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Refolding of Misfolded Inclusion Bodies of Recombinant α-Amylase: Characterization of Cobalt Activated Thermostable α-Amylase from *Geobacillus* SBS-4S

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SM and AJ performed experimental work. MT planned and supervised the study and provided guidance for manuscript writeup. SF, ARA and NR facilitated for the conduction of experiments. BM and MW helped during manuscript writeup.

Key words
Geobacillus, SBS-4S, E.coli, Refolding, α-amylase, AMYSBS.

ABSTRACT

The present study deals with the production, refolding and characterization of recombinant α-amylase (AMY_{SBS}) from *Geobacillus* SBS-4S. AMY_{SBS} exhibited a highest identity of 99.78% with *Geobacillus thermoleovorance* GTA. *E.coli* BL21-CodonPlus (DE3) cells were used as host for expression studies of AMY_{SBS}. Recombinant AMY_{SBS} produced as inclusion bodies was transmitted to soluble active form by denaturing the insoluble protein using 8M urea followed by refolding through gradual dialysis. The refolded enzyme exhibited optimum activity at 55 °C between pH 8-9. The effect of metal ions on the activity of AMY_{SBS} showed that Co^{2+} remarkably enhanced the enzyme activity and 500µM was recorded as optimal Co^{2+} concentration for the maximal activity of AMY_{SBS}. Presence of ionic (SDS) and nonionic (Tween-20, TritonX-100) detergents showed an enhancing effect on the activity of AMY_{SBS}. Stability studies of AMY_{SBS} exhibited that enzyme was quiet stable at 55 °C. Kinetic studies demonstrated the K_m and V_{max} values of 6.67mg/ml and 2500µmol min^{-1} mg^{-1}, respectively when starch was utilized as substrate. To best of our knowledge this is the highest activity among the reported recombinant amylases from genus *Geobacillus*. Laboratory scale production of reducing sugars from cloth-starch makes AMY_{SBS} a suitable candidate to be used in Textile industry.

INTRODUCTION

Amylases are widely distributed hydrolytic enzymes involved in the cleavage of α 1-4 glycosidic linkage in starch and other related carbohydrates (Han *et al*., 2013). Starch is a tasteless polysaccharides produced by all green plants and its structure comprises of monomeric glucose units linked each other through α 1-4 glycosidic linkage (amylose) and α 1-6 glycosidic linkage (amylopectin) (Hemamalini and Dev, 2017). Microorganisms produce amylases to utilize the starch as carbon source in order to fulfil their energy requirement (Onodera *et al*., 2013). The amylases are required for various industries including the liquefaction and saccharification of starch granules, bakery, as desizing agent in textile and paper industry, brewerage, detergent and pharmaceutical industry and for the production of biofuel (Qi *et al*., 2012; Chang *et al*., 2013; Saburi *et al*., 2013; Basma *et al*., 2015).

On the basis of amino acid sequence, the amylases/ glycosyl hydrolases (GH) can be classified into more than 100 families (http://www.cazy.org/) (Onodera *et al*., 2013). Starch hydrolyzing enzymes including endoamylase, exoamylase, debranching enzyme and transferase belong to three glycosyl hydrolases families GH13, GH70 and GH77. Most of α-amylases belong to family GH13. Crystal structures of amylases from this family demonstrated the presence of catalytic triad and one arginine residue which are conserved and liable for activity of these enzymes (Matsuura *et al*., 1984; Buisson *et al*., 1987). The catalytic site consists of an aspartate residue (catalytic nucleophile), a glutamate residue (general acid/base) and aspartate residue (transition state stabilizer) (Uitdehaag *et al*., 1999). The fourth conserved arginine is located two amino acids next to catalytic nucleophile (Gregor *et al*., 2001).

Previously the production of amylases have been reported from animals, plants and microorganisms (Pandey *et al*., 2000; Qi *et al*., 2012; Ozturk *et al*., 2013; Sing and Kayastha, 2014; Qin *et al*., 2014; Li *et al*., 2017).

