Original Research Article

Purification and characterization of α-amylase from a novel thermoalkalophilic strain of *Bacillus sonorensis* GV2 isolated from mushroom compost

Abstract

A novel thermoalkalophilic α-amylase producing bacterial strain *Bacillus sonorensis* GV2 |KJ775811.1| was isolated from mushroom compost. The purification of α-amylase was performed through different chromatography techniques. After purification with SDS-PAGE and Sephadex G-75 gel filtration, the molecular weight of monomeric α-amylase was found to be 45 kDa. The enzyme showed an optimal activity over a wide range of temperature and pH of 35-60 ^{o}C and 7-11, respectively. The effect of divalent ions i.e. Mg^{2+} and Ca^{2+} showed a positive increase in enzyme activity. This enzyme is unique in a sense that it also exhibited a considerable raw corn starch hydrolyzing activity at 55^{o}C. The end products when subjected to TLC which were identified as main maltooligosaccharides, proving the endo action of an enzyme. The V_{max} and K_m values of *Bacillus sonorensis* GV2 α-amylase were found to be 1347 µmol/mg/min and 3.46 mMol/ml. The MALDI peptide mass fingerprint analysis of the reduced and carboxymethylated amylase digested with chymotrypsin indicated that this partial amino acid sequence was homologous by a score of 6 with UDP transferalyase. All these findings suggests about the potential role of this α-amylase for raw starch degrading applications in the relevant industry.

Keywords: Purification; characterization; α-Amylase; Mushroom compost

1. Introduction

Enzymes are biological catalysts which are indispensable components of biological reactions. Among the different enzymes that are widely used in industries, α-Amylase has ever been in increasing demand due to its crucial role in starch hydrolysis [1]. Microbial amylases are most important hydrolytic enzymes which share one-quarter of world enzyme market [2]. Starch is a polysaccharide composed of two types of polymers – amylose and amylopectin. Amylose constitutes 20-25% of the starch molecule which is a linear chain consisting of repetitive glucose units linked by α-1, 4-glycosidic linkage [3]. Amylopectin constitutes 75-80% of starch and is characterized by branched chains of glucose units. These enzymes randomly cleave internal glycosidic linkages in starch molecules to hydrolyze them and yield dextrins and oligosaccharides. There are two types of hydrolases: endo-hydrolase and exo-hydrolase. Endo- hydrolases act on the interior of the substrate molecule, whereas exo-hydrolases act on the terminal non reducing ends [4]. Amylases are of the most important enzymes used in a wide number of industrial processes such as food, fermentation and pharmaceutical industries due to their low cost, large productivity, chemical stability, environmental protection, plasticity and vast availability [5]. They are mainly employed for starch liquefaction to reduce their viscosity, production of maltose, oligosaccharide mixtures, high fructose syrup and maltotetraose syrup. In detergents production, they are applied to improve the cleaning effect and are also used for starch de-sizing textile industry [6].

Keeping in view the importance and industrial application of amylase, the present study was undertaken with production, purification as well as biochemical characterizations of an extracellular α-amylase enzyme produced by a novel strain of thermoalkalophilic isolate *Bacillus sonorensis* GV2 isolated from mushroom compost.

2. Materials and Methods

2.1 Microorganism and Culture Conditions

Bacillus sonorensis GV2 a novel thermoalkalophilic bacterium accession no. [KJ775811.1 from NCBI-US] used in the study was isolated from the mushroom compost collected from Directorate of Mushroom Research, Chambaghat, Solan, Himachal Pradesh, India. The culture was grown at 50 ±2 °C for 24 h in starch agar medium (peptic digest of animal tissue-5.0 g, yeast extract-1.5 g, beef extract- 1.5 g, starch soluble -2.0 g, Sodium chloride-5.0 g, agar-15.0 g, distilled water -1000 ml, pH -9.0 ±0.1). The pure line culture was maintained on starch agar slants and preserved in refrigerator at 4 °C and subcultured once in a month.

2.2 Production and purification of extracellular enzyme by microbial isolates

2.2.1 Inoculum preparation

Bacillus sonorensis GV2 was grown in 100 ml of starch broth at 50±2°C for 24 h. As soon as the substantial growth was observed in the broth, the optical density was set to 1.0 O.D.

