwide enzyme production and represent one of the largest and most successful applications of modern industrial biotechnology (Rao et al. 1998).The use of enzymes in detergent formulation not only enhances the detergent ability to remove tough stains but also makes the detergent environmentally safe. Out of the vast pool of enzymes exploited in detergent industries worldwide, there is always a need of new enzymes with novel properties that can further enhance the wash performance of currently used enzyme-based detergents (Gupta et al. 1999). Amylases are used in the formulation of enzymatic detergent, and 90% of all liquid detergents contain these enzymes (Hmidet et al. 2009). These enzymes are used in detergents for laundry and automatic dishwashing to degrade the residues of starchy foods. Most of the reported cold-active enzymes for detergent application were originated from Antarctic ecosystem and were mainly proteases (Gerday et al. 1997; Russel et al. 1997).

Comparatively little information is available on cold-active α-amylases from microorganisms isolated from glacier regions, which may be an ideal habitat for cold-adapted microorganisms. The

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present study includes production optimization, purification and biochemical characterization of cold-active α-amylase produced by novel psychro-tolerant *Bacillus cereus* strain GA6 isolated from soil of Gangotri glacier, Western Himalaya, India and its application in detergent formulation for cold washing.

Materials and Methods

Isolation of cold-active α-amylase producing bacteria

In order to isolate psychro-tolerant bacteria, twenty five soil samples were collected from vicinity of Gangotri glacier, Western Himalaya, India during winter season of 2011. The glacier is situated in between 30°44'–30°56'N and 79°04'–79°15'E having temperature 2-5 °C in summer and subzero in winter. The samples were serially diluted in sterile cold saline solution and subjected to isolation of α-amylase producing microbes at low temperature (Mishra and Behera 2008). Production of α-amylase was carried out by using the modified method of Abe et al. (1988). Protein concentration was determined by the method of Lowry et al. (1951) using BSA as standard.

Alpha-amylase assay

Amylolytic activity with starch (Himedia) as a substrate was assayed by the method of Swain et al. (2006). This amylase assay was based on reduction in blue color intensity resulting from enzyme hydrolysis of starch. The reaction mixture consisted of 0.2 ml enzyme (cell free supernatant), 0.25 ml of 0.1% soluble starch solution and 0.5 ml of phosphate buffer (0.1M, pH 6.0) incubated at 50°C for 10 min. The reaction was stopped by adding 0. 25 ml of 0.1N HCl and color was developed by adding 0.25 ml Gram's iodine solution. The optical density of blue color solution was determined by using spectrophotometer at 690 nm. The activity was expressed in units. One unit of enzyme activity is defined as the quantity of enzyme that causes 0.01% reduction of blue color intensity of starch-iodine solution at 50°C in one min per ml.

Identification of potential isolates

The potential isolate was identified and characterized by using Bergeys Manual of Systematic Bacteriology (Garrity 1985). The identity of the isolate was further confirmed by 16S rRNA analysis as described in our previous article (Roohi et al. 2011).

Optimization of media and fermentation conditions for enzyme production

The effect of incubation period on enzyme production and cell growth was determined by inoculating production media with bacterial isolate (1%) and incubated at 20±2 °C for different time intervals (24-168 h) at 120 rpm. Alpha-amylase production was estimated after every 24 h for 8 days using the method of Swain et al. (2006). Growth kinetics was obtained by measuring the cell density at 660nm. To determine optimum temperature and pH for fermentation, the inoculated media was incubated at different temperatures (4-50 °C) and pH (pH 5-12) in a rotary shaker (120 rpm) and also in static condition. The effect of additional supplements (1%) in production media such as glucose, sucrose, maltose, lactose and glycerol as a carbon source; and glycine, casein, yeast extract, ammonium acetate and ammonium sulphate as a nitrogen source were evaluated in terms of enzyme yield. To assess the impact of metals, media was supplemented with maximum tolerance level of different metal ions and incubated under optimized conditions for 48 h. The heavy metals used were Ca^{2+}, Cu^{2+}, Zn^{2+}, Fe^{2+}, Mg^{2+} and Hg^{2+}. Amylase activity was measured as per standard protocol (Swain et al. 2006).

Extraction and purification of cold-active α-amylase

The isolated strain *Bacillus cereus* GA6 was grown in optimized conditions and cells were removed by centrifugation at 10000g for 10 min at 4°C. Cell-free supernatant was subjected to ammonium sulfate precipitation (10-80% w/v) and the precipitate was resuspended in 0.1M phosphate buffer (pH 6) that was dialyzed against the same buffer for 24 h. The amount of salt required for percentage saturation was calculated by the method given by Deutscher (1990). The partially purified protein was subjected to ion exchange chromatography on a DEAE cellulose column (Bangalore Genei, India) that was pre-equilibrated with phosphate buffer (0.1M, pH 6). The protein was eluted (flow rate 60 ml/h) with a linear gradient of NaCl (0.1 to 1M) in the same buffer. A total of 40 fractions were collected and assayed for protein and enzyme activity. All subsequent steps were carried out at 4°C. Fractions having highest amylase activity was desalted and concentrated by dialysis and lyophilization, respectively.

