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Purification and characterization of Thermostable and Calcium independant α- Amylase from *Streptomyces griseus* TBG19NRA1

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Abstract

The alpha amylase is one of the most important industrial enzyme which has wide range of commercial interest. The amylolytic enzymes active at high temperatures (90^{o}C) and calcium independent would benefit starch-processing industries. A mesophilic isolate of *Streptomyces griseus* TBG19NRA1 produces extracellular thermo stable α-amylase. The strain showed maximum α-amylase activity at the end of 48 h of incubation at pH 7. The enzyme was purified and molecular weight of 60 kDa was obtained. The purified α-amylase reported maximum relative activity at 80^{o}C without the addition of $CaCl_2$ and revealed the enzyme is thermostable in nature. The amylase activity was strongly stimulated by Fe^{3+} and Mg^{2+} (5 mM). The chemical inhibitors and denaturants like DTT, BME and EDTA slightly stimulated enzymatic activity. The analysis of kinetic showed that the enzyme resulted *Km* of 1.6 mg/mL and *V* max of 28 mg/mL/min at pH 7. The Peptide mass fingerprinting using LC-MS analysis and subsequent Uniprot database search confirmed the enzyme as α-amylase. The present studies focus on the optimization of production conditions and purification of thermostable α-amylase from *S. griseus* TBG19NRA1.The purified alpha amylase were thermostable at 80°C and calcium independent in nature. The enzyme purifies were suitable in liquefaction of starch in high temperature and in starch based industrial applications.

The alpha amylase (endo-1,4-α-d-glucan glucanohydrolase, E.C 3.2.1.1), is one of the widely distributed hydrolyses in nature, belongs to family 13 of the glycoside hydrolase group of enzymes. This is an extra cellular endo-enzyme which cleaves internal α-1,4-glycosidic linkage in starch and resulted byproducts such as glucose, maltose, and dextrins[37]. This industrial enzyme shares approximately 25–33% of the world enzyme market, in second place after proteases and have wide range of commercial interests in starch liquefaction process, toxic water removal, brewing, alcohol production, baking, textile production, paper recycling and other industries[31,42]. A huge number of α-amylases obtained from different sources including various groups of microorganisms, have been available as of now but many of them are not meeting the industrial demands. Amylolytic enzymes are mainly characterized from bacteria like *Bacillus* spp. and filamentous fungi like *Apergillus* spp [31].

The α-amylase from *Bacillus licheniformis* (BLA) has been most widely used enzyme for processing starch since 1980 (Wang *et al.*)[40]. This enzyme requires the addition of calcium (Ca^{2+}) for its thermostability and functions optimally at 90°C and pH 6–7 (Violet and Meunier)[39]. So use of BLA increases the number of industrial process steps and cost as above conditions are significantly different in normal starch liquefaction process. The industrial starch liquefaction process, dissolving starch fraction requires a pH of ~4.5, and the next processing step involves liquefaction of starch to glucose oligomers, ideally performed at pH 4.5 and 105 °C. This has prompted the search of novel α-amylases with thermostability, calcium independency, pH tolerance, oxidant stability and very high starch hydrolyzing efficiency.

The members of actinobacteria are emerging as an alternative source of α-amylases as evidenced from literature survey. α-Amylases are reported from many species of *Streptomyces*, includes *S. praecox* [33] *S. hygroscopicus*[23] *S. albus*[6] *S. griseus* IMRU3570 (Vigal *et al.*)[38] *S. thermocyaneo-violaceus*[15]. The continual search for the novel enzymes with robust characters leads to the isolation, characterization and purification of thermostable α-amylases enzymes from *S. erumpens* MTCC 7317 (Kar *et al.*)[17] thermophilic actinomycete like *Streptomyces* sp. MSC702 (Singh *et al.*)[31] and haloalkaliphilic marine *Streptomyces* strain A3 (Chakraborty *et al.*)[9]. Actinomyces such as *S. avermitilis, Streptomyces strain* A3 and *S.* sp Al-Dhabi-46 had been reported for the production of amylases[2,14,27]. Though the amylolytic enzymes active at high temperatures (90°C) would benefit starch-processing industries and several thermophlic organisms are versatile producers of the thermostable α-amylases, the production processes at higher temperatures will require new process design[20].

The processes using thermophiles still lack the maturity of classical processes with mesophilic microorganisms[7]. This observation highlights the importance of searching for

thermostable and calcium independent α-amylases from mesophilic organisms. The *Streptomyces griseus* TBG19NRA1 (Synonym: *S. setonii*) is a mesophilic actinomycete strain isolated and characterized from the forest soil collected from Neyyar wild life sanctuary of Western Ghats of Kerala. This strain is producing an antimicrobial metabolite at its optimum conditions where starch is used as a sole carbon source Shiburaj and Abraham[29]. This observation leads to the identification of its ability produce amylase enzymes. The present studies focus on the optimization of production conditions and purification of thermostable α-amylase from *S. griseus* TBG19NRA1.

Microorganism and culture conditions:

A mesophilic actinomycete, *Streptomyces griseus* TBG19NRA1 (MTCC 3756) isolated from forest soils collected in Neyyar WLS of Kerala, India was used in this study. The strain was maintained on Sabouraud's agar plates at 4°C. All the culture media were autoclaved at 121°C (15 lbs) for 20 min.

Improvement of α-Amylase production:

The amylase activity of the strain was demonstrated on starch-casein agar plate containing 1% soluble starch after 4 days of incubation at 30°C, the plates were stained with Gram's iodine solution (0.2% I_2 and 2% KI). α-Amylase production in submerged fermentation (SmF) was carried out in 250mL Erlenmeyer flask using basal medium containing 6.0 g Na_2HPO_4, 3.0 g KH_2PO_4, 5.0g NH_4Cl, 0.5 g NaCl, 0.15 g $CaCl_2$, 0.25 g $MgSO_4\,7H_2O$, 0.2 g Casein hydrolysate, 0.10g Yeast extract, 10 g Starch per liter of distilled water at pH 7.0. Cotton plugged flask was autoclaved at 121°C for 20min and cooled. The medium was inoculated with 1% inoculum and incubated at 30°C and 120 rpm. The physiological parameters like, the effect of incubation period and initial pH of the medium were investigated by analysing amylase activity and total protein at different time points and at a varying pH (4-10). Samples were harvested by filtering through Whatman filter papers 1 and centrifuged at 10,000 g for 10 min at 4°C; the cell-free supernatant (crude enzyme) was used for α-amylase assay.