1.2.2. Enzyme production

5 ml of inoculum was added to 45 ml of starch medium [7] which comprised (g/l): starch, 10.0; yeast extract, 5.0; peptone, 2.0; KH_2PO_4, 0.5; $CaCl_2.2H_2O$, 0.1; $MgSO_4.7H_2O$, 0.12; NaCl, 1.5, pH, 9.0 in 250 ml of Erlenmeyer flasks. An uninoculated culture medium was kept as a control. The inoculated and uninoculated enzyme production media were then incubated for 3 days at 50±2°C at 120 rpm. After incubation, the culture contents were centrifuged at 12,000 rpm for 20 min (4°C). The supernatant thus obtained was used as the crude enzyme for quantifying extracellular amylolytic activity.

2.2.3 α-Amylase assay [8]

1 ml of enzyme solution with 0.2% starch was incubated at 37 °C for 30 min. To this, 5.0 ml of 1N acetic acid was added to stop the reaction, followed by its dilution to 200 ml with distilled water. Finally, 5.0 ml of iodine reagent was added and the preparation was read at 580 nm. One International Unit (IU) of amylase activity is defined as the disappearance of an average of 1µmol of iodine binding starch material per minute in the assay reaction.

2.2.4 Protein assay [9]

To 0.1 ml of culture supernatant, 2.5 ml of Lowry's alkaline reagent was added, mixed and allowed to stand for 10 min. Diluted (1N) Folin Ciocalteau's reagent (0.25 ml) was added. The contents were shaken quickly and allowed to stand for 30 min for maximum colour development. The absorbance of the reaction mixture was read at 670nm against a reagent blank.

2.3 Purification of α- amylase enzyme

The crude enzyme solution (250 ml) was precipitated with solid ammonium sulfate (0-80 % saturation) at 4^0C. The preparations were kept overnight at 4°C and then centrifuged that resulted in the separation of precipitates and supernatants. The precipitate obtained after centrifugation was then resuspended in a minimum volume of Tris HCl buffer (20 mM, pH 8.0) separately and were refrigerated for further use. The enzyme solution was dialyzed against the same buffer overnight at 4 ^{0}C using 14 kDa cut-off dialysis membrane. The dialyzed α-amylase sample was then applied on to a Sephadex G-75 column, pre-equilibrated with 20mM Tris HCl (pH-8.0). The column was washed with 500 ml of equilibration buffer and the bound protein eluted with the same buffer. Fractions (2.0 ml) were collected at a flow rate of 2ml/3.5min and assayed for enzyme activity. The active fractions which showed higher extracellular α-amylase activity were pooled and purified enzyme was kept under refrigeration for further use.

The Concentrated supernatant containing enzyme dissolved in 20mM Tris HCl buffer (pH 8.0) was loaded on a DEAE-cellulose glass column. The column was eluted at a flow rate of 0.2 ml/min, with a linear NaCl gradient from of 0.1 M, 0.2 M…., 1.0 M in 20mM Tris HCl buffer (pH 8.0) and the protein content was measured at 280 nm. Active fractions were pooled and used for electrophoresis analysis.

2.4 Estimation of molecular weight of α-Amylase [10]

SDS-PAGE was performed using 12% polyacrylamide gel under non-reducing conditions. The protein bands were visualized by staining coomassie brilliant blue. The molecular weight of the purified enzyme was determined by comparing with Rf valves of standard molecular weight markers, PAGE mark^{TM} Protein Marker (14.3-97.4 kDa; G-Biosciences).

2.5 Zymography of α-Amylase

Zymogram analysis was performed by using the same conditions that of Native PAGE. Amylase activity was detected by incubating the native agarose gel into 1% starch solution at 50 ^{o}C for 1h. The gel was removed and then stained with iodine reagent. Amylase activity was visible as transparent bands on the dark blue background. After this, agarose gel bands were cut and placed onto starch agar plates and incubated at 50 $^\circ$C for 24h, then flooded with iodine reagent. Amylase containing agarose gel gave a clear halo around the gel in the blue background.