Characterization of purified α-amylase

Determination of optimum pH and pH stability

The effect of pH on the activity of α-amylase was measured by using the following buffers; 0.2M acetate (pH 4-5), 0.1M phosphate (pH 6-8), 0.05M borax-NaOH buffer (pH 9-10) and 0.05M sodium hydrogen phosphate buffer (pH 11-12) (Aneja 2003). Reaction mixtures were incubated at 20°C for 30 min and the respective activity was measured. To determine pH stability, the enzyme was pre-incubated at different pH (4-12) for 1 h but without substrate and then estimated the residual activity as per standard assay procedure.

Determination of optimum temperature and thermal stability

The effect of temperature on the enzyme activity was determined by performing the previously described standard assay procedure within a temperature range of 4-45 ºC by incubating the reaction mixture for 1 h. Thermo-stability of the enzyme was examined by pre-incubating the enzyme for 3 h without substrate within a temperature range of 4-45 °C and residual enzyme activity was determined.

Effect of metal ions and inhibitors

The effect of metal ions and inhibitors on the activity of purified α-amylase was investigated by pre-incubating the enzyme with different metal ions (Ca^{2+}, Zn^{2+}, Hg^{2+}, Co^{2+}, Ba^{2+}, and Cu^{2+}; 5mM) and inhibitors (EDTA, H_2O_2 and Urea, 1-10mM; $CuSO_4$ and SDS, 0.1-1%) for 30 min at 20±2 °C.

Effect of substrate concentration on α-amylase activity and their kinetic studies

To determine K_m and V_{max} for enzyme-substrate catalyzed reaction; 0.1, 0.2, 0.5, 0.7, 1.0, 1.2 and 1.4 mg/ml soluble starch in 0.1M phosphate buffer (pH 6.0) was mixed with purified α-amylase (5662 units) and the mixture was incubated at 22°C for 30 min and the reaction was stopped immediately by heating at 100°C for 2 min. K_m and V_{max} values were obtained from Lineweaver-Burk plot (1/[V] ml/Units versus 1/[S] ml/mg) and expressed as the mean of the three different experiments.

Thin-Layer Chromatography (TLC) analysis

The hydrolysis products of starch were identified by TLC on silica gel plates using the method of Zhang and Zeng (2008). Soluble starch (1%) were hydrolyzed by incubating with amylase in 0.1M phosphate buffer, pH 6 at 20°C for 6 h. Aliquots (5 ml) of the reaction mixtures were chromatographed on a silica gel with chloroform–acetic acid–ddw (18:21:3, v/v), and the products were detected by spraying the gel with aniline-diphenylamine-phosphate followed by baking at 120°C for 30 min. Standards used were Glucose (S1), maltose (S2), maltotriose (S3) and maltotetraose (S4).

SDS–PAGE and zymogram analysis

The molecular weight of purified amylase was determined by SDS-PAGE. Electrophoresis was performed by the method of Laemmli (1970). The gel was stained with Coomassie Brilliant Blue R-250 and the relative molecular mass of the protein was calculated using standard protein markers (Sigma), run simultaneously. SDS-PAGE-zymogram was performed for identity confirmation by the proposed method of Lin et al. (1998).

Evaluation of enzyme for use in detergent formulation

Compatibility and stability analysis

Application of cold-active purified α-amylase as a detergent additive was studied by using the method of Kuddus and Ramteke (2011). The enzyme compatibility with commercially available laundry detergents was studied. The used detergents were Ariel and Tide (Procter and Gamble, India), Surf Excel and Wheel (Hindustan Lever Ltd., India) and Ghari (Rohit Surfactants Private Limited). The detergent solution (1% w/v) was boiled for 15 min to inactivate the enzymes that could be part of their formulation (enzyme assay was performed to check the activity). The cold-active amylase (10 mg/ml) was then incubated with detergent solution for different time intervals (0.5-3 h) at 22±1 ºC and the residual activity was determined in comparison to control (without any detergent).

Wash performance analysis

Wash performance analysis of purified enzyme was studied on white cotton cloth pieces (8x8 cm) stained separately with food gravy and mixture of baby food and chocolate and then left for overnight. The following sets were prepared for cold-washing:
a) Flask containing distilled water (100 ml) + stained cloth (with food gravy and mixture of baby food and chocolate, independently).
b) Flask containing distilled water (98 ml) + stained cloth + 2 ml Tide detergent (1% w/v).
c) Flask containing distilled water (96 ml) + stained cloth + 2 ml Tide detergent (1% w/v) + 2 ml purified α-amylase (4774 units/ml).
The flasks were incubated for 30 min (22ºC) in shaking condition (100 rev/min) and rinsed with cold water then air dried. The wash performance was analyzed by measuring relative reflectance of cloth pieces using digital reflectance meter (Aimil Ltd., New Delhi) to test enzyme efficiency for stain removal. Untreated stained cloth pieces were taken as control (Kuddus and Ramteke 2011; Beg and Gupta 2003; Adinarayana et al. 2003).