Amylase Assay and protein Determination:

The α-amylase activity estimation were done by analysing reducing sugar released during hydrolysis of 1.0% (w/v) starch in 0.1M phosphate buffer (pH 7.0) by enzyme (cell-free supernatant) incubated at 80°C for 10min.The amount of reducing sugar level released in the mixture was determined by the dinitrosalicylic acid (DNS) method Miller[24]. Absorbance at 550 nm was recorded by using UV-visible spectrophotometer (UV-1700 Pharmaspec Shimadzu) and activity was calculated from a standard curve using maltose as the standard. One unit (U) of enzyme activity was defined as the amount of enzyme required for the liberation of 1 μmol reducing sugar as maltose per minute under standard assay conditions. Total protein were estimated by using BSA (bovine serum albumin) as standard, as described by Lowry *et al.*,[22]. All experiments were carried out in triplicate and the data presented are average values.

Amylase purification:

The various steps of enzyme purification were carried out at 4°C unless otherwise mentioned. The crude enzyme was treated with solid ammonium sulphate with continuous overnight stirring and separation into the following saturation ranges: 0–20%, 20–40%, 40–60%, and 60–80%. The precipitates collected by centrifugation (10,000 g for 15min) were dissolved in 0.1Mphosphate buffer pH 7.0. The enzyme solution was dialysed against the same buffer for 12 h with several changes to remove the salt. The desalted enzyme solution was applied to a Sephadex G-100 column (30 cm×1.5 cm) previously equilibrated with the same buffer. The active fractions were eluted, pooled, and analyzed for amylase activity. The molecular weight of α-amylase was determined by comparing its electrophoretic mobility with that of standard proteins having known molecular weights on a 12% SDS-PAGE. A zymogram analysis was done by washing the gel twice with 0.5% (*v*/*v*) Triton X-100 for 15 min to remove SDS. After washing of SDS, the gel was incubated in 2.0% soluble starch in buffer at 50°C for 20 min. The gel was then kept in the same buffer for 10 min at 50°C and then stained with iodine solution (0.3% I_2 in 3% KI) for 3 min. Enzyme activity was visible as a pale yellow band in dark colored gels [25].

Tryptic Digestion and LC-MS/MS Analysis:

In-gel Tryptic digestion was done essentially as described earlier with minor modifications[5]. The single band of purified α-amylase obtained on 10% SDS-PAGE was cut into 1 mm^2 pieces, were destained three times (15 min each change) using 25 mM ammonium bicarbonate made in 50% acetonitrile. After dehydration in 100% acetonitrile for 15 min, the gel pieces were dried under vacuum for 30 min. Dried gel pieces were rehydrated for 30 min on ice with 600 ng of trypsin (Invitrogen) dissolved in 5 µl of 100 mM ammonium bicarbonate in 10% acetonitrile. The reduced proteins were subsequently alkylated by incubating gel pieces with 20µl of 40mM ammonium bicarbonate in 10% acetonitrile and incubated at 37°C for 16 h. After reduction and alkylation peptides were extracted from gel pieces using 25µl of 0.1% trifluoroacetic acid (TFA) in 60% acetonitrile and then with 20µl of 100% acetonitrile. Extracted peptides were vacuum dried, desalted using C18 tips dried and stored at 4°C. Dried tryptic peptides were suspended in 10 µl of 0.1% formic acid (FA) and finally analyzed using LC-MS/MS.

Tryptic digested peptides were pooled and analyzed using a Thermo Easy nLC 1000 (Thermo, USA) coupled to Orbitrap Velos Pro mass spectrometer (Thermo, USA) as described earlier[29]. The peptide sequences from the MS/MS spectra acquired from an Orbitrap Velos Pro mass spectrometer were analyzed by Proteome Discoverer v1.4 using Mascot (Matrix Science, London, UK; version 2.4.1.0) and the inbuilt Sequest HT search algorithm. Both Sequest HT and Mascot were configured to search the complete proteome of alpha amylase protein sequences in *Streptomyces* (A0A059WI20 *S. albulus*/ M3G0D4 *S. bottropensis* ATCC 25435/ D9WE18 *S. himastatinicus* ATCC 53653/ D9Y0J5 *S. griseoflavus* Tu4000/H1Q7Z5 *S.*

coelicoflavus ZG0656/ L1KPE8 *S. ipomoeae* 91-03) downloaded from the Uniprot database. The sequences were downloaded from the Uniprot website using the download option - "FASTA (canonical & isoform)". Essential peptides with a high probability (MASCOT scores exceeding threshold; $p < 0.05$) were referred to as hits. Scores thus obtained were derived from peptide ion scores as a non-probabilistic basis for ranking proteins.

Characterization of α-Amylase :

Effect of Temperature and pH on Enzyme stability:

The optimum incubation temperature was examined by carrying the enzyme-substrate reaction for 10min at different temperatures (30–100°C) keeping constant pH7.0 (0.1Mphosphate buffer), with or without $CaCl_2$ (0.2 M) and compared with standard amylase of *Bacillus licheniformis* (Sigma-Aldrich). Further optimum reaction time was determined by carrying the enzyme-substrate reaction at optimum temperature (80°C) and constant pH 7.0. The pH optima of the α-amylase were estimated by preparing the reaction mixture with various pH buffers and assayed for 10 min at 80°C. Three buffers (0.1M) were used for different pH, that is, phosphate-citrate buffer for pH 3.0, 4.0 and 5.0, phosphate buffer for pH 6.0, 7.0 and 8.0, and glycine-NaOH buffer for pH 9.0 and 10.0. Enzyme activity was expressed as percentage relative activity.