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The microbial enzymes are preferred due to their ease and economic production (Pandey *et al*., 2000; Subash *et al*., 2017). Among α-amylase producing microorganisms, *Bacillus* sp. are the most extensively studied microbes due to the production of thermostable enzymes (Prakash and Jaiswal, 2010) while α-amylases from some other bacteria with special properties have been reported (Bai *et al*., 2012; Kumar and Khare, 2012; Li *et al*., 2017).

Geobacillus are gram positive, endospore forming bacteria, having ability to grow at higher temperatures ranging 37 to 75°C where most of other species fail to survive (Nazina *et al*., 2001). The enzymes produce by *Geobacillus* are thermostable having ability to show resistance against extremes of pH, chemical denaturants, organic solvents and detergents (Jorgensen *et al*., 1977). *Geobacillus* SBS-4S was isolated and characterized from hot spring present in Northern areas of Pakistan. This strain has ability to produce several industrially important enzymes (Tayyab *et al*., 2011a). Previously the production and characterization of lipase and carboxypeptidase (Tayyab *et al*., 2011a, b) from this strain have been reported. Current study deals with the characterization of recombinant α-amylase from this strain.

MATERIALS AND METHODS

Microbial culture of *G.* SBS-4S was utilized for the isolation of genomic DNA (Kronstad *et al*., 1983). Nanodrop (Thermo Scientific, Wilmington, USA) was utilized for the DNA quantification.

PCR amplification of AMY_{SBS} gene

The gene was amplified using AMY-N (5'-**CATATG**G CGGAAAAAGAAGAACGGACGTGGC) and AMY-C (5'-CTATTCCGGCATCCGCTTCGCCCGTTTTT) as forward and reverse primers, respectively, using genomic DNA from *G.* SBS-4S as template. The bold sequence in forward primer was the unique restriction site of *Nde*I. Amylase gene sequence of *Geobacillus kaustophillus* was utilized for designing the primers, as this is the closest homologue of strain SBS-4S on the basis of 16S rRNA. The amplified PCR product was purified using DNA purification kit (GeneAll, Seoul, Korea).

Cloning of AMY_{SBS} gene in pTZ57R/T

The purified PCR product was ligated in the pTZ57R/T using InsTAclone PCR Cloning Kit (Thermo Scientific, Life Sciences, USA) and this ligated material (pTZ-AMY) was utilized for the transformation of *E. coli* DH5 α competent cells and selection of positive clones was done on the basis of blue/white screening. Plasmid DNA from selected clones was isolated by alkaline lysis method (Sambrook and Russell, 2001). Restriction digestion using *Nde*I and *Hin*dIII endonucleases was performed to check the presence of insert in the recombinant plasmid (Sabir *et al*., 2017).

DNA sequencing and phylogenetic analysis

The positive clone after restriction analysis was utilized for DNA sequencing (Sanger *et al*., 1977). The DNA sequence was submitted in DNA Data Bank, Japan (Accession No. AB971162) and was used for homology and comparative analysis using NCBI BLAST and Clustal Omega Software (Altschul *et al*., 1990; Thompson *et al*., 1994). MEGA 4 software was utilized for the construction of phylogenetic tree (Tamura *et al*., 2007).

Expression studies of AMY_{SBS} gene

The AMY_{SBS} gene was transferred from pTZ-AMY to pET-21a already restricted with the same restriction endonucleases. The ligated vector (pET-AMY) was utilized for the transformation of DH5 α cells. The restriction analysis of pET-AMY was done to analyze the presence of insert in the vector. BL21-CodonPlus (DE3) was used as expression host after transformation using pET-AMY and these transformed cells were utilized for the production of recombinant amylase.

The overnight grown transformed BL21-CodonPlus (DE3) cells were diluted 100 times with fresh Lauria berteni Medium (1% Tryptone, 0.5% Yeast extract, 0.5% NaCl) and was incubated at 37°C till the OD at 660nm reached to 0.4. The cells were induced with 0.1mM Isopropyl-β-D-thiogalactopyranoside (IPTG) followed by incubation for another 4.5h at 37 °C. Cells were harvested by centrifugation (Z300K, Hermle, Germany) at 8,000 rpm for 15 min and re-suspended in 50mM Tris-HCl buffer (pH 8) and were lysed by sonication. The production of soluble or insoluble AMY_{SBS} was examined by SDS-PAGE analysis (Laemmli, 1970). Expression of AMY_{SBS} was also examined at low temperature, for this, the inoculated medium after induction with IPTG, was incubated overnight at 20°C.