Statistical analysis

All the experiments were carried out in triplicates and the standard error in results was within 5%. Appropriate controls were maintained in all the experiments.

Results

Psychrotrophic α-amylase producing bacteria

Total thirty prominent bacterial colonies were screened from soil of Gangotri glacier, producing cold-active extracellular α-amylase. Depending upon larger hydrolysis zone on starch agar media at 15±2 °C, six isolates were subjected to enzyme production in amylase producing broth media. On the basis of maximum enzyme production (3000 units) one potential isolate, designated as GA6, was selected for further studies.

Identification of potential isolate

The isolated cold-active α-amylase producing bacterium was identified on the basis of 16S rRNA gene alignment with BLAST search from the GenBank database at NCBI (MIDILABS Inc., USA) that showed 99% homology with *Bacillus cereus*. The phylogenetic tree (Fig. 1) was constructed using software MEGA 4.1 (Tamura et al. 2007). Final sequence of GA6 was submitted to GenBank having an accession no. HQ832575. The strain was Gram-positive, rod shape, aerobic, non-motile, spore forming and catalase positive with opaque colonies and could grow up to 5 mm diameter in size. The organism grew well in the pH range of 7-11 and temperature range of 4-37 °C but did not grow above 40°C. The isolate could tolerate up to 8% NaCl concentration.

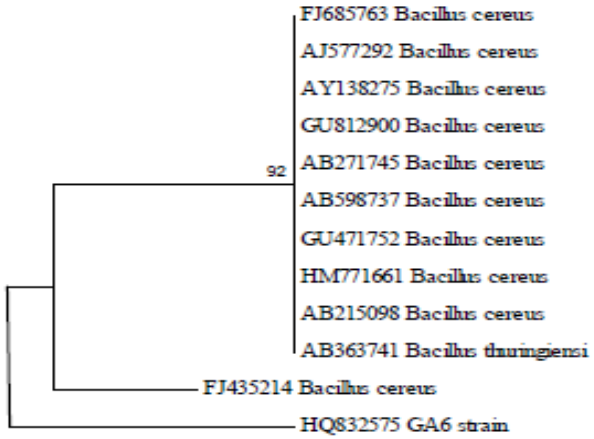

Fig. 1. Phylogenetic tree showing the homology of strain GA6 with *Bacillus cereus*

Optimization of α-amylase production

Alpha amylase production by strain GA6 began after 48 h and reached maximum at 96 h (4364 units) in logarithmic phase at 15±2 °C (Fig. 2). However, the cell growth was maximal at 120 h of incubation which indicate that the production of enzyme was independent to cell growth. Optimum temperature was found to be 20°C over 96 h of incubation (Fig. 3). However, there was continuous decline in enzyme production with increase in incubation temperature and it was totally inhibited at 50°C. The pH of the culture media strongly affects many enzymatic reactions and transport of compounds across the cell membrane and from Fig. 3 it was clear that amylase production was maximum at pH 10 (4732 units).

Enzyme production leads a four-fold boost in shaking condition (4662 units) at 120 rpm as compared to unperturbed condition (1204 units) at 20°C after 48 h incubation, established the necessity of

aeration for growth of the strain. Enhanced production of amylase was achieved with glycerol (4744 units), ammonium acetate (4746 units) and Ca^{2+} (4780 units) while lactose, glycine and Fe^{2+} worsened the production (Table 1).

Purification and characterization of α-amylase

Cold-active α-amylase was purified to homogeneity by precipitating with ammonium sulphate (50%) and using a single step ion-exchange chromatography on a DEAE-cellulose (Fig. 4). Enzyme

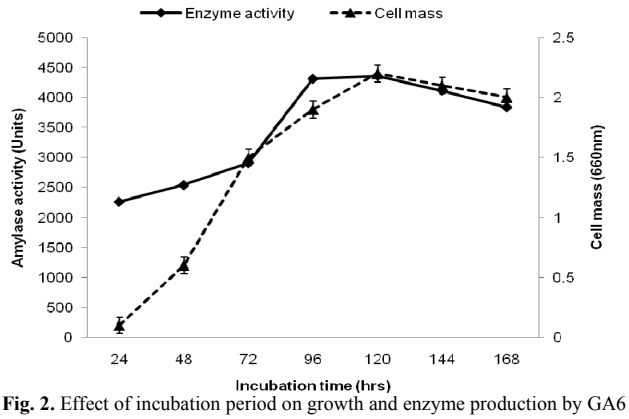

Fig. 2. Effect of incubation period on growth and enzyme production by GA6 at 15±1°C