Effect of various reagents on Enzyme activity :

Effect of various additives such as salts of 8 metal ions (5mM) (Na^+, K^+, Mn^{2+}, Mg^{2+}, Ba^{2+}, Zn^{2+}, Fe^{3+} and Ca^{2+}) and different reagents like DTT, EDTA, SDS, Urea, 2-Mercaptoethanol and PMSF (5mM) on enzyme activity was tested by incorporating 1mL solution of each additive in enzyme substrate reaction mixture. The reaction was carried out for 30 min. Enzyme activity was measured under standard assay conditions. Enzyme activity were determined as the percentage of relative activity of the control (without additives) are considered as having 100%.

Steady State kinetics measurement:

Determination of the kinetic parameters for the hydrolysis of α-amylase activity were calculated according to the method of Lineweaver-Burk plot by using the starch as substrate in concentrations ranged from 0.025 - 2 mg/mL. Values of maximum rate; V max (mU/mg/min) and Michaelis-Menten constant; Km (mg/mL) were determined and all the reactions were carried out at 80°C and pH of 7.0.

The Microbial strain and Amylolytic activity:

The *Streptomyces* strain used in this study was isolated previously from the forest soil Shiburaj and Abraham[30]. The strain was identified as and identified as *S. griseus* TBG19NRA1 (synonym: *S. setonii*) based on morphological, biochemical and 16S ribosomal ribonucleic acid (rRNA) gene sequencing and deposited to Microbial Type Culture Collection and Gene Bank (MTCC 3756), CSIR-IMTECH, Chandigarh, India. The 16S rRNA

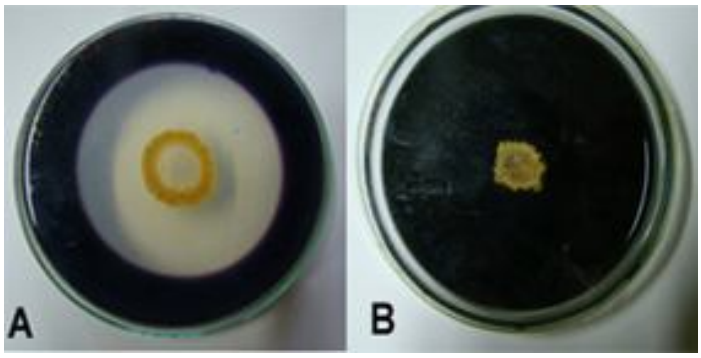

Figure 1: Amylolytic Activity of *S. griseus* TBG19NRA1: Four days growth on starch-casein agar plate with 1% starch, stained with Gram's iodine solution. A- *S. griseus* TBG19NRA1, B - *S. peucetius* (MTCC 3000) as negative control

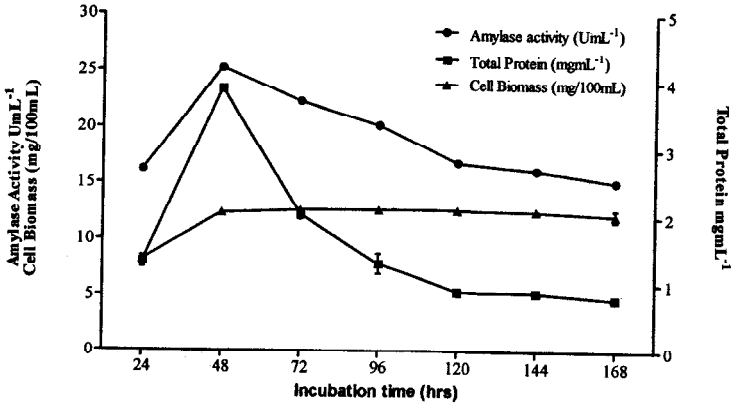

Figure 2: Effect of Incubation time on α-amylase activity, total protein production and growth of *S. griseus* TB19NRA1 under submerged condition.

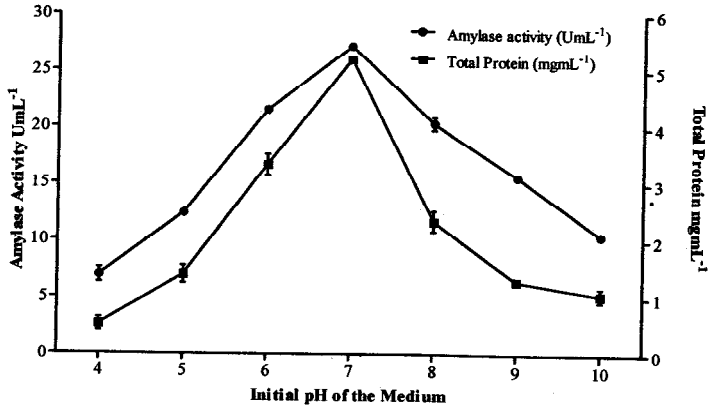

Figure 3: Effect of initial pH (4-10)on the α-amylase and total protein production by *S. griseus* TBG19NRA1 under submerged condition.

gene sequence of organism was deposited in GenBank with accession number KX269853. The amylolytic activity of the strain was demonstrated on starch-casein agar plate containing 1% soluble starch after 4 days of incubation at 30°C. The clear zone around the bacterial growth after staining with Gram's iodine solution indicated the high amylolytic activity of the strain (figure 1).