Refolding and purification of AMY_{SBS}

The insoluble AMY_{SBS} produced in the form of inclusion bodies was denatured using 8M urea in Tris-HCl buffer (pH 8). The soluble (denatured) protein was separated from insoluble material by centrifugation and the soluble protein was transferred to dialysis tube and the urea was removed by fractional dialysis. The urea free protein sample was centrifuged and supernatant was utilized for the purification. Initially the sample was loaded on pre-equilibrated DEAE-Sephadex column and the unbound protein was removed by washing the column with 50mM Tris-HCl buffer (pH 8) and the elution was done with NaCl gradient. Molecular mass of AMY_{SBS} was determined by Sephadex G-75 size exclusion column chromatography. The elution was done with 50mM Tris-HCl buffer (pH 8).

Protein contents of fractions were determined by Bradford (1976) method and the purity was analyzed by SDS-PAGE.

Activity assay

Activity assay mixture (500 µl) was prepared by taking 200 µl of 50mM Tris-HCl buffer (pH 8), 200 µl of 1% starch dissolved in same buffer and 100 µl enzyme solution. The assay mixture was incubated at 55 °C for 30 min and the release of reducing sugars was estimated at 540 nm using Dinitrosalicylic acid (DNS) method (Shah *et al*., 2014). Standard curve was prepared for glucose and was utilized for the calculation of activity units. One unit of enzyme activity was defined as the amount of enzyme required to release 1 µmol of reducing sugar per min.

Effect of temperature and thermo-stability studies of AMY_{SBS}

Effect of temperature on the activity of AMY_{SBS} was examined at pH 8 using 50 mM Tris-HCl buffer by incubating the reaction mixture at various temperatures ranging 40-70 °C. Thermostability studies of AMY_{SBS} was done at 55 and 60 °C. The enzyme was incubated at the selected temperature and enzyme fractions were withdrawn after regular intervals and were utilized for activity assay as described above.

Effect of pH, metal ions and detergents on the AMY_{SBS} activity

Effect of pH on the AMY_{SBS} activity was examined by measuring the production of reducing sugars at various pH ranging 4-11 using 50 mM of each of acetate buffer (4-5), phosphate buffer (5-7), Tris-HCl buffer (7-9) and glycine/NaOH (9-11) using 0.4% starch as substrate.

In order to examine the effect of metal ions, the activity assay was conducted in the presence of various metal cations (Ca^{2+}, Mg^{2+}, Co^{2+}, Cu^{2+} and Zn^{2+}) at a final concentration of 1mM. Chloride salts of metal ions were utilized during these studies. Effect of detergents was also examined on the activity of AMY_{SBS}. The activity assay was done in the presence of (0.1%) ionic (SDS) and non-ionic (Tween-20, Tween-80 and Triton X-100) detergents.

Kinetic studies of AMY_{SBS}

AMY_{SBS} activity was recorded with the increasing concentrations of starch (2-10 mg/ml) and the data obtained was utilized for the estimation of kinetic parameters.

Suitability of AMY_{SBS} for textile industries

A piece of cotton cloth (18×9 cm) was incubated at 60 °C with 10% starch for 15 min. The cloth was dried and cut into two equal pieces (9×9 cm). One piece was incubated with enzyme at 55 °C for 30 min in 50 mM Tris-HCl buffer and released reducing sugars was estimated as mentioned above. Second piece was used as negative control and was treated under the same above mentioned conditions except enzyme.