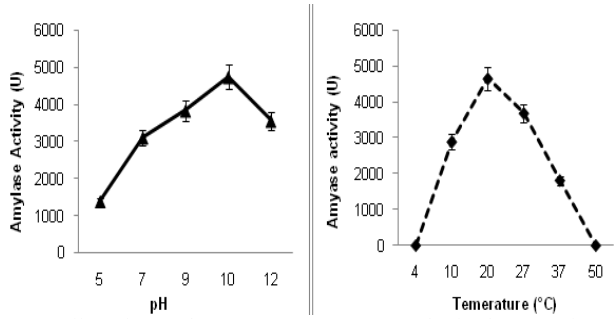

Fig. 3. Effect of pH and temperature on enzyme production by GA6 after 96 h of incubation

Table 1: Effect of carbon, nitrogen and metal ions on α-amylase production by GA6

S. No.	Additional supplements (1%)	α-amylase activity (units)
	Control	3225
	Carbon source	
1	Glucose	1476
2	Sucrose	1952
3	Maltose	1952
4	Lactose	1238
5	Glycerol	4744
	Nitrogen source	
1	Casein	1746
2	Glycine	317.4
3	Yeast extract	1905
4	Ammonium acetate	4746
5	Ammonium sulphate	476.1
	Metal ions	
1	$FeCl_3$	462
2	$CuSO_4$	625
3	$CaCl_2$	4560
4	$MgCl_2$	3345
5	$ZnSO_4$	1250
6	$HgCl_2$	1250

was eluted from the column as unbound fractions with 0.7M NaCl gradient. The α-amylase exhibited a specific activity of 175.92 U/mg, corresponding to a purification factor of 7.9-fold and a total yield of 53.33% (Table 2).

Optimum pH and pH stability

The effect of pH on α-amylase activity is shown in Fig. 5. The activity increased almost linearly from pH 5 to 9 and it was optimum at pH 9. However, 89 and 90% of the total enzyme activity was manifested at pH 8 and 10, respectively. At pH 7 and 11 the activity was 66 and 54%, respectively. The pH stability result suggests that enzyme was relatively stable over a pH range of 8 to 11 and retained about 80, 82 and 65% residual activity at pH 8, 10 and 11, respectively.

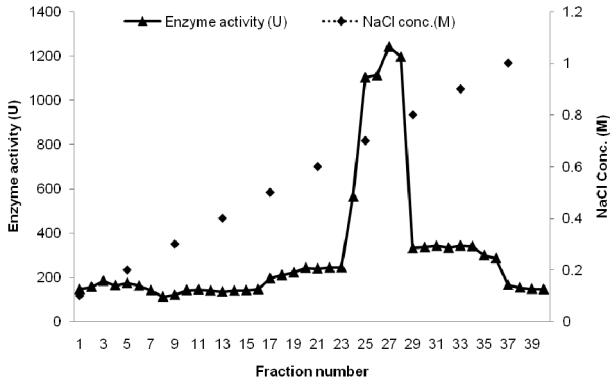

Fig. 4:. The elution profile of α-amylase from *B. cereus* GA6 by using DEAE cellulose column

Table 2. Purification of amylase from *B. cereus* GA6

Purification steps	Total activity (Units)	Total protein (mgL^{-1})	Specific Activity (Umg^{-1})	Purification (factor)	Yield (%)
Crude enzyme	5010	225	22.26	-	100
$(NH_4)_2SO_4$ precip^n (50%) (dialyzed)	3894	64	60.84	2.73	77.72
DEAE-cellulose pool (lyophilized)	2674	15.2	175.92	7.903	53.33

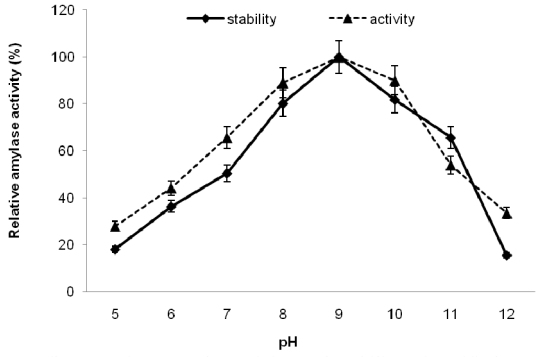

Fig. 5. Influence of pH on the activity and stability of purified α-amylase from *B. cereus* GA6

Temperature optima and thermal stability

The activity and stability of the lyophilized enzyme preparation assayed at temperatures ranging from 4-45 ºC at pH 9 are shown in Fig. 6. Enzyme activity increased with temperature and was found optimum at 22ºC. Activity decreased sharply above 37°C and becomes almost zero at 45ºC. Enzyme showed stability between 4-

37 ºC. The enzyme retained 56, 78 and 80% stability at 4ºC, 15ºC and 28ºC, respectively of that at 22ºC.