Production and purification of α-Amylase:

The strain TBG19NRA1was inoculated on production media and incubated for 7 days (30°C at 120 rpm). Time profiles of enzyme activity and growth are shown in (Figure 2). The α-Amylase production of the strain initiated in its early log phase (24h), attain its maximum production at 48h (25 U/mL) and declined thereafter. The result suggested that α-amylase production of the strain is independent of growth phase and according to Dona *et al.*,[11] the enzymatic activity result in the accumulation of sugars over a critical concentration in the medium may inhibit the enzyme production further in the stationary phase. The Similar results were also observed in the α-amylase production in *S. rimosus*[41] and in thermophilic *Streptomyces* sp. MSC702 (Singh *et al.*)[32]. While in *S. erumpens*, it is reported be at 36 h of incubation[17]. Prolonged time of incubation were observed in *S. griseoflavus*[35] and *S. clavifer*[1] at 68 h and 96 h respectively. The pattern of amylase activity was highly influenced by the initial pH of the medium (Figure 3). The strain showed maximum activity (27.1 U/mL) at pH 7.0. Amylase production in *Streptomyces* sp. MSC702 (Singh *et al.*)[32] and *S. erumpens*[17] MTCC 7317 also maximum at neutral pH and these results agreed with our observation. The optimum temperature for the α-amylase activity from strain TBG19NRA1 was observed at a temperature of 30°C (data not shown).

Some of the industrial enzymatic processes are carried out using microbial whole cells but more efficiency can be achieved using purified enzymes. Extracellular α-amylase from *S. griseus* TBG19NRA1 was purified by combination of ammonium sulfate precipitation, dialysis and gel filtration chromatography through Sephadex G100. The purification scheme and yield is summarized in Table 1. α-Amylase was purified using 60% saturation with $(NH_4)_2SO_4$ having yield up to 39% (7.4 U/mg) with a 1.48 fold purification. After Sephadex G-100 filtration the specific activity of α-amylase increased up to 34.7 U/mg with 7 fold purification having yield of 14.5%. Whereas Singh *et al.*,[32] recovered 56.58% of enzyme with 2.98 fold purification by 40-60% $(NH_4)_2SO_4$ precipitation in *Streptomyces* sp. MSC702. Chakraborty *et al.*,[9] succeeded to purify the alpha enzyme with Sephadex G-75 column chromatography which resulted 43.92 fold purification with a yield of 20.27% in *Streptomyces* strain A3. In the present work, the yield is comparatively less compared to previous reports.The purified enzyme appeared as a single band in 12% SDS-PAGE with a molecular weight of 60 kDa confirmed the high purity of the enzyme and it match with the activity band of α-amylase observed on the zymogram (Figure 2). The molecular weights microbial α-amylases, irrespective of their difference in other characters, are usually in the same range 40-70 kDa[13].

Table 1. Purification profile of á-amylase produced by *S. griseus* TBG19NRAI.

Purification Steps	Volume (ml)	Total activity (Units)	Total protein (mg)	Specific activity (units/mg protein)	Yield (%)	Fold of Purification
Culture Filtrate	200.00	8140 .00	1652	5	100.00	0
Ammonium sulphate (60%)	10.00	3165 .00	425.00	7.4	39	1.48
Dialysis	5.00	1390.00	142.5	9.75	17.07	1.95
Sephadex G-100	5.00	1182.00	34	34.76	14.5	7

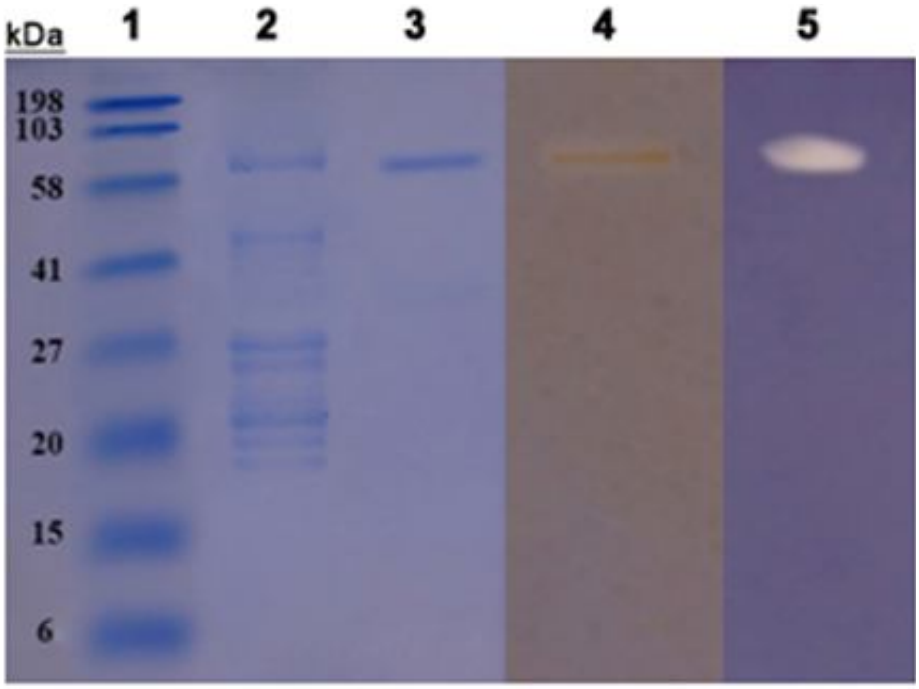

Figure 4 SDS-PAGE pattern of the purified α- amylase from *S. griseus* TBG19NRA1. The lanes represent, **M:** Molecular weight markers, myosin (198 kDa); β-galactosidase (103 kDa); Bovine serum abumin (58 kDa); Ova albumin (41 kDa) and Carbonic anhydrase (27 kDa); Soybean trysin inhibitor (20 kDa); Lysozyme (15kDa); Aprotinin (6kDa) **Lane 1:** Crude enzyme**; Lane 2** : purified amylase; **Lane 3** activity staining; **Lane 4** silver staining.