RESULTS

Cloning of AMY_{SBS} gene

PCR resulted in the amplification of approximate1.5 kb AMY_{SBS} gene. The AMY_{SBS} gene was ligated in pTZ57R/T. Restriction digestion with *Nde*I and *Hin*dIII resulted in the liberation of insert from the pTZ-AMY which confirmed cloning of AMY_{SBS} gene in pTZ57R/T. The cloned fragment was sequenced. DNA sequence comparison of AMY_{SBS} gene (AB971162) showed sequence similarity (identity) of 99.86% with *G. thermoleovorans* CCB-US3-UF5 (CP003125), 99.64% with *Anoxybacillus amylolyticus* (AB908318), 99.43% with *Geobacillus* sp. GXS1 (FJ481119), 94.11% with *G. kaustophilus* (BA000043) and 92.26% with *Geobacillus* sp. GHH01 (CP004008).

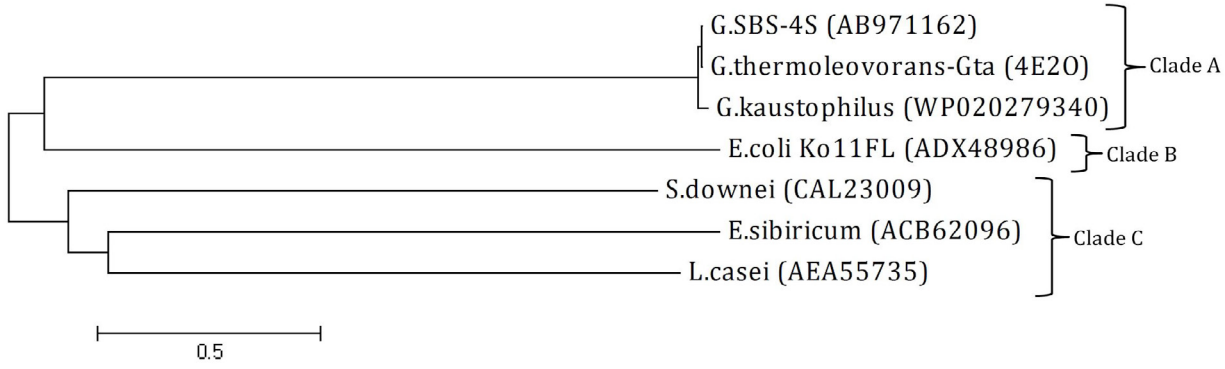

Fig. 1. Phylogenetic tree: The tree was constructed using amino acid sequence of amylase from *Geobacillus* SBS-4S and reported sequences from NCBI GenBank. The name at the end of each branch present the bacterial strain with accession number from which the amylase sequence was originated. Clades A, B and C present the GH family 13, 77 and 70, respectively. The tree was constructed at a genetic distance of 0.5 using Mega 4 software.