Stability of enzyme with metal ions and inhibitors

This cold-active α-amylase showed 185% catalytic activation in presence of 5mM Ca^{2+} (Table 3). Strongest inhibitory effect was observed in the presence of Fe^{2+} and Zn^{2+} where activity becomes zero. Cu^{2+}, Ba^{2+}, Co^{2+}, and Hg^{2+} also inhibited the enzyme activity and only 38.95, 58.82, 76.46 and 36.46 % activity remained, respectively. Enzyme showed remarkable resistance against EDTA, SDS and Urea as more than 50% activity still remained when incubated for 30 min at 20ºC while $CuSO_4$ (1%) and H_2O_2 (10mM) obtained as a potent inhibitors.

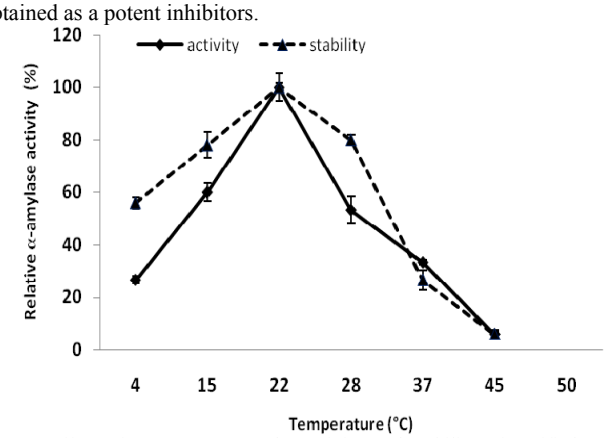

Fig. 6. Effect of temperature on the activity and stability of purified α-amylase from *B. cereus* GA6

Table 3. Effect of metal ions and inhibitors on the activity of purified amylase

Inhibitor	Concentration	% Relative activity
Control	-	100
Ca^{2+} ($CaCl_2$)	5mM	185
Ba^{2+} ($BaCl_2$)	5mM	58.82
Co^{2+} ($CoCl_2$)	5mM	76.46
Hg^{2+} ($HgCl_2$)	5mM	36.46
Cu^{2+} ($CuSO_4$)	5mM	38.95
Zn^{2+} ($ZnCl_2$)	5mM	0
Fe^{2+} ($FeSO_4$)	5mM	0
H_2O_2	1mM	25
	10mM	0
EDTA	1mM	70
	10mM	45
$CuSO_4$	0.1%	10
	1%	0
Urea	1M	60
	10M	35
SDS	0.1%	50
	1%	25

Effect of substrate concentration on α-amylase activity and their kinetic studies

When activity of purified cold-active enzyme was measured with different concentration of starch (0.1 to 1.4 mg/ml) in optimized conditions, it was observed that amylolytic activity increased with substrate concentration linearly and reached to maximum at 1 mg/ml and then becomes constant (figure not shown). A Lineweaver-Burk plot (Fig. 7) indicates that this enzyme has apparent K_m and V_{max} values of 0.27 mg/ml and 2600 Units/ml, respectively for the hydrolysis of soluble starch.

TLC analysis

TLC was used to analyze the hydrolysis patterns of soluble starch digested by the purified amylase (Fig. 8). The main hydrolysis products of soluble starch were mainly glucose, maltose, maltotriose and a little of maltotetraose also. This hydrolysis patterns revealed that amylase from *B. cereus* GA6 is a typical alpha-amylase as it hydrolyze the α-(1,4)-glycosidic linkage only.

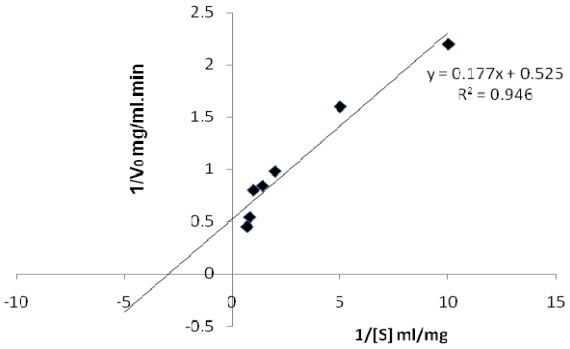

Fig. 7. Lineweaver-Burk plot for the hydrolysis of starch by purified α-amylase from *B. cereus* GA6

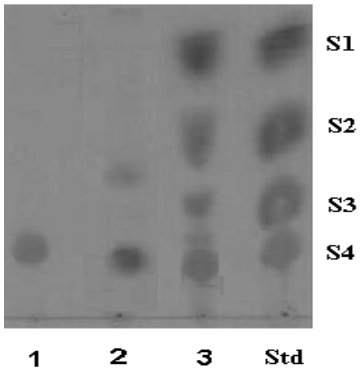

Fig. 8. TLC analysis of hydrolysis products of soluble starch digested by *B. cereus* GA6 α-amylase. (Lane 1: product generated after 0.5 h; lane 2: product generated after 2 h; lane 3: product generated after 6 h; Std: standard marker, S1. Glucose, S2. Maltose, S3. Maltotriose, S4. Maltotetarose)

Molecular weight

For determination of molecular weight, enzyme preparations and known molecular weight markers were subjected to SDS–PAGE. The purified protein showed a single band equal to a molecular mass of about 55 kDa on SDS-PAGE confirming that enzyme is composed of single polypeptide chain and activity staining gel confirming high enzyme purity (Fig. 9).