Optimum conditions of enzymatic activity :

The enzymatic activity of the purified α-amylase from *S. griseus* TBG19NRA1 was determined at varying temperature from 50 to 100°C with or without $CaCl_2$. The results were compared with that of standard α-amylase from *Bacillus licheniformis* (BLA) purchased from Sigma-Aldrich. The optimum temperature for the α-amylase activity from *S. griseus* TBG19NRA1 is ranging from 70-90°C (figure 5) with a maximum relative activity (98.7%) at 80°C without the addition of $CaCl_2$. The relative activity was 92.3% and 56% at 90°C and 100°C respectively. This has revealed the enzyme is thermophilic in nature. At temperature of 90°C, the retained relative activity was 92.3%, where the standard α-amylase (BLA) with $CaCl_2$ (0.2 M) is having 100% relative activity. The previous reports shows that optimum temperature for the α-amylase activity from *Streptomyces* sp. MSC702 was 50–75°C with maximum activity at 55°C (Singh *et al.*)[32]. The data from present study provide lines of evidence that α-amylase from *S. griseus* TBG19NRA1 could be a good candidate for the efficient liquefaction of gelatinized starch. There is a remarkable decrease in enzymatic activity of α-amylase from *S. griseus* TBG19NRA1 with the addition of $CaCl_2$ (0.2 M), showing the calcium independent nature. Most of the amylases reported till date are Ca^{2+} ion dependant and $CaCl_2$ known to be a stabilizer for amylases isolated by various microorganisms. Similar results are also observed in *Streptomyces* sp. MSC702 where Ca^{2+} ions are inhibitory[32] and *Streptomyces* strain A3 where Ca^{2+} ions are neither inhibitory nor potentiating amylase activity Chakraborty *et al.*,[9]. The results suggests suitability of *S. griseus* α-amylase in fructose syrups manufacturing industry, where glucose isomerase being used along with α-amylase are and $CaCl_2$ inhibit enzymatic activity of glucose isomerase. In starch based industrial processes, removal of metal ions is both cost and time consuming[18] and so the use of Ca^{2+} independent α-amylases at high temperatures would be highly favoured.

The enzyme-substrate reaction was maximally active within the range of 30 min to 150 min (>60% relative activity) with maximum α-amylase activity achieved in 60 min at 80°C (Figure 6). There was a remarkable decrease in α-amylase activity after 180 min incubation. In comparison with earlier reports on the thermostable α-amylases from *Streptomyces* spp., the present one shows more stability at high temperature up to 150 min.

The optimum pH for α-amylase activity from *S. griseus* TBG19NRA1 ranged from pH 6.0 to 9.0 (retained >80% activity) with a maximum activity at pH 7.0 (Figure 7). Although a decline in enzyme activity was observed above pH 9.0 and below pH 6.0, the enzyme was still active at pH 5 and pH 10 retaining its 74.4 % and 48% activity respectively. Similar to the present study, a wide range of pH stability (pH 3.0–8.0) was observed with α-amylase of *Streptomyces* sp. MSC702 , with maximum activity at pH 5.0 Singh *et al.*,[32] Ammar *et al.*,[4] observed stability at acidic to neutral pH (5.0–7.0) for *Streptomyces* sp. α-amylase. But it was reported the stability of α-amylases were at alkaline pH range (7.0–11.0) for *Streptomyces*

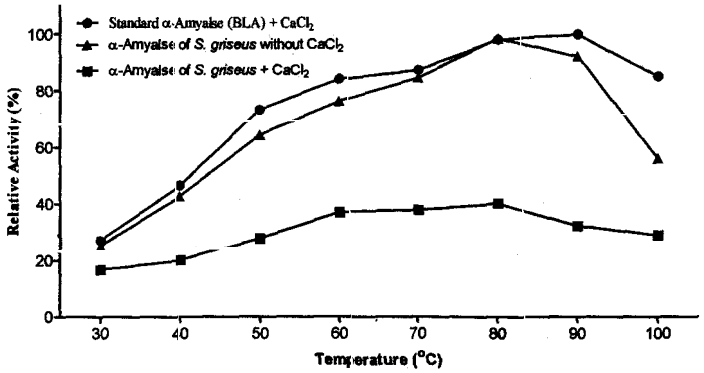

Figure 5: Effect of Temperature (10 min) on activity of the purified α-amylase from *S. griseus* TBG19NRA1 with or without $CaCl_2$ in comparison with standard α-amylase (BLA). The relative activity (%) activity was calculated taken the amylase activity of BLA at 90°C as100%.

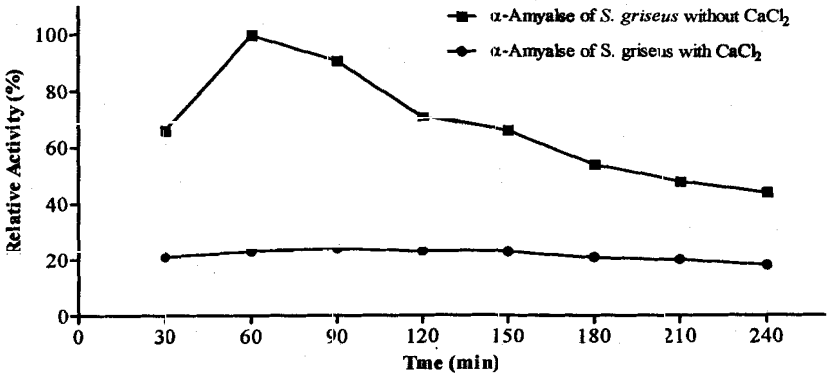

Figure 6: Effect of different incubation periods on enzyme activity at 80°C on the purified α-amylase from *S. griseus* TBG19NRA1 with or without $CaCl_2$.

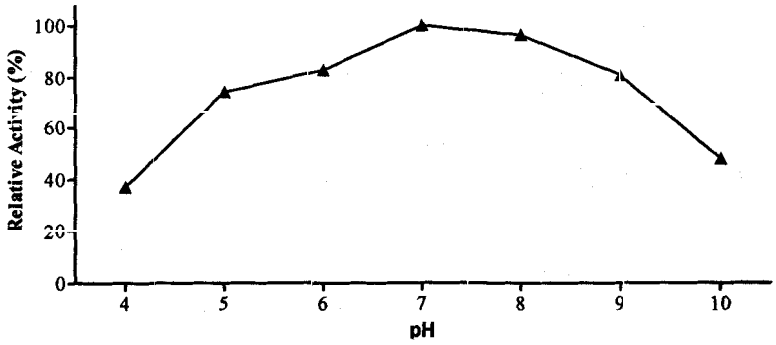

Figure 7: Effect of pH on the activity of the purified α-amylase from *S. griseus* TBG19NRA1with 10 min incubation at 80°C.

sp. D1 [9] and *S. gulbargensis* Syed *et al.*,[34]. The α-amylases acting on a wide pH range (5-10) is desired to satisfy all applications either acidic as glucose syrup production or alkali as detergents industries[12].