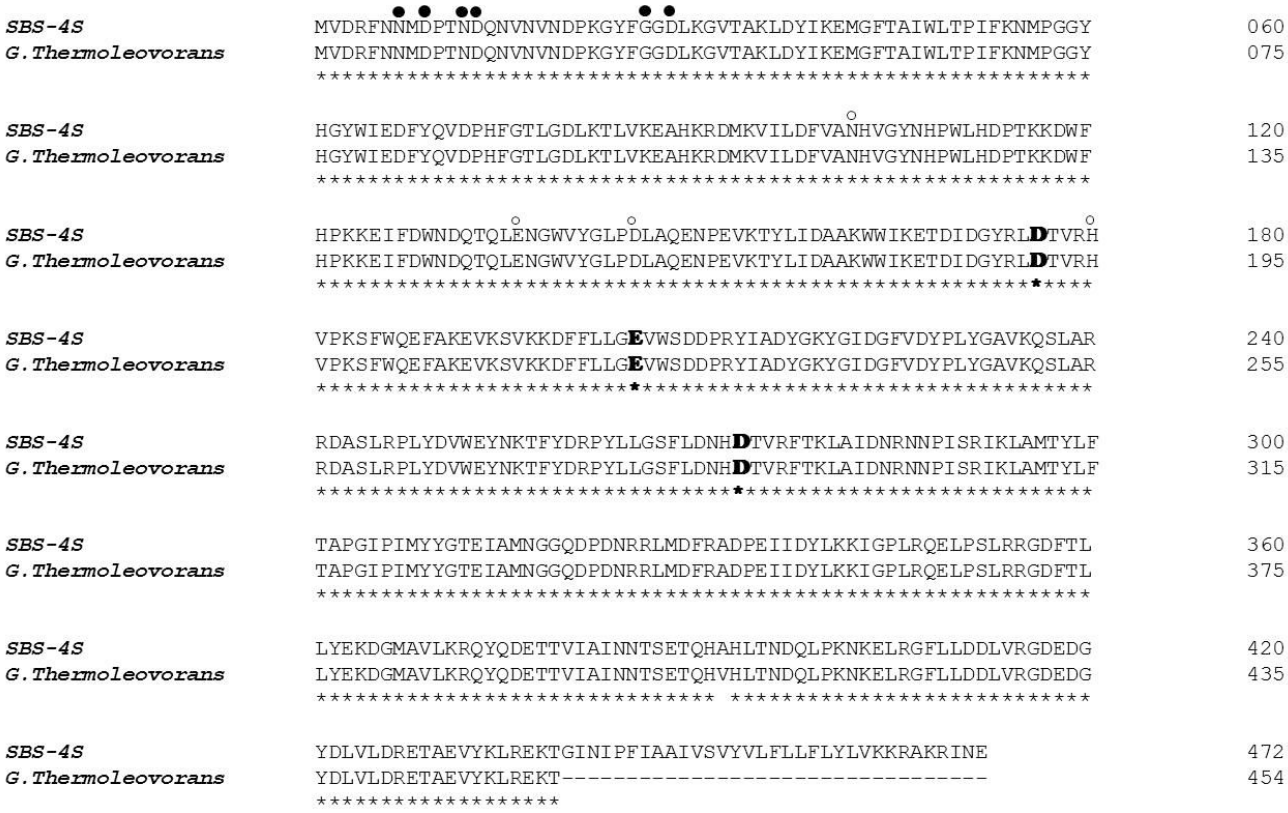

Fig. 2. Amino acid sequence comparison of AMY_{SBS} (AB971162) with its closest homologue *G. thermoleovorans* (4E2O) that has been characterized. Identical amino acids are shown by asterisks below the sequence. The names at left hand side, indicates the organism from which the sequence originated. The active site residues are shown by bold letters. The open and closed circles above the sequence represent the amino acids involved in the binding of metal ions.

Phylogenetic analysis of AMY_{SBS}

Phylogenetic analysis on the basis of amino acid sequence of AMY_{SBS} with the reported amylases indicated that AMY_{SBS} clustered with various members of genus *Geobacillus* in clade A (Fig. 1). Among the characterized members of *Geobacillus*, GTA amylase from *G. thermoleovorance* CCB-US3-UF5 was recorded to be the closest neighbor of AMY_{SBS} as both the amylases shared a sequence identity of 99.79% on the basis of amino acid sequence. This analysis indicated that the GH family 13 (clade A, Fig. 1) and 77 (clade B, Fig. 1) has been evolved from a common ancestor whereas these two families share less homology with GH family 70 (clade C, Fig. 1).

Comparative analysis of AMY_{SBS}

Comparative analysis of AMY_{SBS} amino acid sequence with various members of *Geobacillus* showed sequence identity of 99.79% with *G. thermoleovorans* CCB_US3_UF5 (4E2O) and *Geobacillus* sp. MAS1 (WP023633941); 98.94% with *Geobacillus* sp. GXS1 (ACK58047); 98.09% with *Geobacillus* sp. WSUCF1 (WP020755052); 97.03% with *G. kaustophilus* (WP020279340) and 96.4% with *G. sp.* GHH01 (WP015374071). *G. thermoleovorans* was the only reported member from this genus with the fully characterized recombinant amylase (GTA) which belongs to GH family 13. Sequence comparison demonstrated the conserved amino acids for incorporation of Metal-I (Asn^{7}, Asp^{9}, Asn^{12} and Asp^{13}) and Metal-II (Asn^{102}, Glu^{136}, Asp^{145} and His^{180}) while three amino acids Asp^{176}, Glu^{205} and Asp^{273} (AMY_{SBS} numbering) were active site residues essential for the activity (Fig. 2).