Detergent Compatibility and stability analysis

Cold-active α-amylase purified from *B. cereus* GA6 showed excellent stability and compatibility with a wide range of locally available commercial detergents (1% w/v) which is a mandatory requirement for cold-washing. It was most compatible with 'Tide' detergent retaining 92.27% activity after 0.5 hour incubation at 22ºC and it retained 86, 78 and 72% activity after 1.0, 2.0 and 3.0 hour incubation, respectively (Table 4). Next to Tide, the enzyme was compatible with 'Ghari' detergent exhibiting 89% activity after 0.5 hour incubation, and retained 81.8, 74.9 and 70.7% activity after 1.0, 2.0 and 3.0 hours of incubation, respectively at 22ºC.

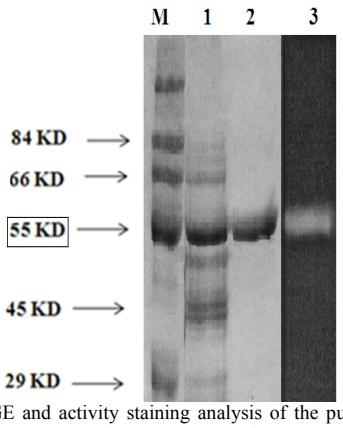

Fig. 9. SDS-PAGE and activity staining analysis of the purified α-amylase from *B.cereus* GA6. (M: Marker; Lane 1: Crude protein; Lane 2: Purified lyophilized α-amylase; Lane 3: activity staining of purified enzyme).

Remaining activity of the enzyme was more than 64% with rest of the tested detergents even after 3.0 hours of incubation.

Table 4. Compatibility of amylase from *B. cereus* GA6 with commercial detergents at 20±1 ºC

Detergents	Relative residual amylase activity (%)			
	0.5 hour	1.0 hour	2.0 hour	3.0 hour
Surf Excel	88.27	80.20	70.5	65
Wheel	85.55	79	71.38	66.22
Tide	92.27	86.11	78	72
Ariel	80.91	77.50	70	64.50
Ghari	89.07	81.80	74.92	70.70

Wash performance analysis at low temperature

The wash performance on fabrics is dependent on efficiency of amylases for the removal of stains. Result suggests that this cold-active alkaline amylase exhibited high efficiency for the removal of stains when used in combination with 'Tide' detergent at 22±2 ºC. The wash performance analysis of mixture of baby food and chocolate stains on cotton fabric showed an increase in reflectance from 56 to 78% when washed with detergent and enzyme (combined) as compared to detergent only (Fig. 10). Similarly increase in reflectance was from 52 to 82% (Fig. 10) for removal of food gravy stain from white cloth, when washed with detergent supplemented with enzyme (Fig. 11). Therefore, it may be recommended that the supplementation of the cold-active alkaline α-amylase isolated from *Bacillus cereus* GA6 could significantly perk up the cleansing of the starchy stains resulting in complete stains removal at low temperature.

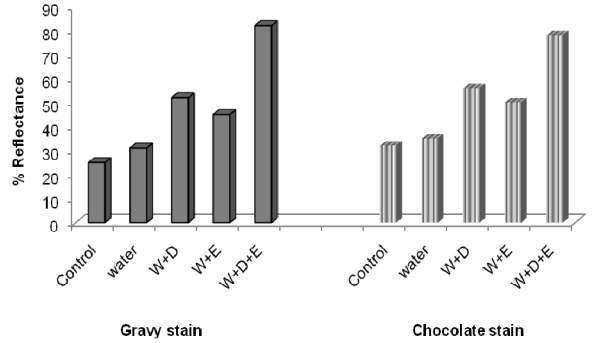

Fig. 10. Removal of food gravy and chocolate stain at 20ºC by cold-active α-amylase (reflectance analysis) (W: Water, D: Detergent, E:Enzyme)

Discussion

In industrial process, bacterial α-amylases are produced mainly from cultures of *Bacillus subtilis* var. amyloliquefaciens (Uhlig, 1998; Goyal et al., 2005). Alpha-amylases from *Bacillus stearotermophilus* and *Bacillus licheniformis* are well characterized and are profoundly used in the starch-processing industry. Since, alkali-tolerance characteristic and stability toward chelators and detergents are important features for use of amylolytic enzymes in detergents and starch-processing, amylases from psychro-tolerant bacteria are of special interest as a source of novel cold-adapted enzymes (Leveque et al. 2000; Saxena et al. 2007).