Effect of metal ions and denaturants *on* α-*Amylase activity :*

The effect of various metal ions on the activity of purified amylase was analyzed. The amylase activity was strongly stimulated by Fe^{3+} and Mg^{2+} (Figure 8), Mn^{2+} showed slight enhancement in enzyme activity. The Na^{+} and K^{+} ions has exhibited 78% retention in the amylase activity, while Ba^{2+}, Ca^{2+} ions inhibited α-amylase activity. Similar results were observed with the α-amylase produced by *Aspergillus oryzae* where Mn^{2+} and Fe^{3+} were stimulated enzymatic activity[25]. Enhancement of amylase activity on metal ions such Mn^{2+}, and $Fe3^{+}$ ions could be based on its ability to interact with negatively charged amino acid residues such as aspartic and glutamic acid[21].

Effect of inhibitors and denaturants on α-amylase activity :

With respect to the effect of chemical inhibitors and denaturants, the activity of 5ØüÞ-amylase was slightly stimulated with dithiothreitol (DTT), β-mercaptoethanol (BME), ethylene diamine tetra acetic acid (EDTA) and Urea, while phenylmethane sulfonyl fluoride (PMSF) and *Sodium dodecyl sulfate* (SDS) inhibited the activity. The β-mercaptoethanol brings change in the conformation of the enzyme in the active site by breaking the disulfide bonds which finally results in loss of enzyme activity. But present study shows addition of 5mM of β-mercapto-ethanol had enhanced the relative activity of

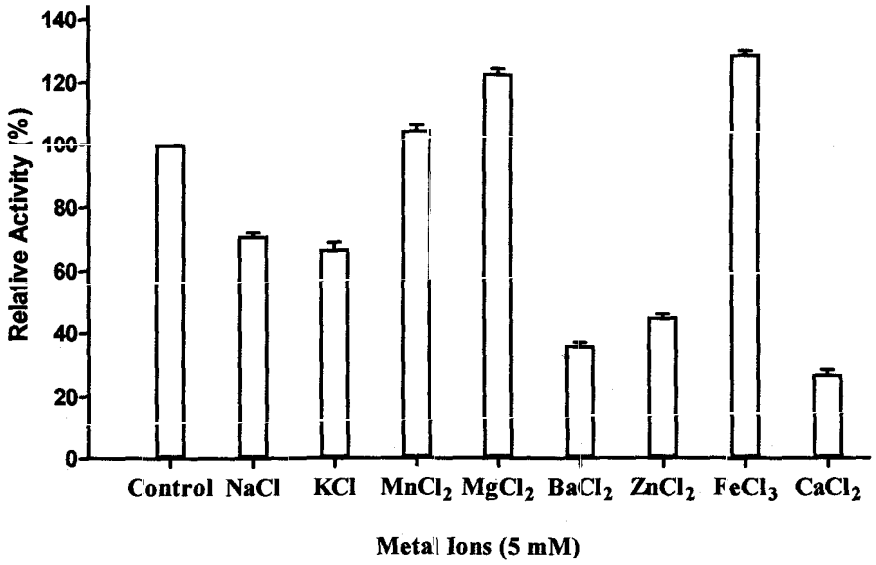

Figure 8: Effect of Metal ions (5 mM) on the activity of the purified α-amylase from *S. griseus* TBG19NRA1with 10 min incubation at 80°C.

enzyme by 115%. Similar findings have been previously reported for amylase from *Thermococcus profundus* DT5432 was slightly stimulated in the presence of dithiothreitol (DTT) and β-mercaptoethanol (Chung *et al.*)[10] and β-mercaptoethanol for thermostable amylases from *Bacillus amyloliquefaciens* (Rai and Solanki)[27]. This indicates that disulfide bonds are not essential for its enzyme activity and cysteine residues (with disulphide bonds between) in the α-amylase do not take part in catalysis. Also, the enzyme activation by dithiothreitol (DTT) and β-mercaptoethanol could be attributed to the reduction in aggregate size by the protection of thiol groups that stabilize the three dimensional structure of enzyme and/or by destroying the intermolecular disulfide linkages[19].

Kinetic determinations :

The enzyme kinetics, *Km* and *Vmax* are significant coefficients in enzyme research and is desirable to choose an catalyst in industry which will have the fastest reaction rate per unit amount of enzyme. The *Km* and rate of reaction for the α-amylase from by *S. griseus* TBG19NRA1 were calculated according to the method of Lineweaver-Burk plot by using the starch as substrate in concentrations ranged from 0.025 - 2 mg/mL. The more firmly the enzyme binds to its substrate, the smaller will be the value of *Km*. The Michaelis-Menten constant (*Km*) and *Vmax* in our investigation was found to be 1.6 mg/mL and 28 mg/mL/min at pH 7 with 0.1 M phosphate buffer (figure 10). The result obtained was more or less close to the *Km* value of 1.9 mg/ml on an alkaline chelator-resistant -amylase from an alkaliphilic *Bacillus* sp. isolate L1711 (Bernhardsdotter *et al.*)[8] and the *Km* value of 0.97 mg/ml obtained on α-amylase produced by thermophilic *B. sphaericus*[3]. The higher *Vmax* and lower *Km* had confirmed the efficiency of this enzyme for diverse applications.