Expression studies of AMY_{SBS} gene

In-order to examine the expression studies, the AMY_{SBS} gene was sub-cloned in pET-21a. The restriction digestion of pET-AMY using *Nde*1 and *Hind*III resulted in the liberation of 1.5 kb AMY_{SBS} gene fragment. SDS-PAGE analysis of expressed protein indicated that almost 95% of the AMY_{SBS} was produced in the form of inclusion bodies (lane 3, Fig. 3) and 5% as soluble protein (lane 4, Fig. 3). Same pattern of production was reported for recombinant lipase from this strain (Tayyab *et al*., 2011a).

The production of AMY_{SBS} was also examined at low temperature (20 °C) but the decrease in temperature could not produce the AMY_{SBS} in active form. It was difficult to purify the AMY_{SBS} from soluble fraction due to its low quantity (lane 4, Fig. 3). The refolding of the AMY_{SBS} resulted in conversion of inactive inclusion bodies to properly folded active protein. The purified protein (lane 5, Fig. 3) after column chromatography was utilized for the characterization of AMY_{SBS}.

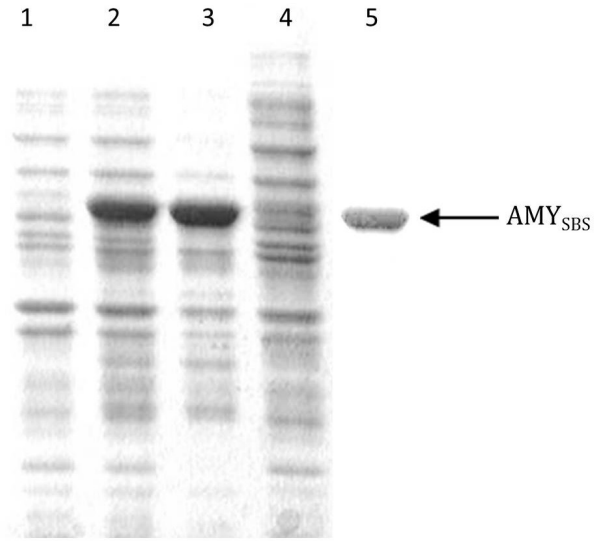

Fig. 3. Coomassie brilliant blue stained sodium dodecyl sulphate polyacrylamide gel showing expression of AMY_{SBS} gene: Lane 1, the soluble portion after lysis of BL21-CodonPlus (DE3) cells transformed with pET-21a without insert (negative control); Lane 2, the total cell protein after lysis of BL21-CodonPlus (DE3) cells transformed with pET-AMY; Lane 3, insoluble portion after lysis of sample in lane 2; Lane 4, soluble portion after lysis of sample in lane 2; Lane 5, purified AMY_{SBS} after column chromatography.

Effect of temperature and pH on AMY_{SBS} activity

Effect of temperature on the AMY_{SBS} activity (Fig. 4A) demonstrated that the activity was increased with the increase in temperature from 40 to 55°C whereas further increase in temperature beyond 55°C resulted in the decreased enzyme activity. The optimal temperature for the activity was recorded as 55°C. Thermostability studies showed that the protein remained stable at 55°C even after half an hour whereas more than 50% residual activity was recorded after 15 min when the protein was incubated at 60°C (data not shown). When the activity was examined at various pH (Fig. 4B), it was observed that increase in pH from 4 to 8 resulted in the increased AMY_{SBS} activity with the optimal activity between pH 8 to 9 in 50 mM Tris HCl buffer, whereas a decline in the activity was recorded at pH above 9.