2.6 Characterization of Purified amylase

2.6.1 Effect of pH on the activity and stability

The effect of pH on enzyme activity was determined by incubating the reaction mixture at various pH ranging from 4.0 to 11.0 at 50±2 ^{0}C for 30 min. The buffers used were Citrate-phosphate buffer (pH 4.0-7.0), Tris HCl buffer (pH 8.0) and glycine-NaOH buffer (pH 9.0-11.0). The enzyme activity was assayed under standard conditions.

2.6.2 Temperature optimum and thermal stability

To evaluate the optimal temperature for the enzyme activity, the assay was conducted at varying temperatures

ranging from 35-121 ^{0}C. To determine the thermostability of enzyme, purified amylase was incubated with Tris HCl (20 mM, 8.0 pH) buffer. The preparations were then incubated at different temperatures ranging from 30, 40 ……121^{o}C for 0-180 min. Half life of the enzyme was determined as the time taken to reduce to half of the original activity.

2.6.3 Effect of metal ions

The purified amylase was incubated with 20mM Tris HCl buffer (pH 8.0) for 30 min with various metal ions (5 mM) K^{+}, Hg^{2+}, Na^{+}, Ca^{2+}, Cu^{2+}, Mn^{2+},Zn^{2+}, Mg^{2+}. The assay was carried out according to the standard assay procedure.

2.6.4 Substrate specificity

The substrate specificity purified amylase in Tris HCl buffer (pH- 8.0) was analyzed by incubating the samples at 50±2 $^{\circ}$C for 30 min with soluble starch, amylopectin, amylose, glycogen, xylose and maltodextrin etc. The standard assay was performed for enzyme activity.

2.6.5 Effect of different concentrations of substrate

To evaluate the effect of different concentrations of a substrate on enzyme activity, varying substrate concentrations ranging from 0.2%, 0.4%..... 2.0% in 20 mM Tris HCl buffer (pH 8.0) were incubated at 50 ±2^{o}C with enzyme for 30 min. The standard assay was performed for enzyme activity.

2.6.6 Effect of media additives

To determine the influence of different additives viz. SDS, EDTA, CTAB, Tween 20, Tween 80, Triton X 100 and Glycerol etc., purified α-amylase in 20mM Tris HCl buffer (pH 8.0) was pre-incubated for 30 min at 50±2 ^{0}C. Activity in the absence of additives was taken as 100%.

2.6.7 Effect of organic solvents on the enzyme activity

Different organic solvents including Acetone, Methanol, Ethanol, Benzene, Chloroform, Xylene were used to investigate the enzyme activity in the presence of organic solvents. Each organic solvent was prepared in the 20mM Tris HCl buffer (pH 8.0). The activity was done under standard assay condition and the sample without organic solvent was considered as 100%.

2.6.8 Shelf stability of amylase

Shelf stability of amylase was determined by pre-incubating the enzyme at 4 ^{o}C in 20mM Tris HCl buffer (pH 8.0). Enzyme activity was determined every 7 days till 186 days.

2.6.9 Raw starch adsorption and hydrolysis

The purified amylase in 20 mM (pH 8.0) Tris HCl buffer was mixed with 0.2 g of raw potato starch and raw corn

starch. The mixture was incubated at 50±2 ^{0}C for 15 min at 120 rpm. After the expiry of the mentioned time, the preparation was centrifuged at 8,000 g for 20 min and amylase activity was estimated by standard procedure. The adsorption rate (AR) was calculated according to the equation:

$$AR\ (\%) = \frac{O - R}{O} X\ 100$$

Where, R and O stand for the residual and original amylase activity, respectively

2.7 Analysis of hydrolyzed products by thin layer chromatography (TLC)

Hydrolysis products of soluble starch by purified α-amylase from both the strains were analyzed by ascending thin layer chromatography using silica gel plates. 1% starch was incubated with purified enzyme separately at 50 ºC for 12h. TLC was run with the solvent system of n-butanol-pyridine water (6:4:3) and a detection reagent comprising 2.0% (w/v) diphenylamine in acetone- 2.0% (w/v) aniline in acetone- 85% (w/v) phosphoric acid (5:5:1, v/v/v).