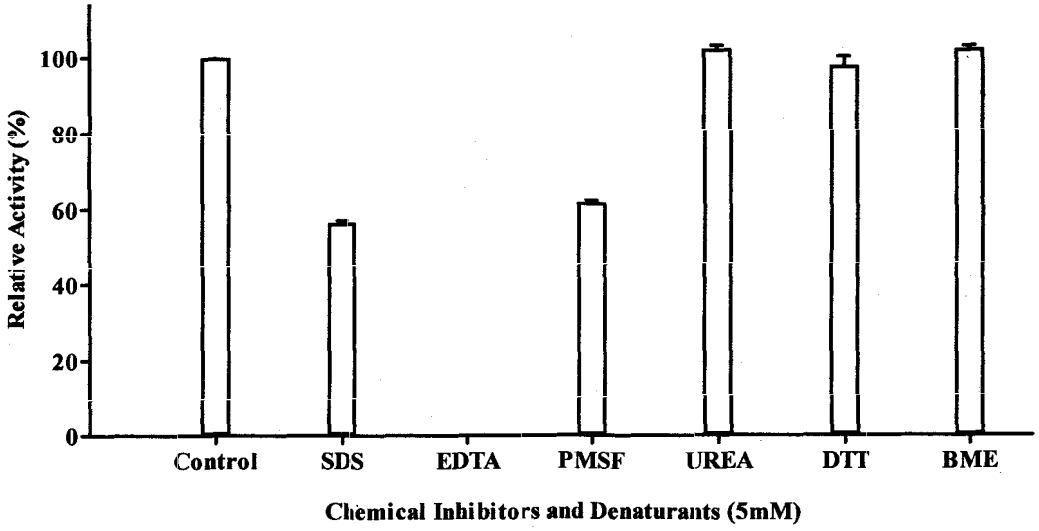

Figure 9: Effect of Chemical inhibitors and denaturants (5 mM) on the activity of the purified α-amylase from *S. griseus* TBG19NRA1with 10 min incubation at 80°C.

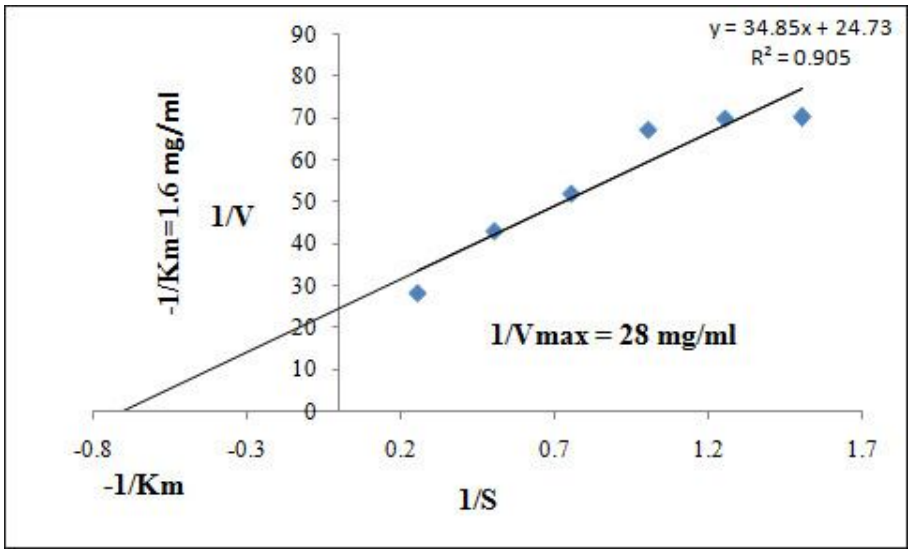

Figure 10: Lineweaver-Burk plot of the reciprocal of initial velocities and starch concentration for determination of Km value of α-amylase.

Identification by peptide mass fingerprinting :

Peptide mass fingerprinting (PMF) has evolved as a powerful tool for identification of protein[28,35]. The enzyme purified from *S. griseus* TBG19NRA1 was digested with trypsin and the peptides were analyzed by nano-RPLC–MS/MS using a Thermo Easy nLC 1000 (Thermo, USA) coupled to an Orbitrap Velos Pro mass spectrometer (Thermo, USA). A peptide spectrum obtained from in-gel tryptic digestion of purified α-amylase gave 14 prominent ions (Figure 11). The raw files acquired from Orbitrap Velos Pro Mass Spectrometer were searched using Proteome Discoverer of version 1.4.1.14 and two search engines (SEQUEST HT and MASCOT) against the complete proteome of α-amylase protein sequences in *Streptomyces* including the iso-forms downloaded from the Uniprot database and the results are given in table 2. The signal 14.78 was further chosen as the target to analyze in the MS mode to identify the precursor ion formed by the ESI ion source. The fragments and fingerprint information of the MS/MS spectrum of the precursor peak were analyzed and the results with scores higher than 54 identified the amino acid sequence of a 18-mer peptide as GIYGTSGSPGHVTSGADK (Figure 12). The peptide sequence were used to search the Uniprot database which identified similarity to α-amylase (Alpha amylase catalytic subunit NCBI A0A059WI20) from *Streptomyces albulus*. The peptide identification with high sensitivity and accuracy is important in mass spectrometry-based proteomics and this result confirmed that this purified amylolytic enzyme is an α-amylase.