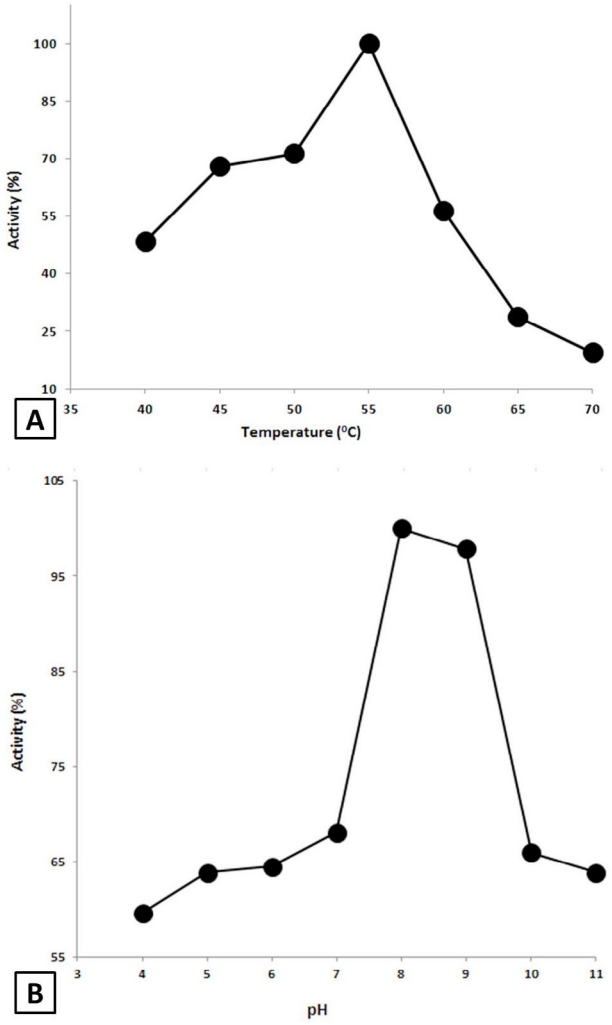

Fig. 4. Effect of Temperature and pH on the AMY_{SBS} activity. **A**, Effect of temperature. The activity was done at various temperatures ranging from 40 to 70°C in 50 mM Tris-HCl buffer (pH 8) using 0.4% starch as substrate. **B**, Effect of pH on the activity of AMY_{SBS}. The activity was examined in 50 mM of each of acetate buffer (4-5), phosphate buffer (6-7), Tris-HCl buffer (8-9) and Glycine/NaOH (10-11) using 0.4% starch as substrate at 55°C.

Effect of metal ions and detergents on the AMY_{SBS} activity

No significant effect on the AMY_{SBS} activity was recorded in the presence of 1 mM Cu^{2+} or Zn^{2+}, whereas slight enhancing effect on the activity was observed when enzyme assay was done in the presence of Ca^{2+} or Mg^{2+} at same concentration. A 3.4 folds enhancement in the activity was recorded in the presence of 1 mM Co^{2+} (Table I) which demonstrated that AMY_{SBS} requires Co^{2+} as cofactor and

500 µM Co^{2+} was recorded as the concentration for the optimal AMY_{SBS} activity. Presence of ionic and non-ionic detergents showed an enhancing effect on AMY_{SBS} activity. Tween 80 and SDS showed a respective increase of 4.3 and 4 times in enzyme activity when used at a final concentration of 0.1% (Table I). AMY_{SBS} activity was slightly enhanced in the presence of Triton X-100.

Table I.- Effect of metal ions and detergents on AMY_{SBS} activity.

Divalent cation or detergent	Relative activity
None	100
Metal[a]	1mM
Zn^{2+}	115
Cu^{2+}	105
Mg^{2+}	169
Ca^{2+}	138
Co^{2+}	346
Detergent	0.1%
Triton X-100	195
Tween 20	133
Tween 80	435
SDS	408

[a]Metal chlorides were used in the essay.

Kinetic studies of AMY_{SBS}

A linear increase in activity was observed when the concentration of starch was increased from 2 to 10 mg/ml. The data was utilized for plotting the Line-Weaver Burk Plot (Fig. 5). The kinetic parameters k_m and V_{max} were recorded as 6.67mg/ml and 2500µmol min^{-1} mg^{-1} respectively. Suitability of AMY_{SBS} for textile industry was examined at laboratory scale. The incubation of AMY_{SBS} with starch containing cloth resulted in the release of 634 µmoles of reducing sugars as compared to control at 55°C.