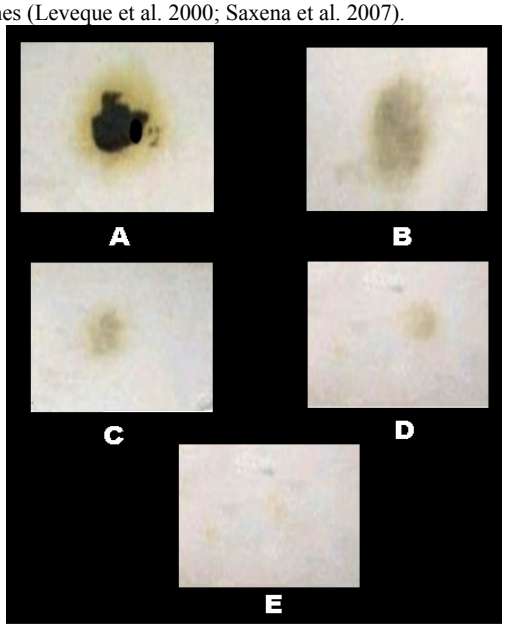

Fig. 11. Wash performance of *B. cereus* GA6 α-amylase in combination with Tide detergent at 20°C. (A) Cloth stained with food gravy; (B) Gravy-stained cloth washed with water only; (C) Gravy-stained cloth washed with enzyme only; (D) Gravy-stained cloth washed with detergent only; (E) Gravy-stained cloth washed with detergent and enzyme

Maximum α-amylase was produced in its logarithmic phase from *B. cereus* GA6 at 15°C at 96 h of incubation. Similar observations were also reported for *Bacillus* sp. (Wijbenga et al. 1991) where maximum amylase production occurred into its late logarithmic growth phase and continued to secrete well into the stationary phase. Gupta et al. (2008) also reported same time duration for highest α-amylase production but in case of *Aspergillus niger*. The optimal temperature for amylase production and growth of the *Bacillus cereus* GA6 were found to be at 20°C. Similar findings were also reported for cold-adapted *Pseudoalteromonas arctica* GS230 (Lu et al. 2010) producing maximum amylase at 20 ºC. However, *Flavobacterium balustinum* A201 (Morita et al. 1997) and *Bacillus* sp. A-001 (Lealem and Gashe 1994) showed its highest enzyme production at 30º and 35ºC, respectively. On pH optimum analyses, *B. cereus* GA6 produced a peak in alkaline side i.e. at pH 10.0. Comparable to the result, optimum pH was 9.0, 8.0 and 7.5 for *Actinomycete* strain AE-19 (Poornima et al. 2008); *Micrococcus antarcticus* (Fan et al. 2009) and *Bacillus* sp. (Lealem and Gashe 1994), respectively. The pH tolerant α-amylase from microbes are commercially important for detergent industry and can be successfully used as an additive to remove starch based stains. Result showing the necessity of Ca^{2+} for production of amylase was in agreement with Vishwanathan and Surlikar (2001) where increased production of *Aspergillus flavus* α-amylase occurs in presence of 5mM Ca^{2+}.

It has been reported that the alkaline amylase of *Bacillus* sp. strain IMD370 showed maximum activity at pH 10 (McTigue et al. 1995). Optimum pH was 8.7 as observed by Igarashi et al. (1998) and Kobayashi et al. (1992) for *Bacillus* sp. KSM-1378 and *Natronococcus* sp. AH-36, respectively. However *B. cereus* GA6 α-amylase showed maximum activity at pH 9.0. The enzyme was highly active at pH range of 7-10 for 1 h at 20ºC. The enzyme was 80% stable between pH ranges 8–11 for 1 h. These findings were highly similar with the literature result (Chakraborty et al. 2009) where α-amylase of marine *Streptomyces* sp. D1, was stable in buffer systems having pH 7–11. These results suggest that the activity of the enzyme is higher in alkaline pH and are pH stable at low temperature, making this enzyme attractive for the detergent industry. This can be further commercially utilized for hide-dehairing process, where dehairing is carried out at pH values between 8.0 and 10.0. Optimal temperature for GA6-amylase was found to be at 22ºC which decreased sharply above 37°C. The temperature profile of Antarctic bacterium, *Alteromonas haloplanctis* (Feller et al. 1994) proposed totally contradictory results where the bacterium faced temperatures close to that of their environment (-2 to 4°C) but the apparent optimal activity temperature was between 30-40°C. Ambiguity was also noticed for *A. psychrolactophilus* (Smith and Zahnley 2005) showing optimum activity between 40-50ºC. *B. cereus* GA6 α-amylase showed good temperature stability from 4-37ºC, giving maximum activity below 25ºC and thereby highly recommended for the category of true psychro-tolerant enzymes (Morita 1975). This cold-active amylase is of considerable interest as it can efficiently hydrolyze starch at ambient to low temperatures, thereby reduces costs in producing alcohol and to use in many other low-temperature applications where industrial fermentation flourishes.