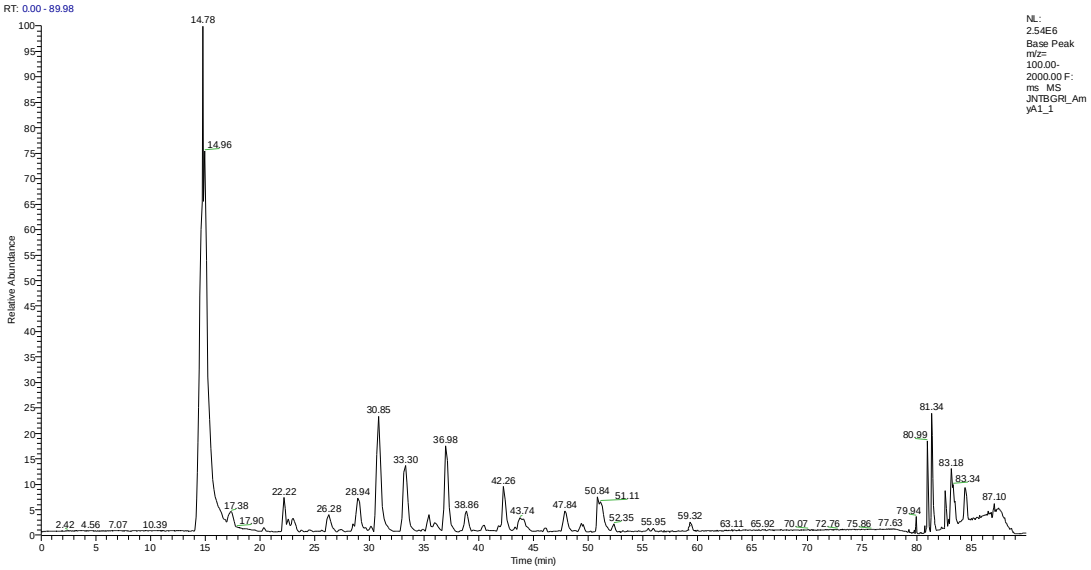

Figure 11: Total ion chromatogram of purified enzyme from *S. griseus* TBG19NRA1

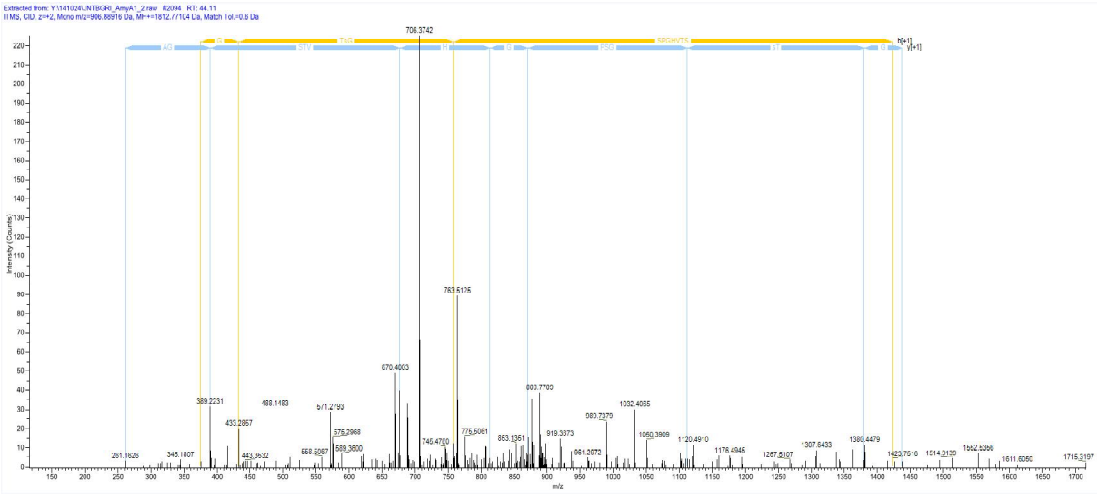

Figure 12: Representative Fragmentation spectrum (MS/MS spectrum) of the precursor peak obtained in LCMS analysis. Sequence selected : GIYGTSGSPGHVTSGADK; G1-Acetyl (42.01057 Da), S6-Phospho (79.96633 Da), Charge: +2, Monoisotopic m/z: 906.88916 Da (-4.51 mmu/-4.98 ppm), MH+: 1812.77104 Da, RT: 44.11 min.

Table 2. The Uniprot database search results of raw files acquired from Orbitrap Velos Pro Mass Spectrometer were searched using Proteome Discoverer of version

Accession	Description	Σ Coverage	Σ# Proteins	Σ# Unique Peptides	Σ# Peptides	# AAs	MW [kDa]	calc. pI
A0A059WI20	Alpha amylase catalytic subunit OS=Streptom-yces albulus	6.22%	1	2	2	579	62.9	5.72
M3G0D4	Alpha-amylase OS=Streptom-yces bottropensis ATCC 25435	4.55%	1	1	1	527	57.7	5.38
D9WE18	Alpha-amylase OS=Streptomyces himastatinicus ATCC 53653	4.28%	1	1	1	538	58.6	5.62
D9Y0J5	Secreted alpha-amylase OS=Streptomyces griseoflavus Tu4000	2.53%	1	1	1	990	106.9	5.78
D9XMJ7	Alpha-amylase OS=Streptomyces griseoflavus Tu4000	1.39%	1	1	1	1803	194.1	5.83
H1Q7Z5	Alpha-amylase Scat Z1 OS=Streptomyces coelicoflavus ZG0656	1.25%	2	1	1	1042	112.9	5.97
L1KPE8	Alpha amylase, catalytic domain protein OS=Streptomyces ipomoeae 91-03	0.99%	1	1	1	1821	196.4	5.47
L7EZQ8	Alpha amylase, catalytic domain protein OS=Streptomyces turgidiscabies Car8	0.66%	1	1	1	1825	196.6	5.78

The present study indicated that *S. griseus* TBG19NRA1 could be a potential actinomycete strain for α-amylase production. The optimum conditions and kinetic properties of enzyme hydrolysis were studied which could be later used as information for future studies. The α-amylase enzyme related to starch degradation was purified and characterized from this mesophilic actinobacteria strain, have a broad range of temperature and pH stability showing its suitability in the industrial applications where temperature and pH plays a critical role. The ability of *S. griseus* TBG19NRA1 α-amylase enzyme to withstand a temperature up to 90-100°C without the addition of Ca^{2+} ions, and acidic to alkaline pH; and temperature optimum for activity (80°C) and stability could suggest that the enzyme has a potential in any of starch liquefaction industries. The enzyme reported in this study can be developed and applied in this technology in the future.

Data availability statement :

The datasets generated for this study are available on request to the corresponding author.

Author contributions :

SS: project acquisition. SS and NSP: trial and project design. BD: trial implementation, data collection, data analysis (statistics and graphics), data interpretations. SS and DB: writing the manuscript.SKP revision of the manuscript. KD : LCMS analysis.

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Conflict of interest :

The authors declare that the research was conducted in the absence of any commercial relationships that could be considered as a potential conflict of interest.

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