DISCUSSION

Aim of the study was to clone and characterize the amylase from locally isolated *Geobacillus* SBS-4S as amylases have vital importance and are required by various industries. On the basis of 16SrRNA gene sequence, *G. kaustophilus* was reported to be the closest homologue of *Geobacillus* SBS-4S (Tayyab *et al*., 2011a) whereas the amylase from this strain (present study) showed maximum identity with *G. thermoleovorans* while the lipase from this microbe was found more closer to *Geobacillus stearothermophilus* (Tayyab *et al*., 2011a).

AMY_{SBS} showed maximal production of 2500 µmol min^{-1} mg^{-1} that is quiet higher as compared to naturally produced 500 µmol min^{-1} mg^{-1} by *G. thermoleovorans* (Maheswar and Satyanarayana, 2007) or 222 µmol min^{-1} mg^{-1} by *Geobacillus* sp. IIPTN (Dheeran *et al*., 2010) or 330 µmol min^{-1} mg^{-1} by *G. thermoleovorans* subsp. (Ilaria *et al*., 2011). Whereas a higher level of production was recorded in some bacilli, that could produce 4,133 U mg^{-1} by *Bacillus subtilis* AX20 (Najafi *et al*., 2005) or 3,239 U mg^{-1} by *Alicyclobacillus acidocaldarius* (Satheesh *et al*., 2010).

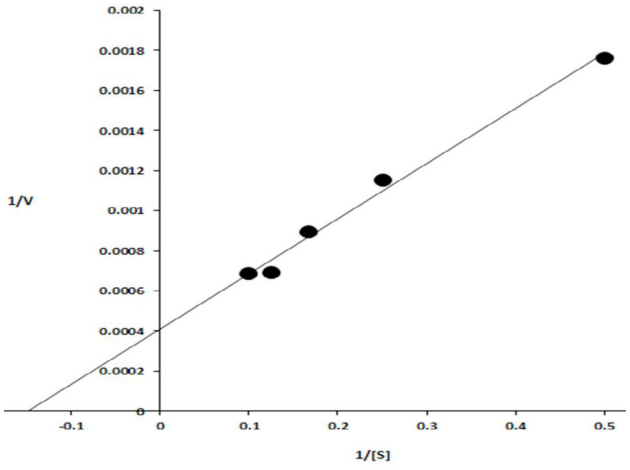

Fig. 5. Lineweaver–Burk plot obtained by taking the inverse of the substrate concentrations (mg/ml) along X-axis and velocities (µmol min^{-1} mg^{-1}) along Y-axis.

Previous reports demonstrated that Ca^{2+} act as cofactor and involved in the stabilization of amylases from *G. Stearothermophilus*, *G. thermoleovorans*, *A. acidocaldarius* and *B. subtilis* (Konsula and Liakopoulou, 2004; Satheesh *et al*., 2010; Ilaria *et al*., 2011; Fincan and Baris, 2014) but AMY_{SBS} showed maximum activity in the presence of Co^{2+} while Ca^{2+} didn't put significant effect on the activity of this enzyme. Same pattern of behavior was reported for amylases from *G. thermoleovorans* and *Anoxybacillus flavithermus* (Maheswar and Satyanarayana, 2007; Aguloglu *et al*., 2014). On the other hand, presence of Ca^{2+} showed an inhibitory effect on the amylase activity from *Anoxybacillus flavithermus* (Aguloglu *et al*., 2014) whereas, both Ca^{2+}or Co^{2+} put inhibitory effect on amylase activity from *Bacillus* sp. TM1 (Sajedi *et al*., 2004).

CONCLUSION

In this study we produced the recombinant α-amylase from locally isolated *Geobacillus* SBS-4S and the insoluble and inactive AMY_{SBS} was refolded to soluble active form

that was utilized for the characterization. AMY_{SBS} showed a high level of activity at a broad range of temperature and pH. Moreover, release of reducing sugars due to hydrolysis of starch from cotton cloth, make it a suitable candidate for its use in textile industry.

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Statement of conflict of interest

The authors declare that there is no conflict of interests regarding the publication of this article.

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