Most α-amylases vary in their response to the chelators, chemical denaturants and metal ions. Almost twice activation was noticed in activity of *B. cereus* GA6 alkaline α-amylase with 5mM Ca^{2+} while Fe^{2+} and Zn^{2+} were proved to be potent inhibitors. Positive effects of calcium were also reported by Arikan (2007) where 130% increment in enzyme activity was noticed in the presence of 5mM Ca^{2+}. Same results for calcium were also reported by Sindhu et al. (2011), Brawn and Kelly (1993) and Dong et al. (1997). An inhibitory effect of Zn^{2+} on the activity of amylases was also observed by Arikan (2007), and Mamo and Gessesse (1999). *B. cereus* GA6 α-amylase has also strong inhibition by 100% with $CuSO_4$ (1%) and H_2O_2 (10mM). In order to have applications in detergent industries, amylase must be stable to various detergent ingredients, such as denaturants and chelators. The amylase from *B. cereus* GA6 exhibited 50% activity when preincubated with 0.1% SDS and 25% activity with 1% SDS at 20ºC for 30 min and also showed considerable resistance against EDTA, SDS and Urea. Several authors (Arikan 2007; Kikani and Singh 2011; Park et al. 2010) reported the existence of inhibitor resistant amylase (SDS, urea, EDTA) but were actually thermophilic or thermostable in nature. To the best of my knowledge this is the first report where cold-active *B. cereus* GA6 alkaline-amylase found to be a chelator resistant at low temperature range so are suitable in liquefaction of starch, in detergent and textile industries and in other industrial applications in fairly cold environments. This resistance, which is essential requirements, suggests that the use of enzyme as effective additive in detergents. 1 mg/ml soluble starch was found optimum for *B. cereus* GA6 amylase and it also showed less K_m (0.27 mg/ml) and high V_{max} (2500 Units/ml), suggesting strong affinity between enzyme and substrate. Our results are confirmed with the results in the literature where α-amylase of halophilic *Bacillus cereus* MS6 showed maximum enzyme activity in 1 to 1.5% of starch concentration (Al-ZaZaee et al. 2011) and a K_m of 0.88 mg/ml was obtained for α-amylase purified from *Streptococcus bovis* JB1 with soluble potato starch (Freer 1993). The results of K_m were in accordance with Li et al. (2011) and Alexsandro et al. (2011) where observed K_m was 0.27 and 0.37 mg/ml, respectively. In other reports, Bano et al. (2011) also observed the K_m and V_{max} value of 2.68 mg/ml and 1773 Units/ml, respectively for α-amylase purified from *Bacillus subtilis*. The hydrolysis pattern of *B. cereus* GA6 amylase confirmed its type alpha as obtained by TLC analysis. These findings were highly similar with the literature results (Zhang and Zeng 2008; Li et al. 2011; Michelin et al. 2010) where glucose, maltose and maltotriose were found as final hydrolysates of soluble starch by purified cold-adapted amylase of *Nocardiopsis* sp. 7326, *Rhizopus oryzae* and *Paecilomyces variotii*, respectively. Molecular mass of 55 kDa having single polypeptide chain of *B. cereus* GA6 α-amylase was confirmed by the Zymogram analysis. Similar findings were also reported for *Bacillus licheniformis* (Haq et al. 2010) and *Pseudomonas* sp. 7193 (Zhang and Zeng 2008).

Compatibility and stability of *B. cereus* GA6 alkaline α-amylase with commercial detergents at 20ºC was excellent and retaining more than 92% activity after 0.5 h of incubation with 'Tide'. A similar compatibility result was obtained for *Bacillus* sp. SMIA-2 amylase retaining 75% of its activity after 20 min. of incubation with ,Tide, but at 50ºC (Carvalho et al. 2008). Therefore, it may be suggested that the cold-active amylase from *B. cereus* GA6 is appropriate as an additive for commercial detergents for low temperature washing.

Conclusions

Cold-adapted *B. cereus* GA6 isolated from Gangotri glacier showed promising production of Ca^{2+}-dependent, alkaline α-amylase that was able to withstand a temperature up to 4°C for 24 h but having temperature optima of 22ºC. Enzyme resistance against chemical denaturants and detergents were interesting properties as very few reports of such effects exist in the literature for psychro-tolerant amylase therefore suggests the potential of this enzyme in detergent industry for laundry and automatic dishwashing at low temperatures and to keep the environment safe and clean. The organism can also find applications in environmental bioremediation at cold regions.

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