2.8 Determination of Kinetic Parameters

Determination of the kinetic parameters for the hydrolysis of *α*-amylase enzyme was calculated according to the method of Lineweaver-Burk plot by using the starch as sa substrate in concentrations ranged from 2.0, 4.0…… 20.0 mg/ml in 20mM Tris HCl buffer (pH 8.0). The kinetic parameters of Michaelis-Menton constant, K_m and maximal reaction velocity, Vmax were determined by linear regression according to Lineweaver and Burk double-reciprocal plot. Catalytic turnover number (K_{cat}) was determined.

2.9 Determination of internal amino acid sequence of amylase by MALDI-TOF mass spectrometry

The peptide mixture (1 µl) was mixed with an equal volume of matrix solution (4 mg/ml α-cyano-4-hydroxycinnamic acid in 50% acetonitrile and 0.1% aqueous TFA) and deposited onto the MALDI target plate using a MALDI-Q-TOF Premier (Waters, Manchester, U.K.) instrument. External calibration covering the m/z 729-3,959 mass range was achieved with a mixture of polyethylene glycols. A single point lock-mass (Waters) correction was used as reference (Glu-fibrinopeptide, m/z 1570.6774; Sigma-Aldrich) and was applied to all spectra. Calibrated spectrawere submitted to database searches (Swissprot, NCBI) using the MASCOT mass mapping software.

3. Results and Discussion

3.1 Production and Purification of amylase

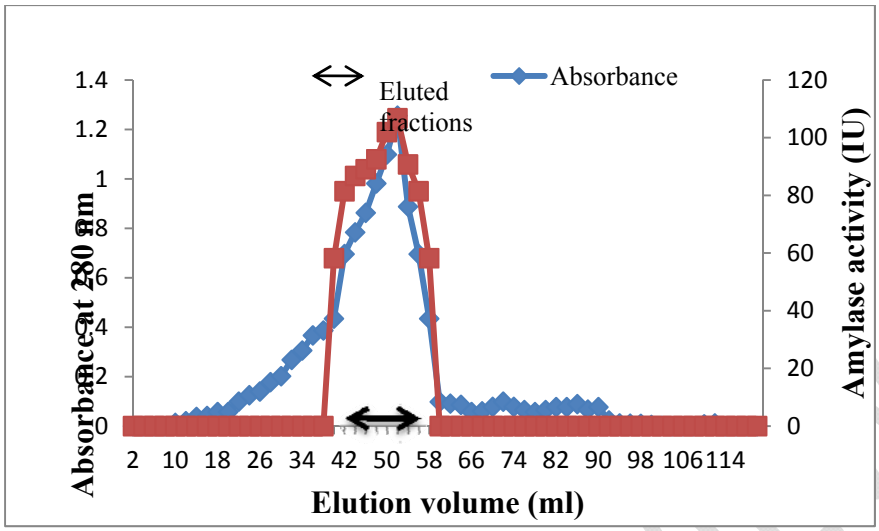
A newly isolated thermoalkalophilic α-amylase producing a bacterial strain of *Bacillus sonorensis* GV2 |KJ775811.1| was isolated from mushroom compost and was subjected to a purification process. For amylase production 5 ml of 1.0 O.D. culture of *Bacillus sonorensis* GV2 was added to 45 ml of starch medium, pH 9.0 in 250 ml of Erlenmeyer flasks and were incubated for 3 days at 50±2°C at 120 rpm crude amylase produced by *Bacillus sonorensis* GV2 under submerged fermentation gave the total activity of 827.70 IU with its specific activity of 2.18

IU/mg and 37.64 mg of protein in crude supernatant. Amylases are produced by number of microorganisms such as *Bacillus subtilis*, *Bacillus* sp. VS04 and *Bacillus* sp. under submerged fermentation [11]. The cell free extracts of *B. sonorensis* GV2 were subjected to sequential ammonium sulphate saturations from 0-80%. *B. sonorensis* GV2 α-amylase got precipitated at 20-70% saturation of ammonium sulphate, with 27.63 mg/g protein and specific activity of 3.21 IU/mg (Table 1) and 1.47-fold purification of enzyme was achieved with 53.73% amylase yield after 70% ammonium sulphate fractionation. In a study, researchers have reported 70% saturation as optimum for precipitation of extracellular α-amylase form *B. subtilis* [12].

Table1. Purification profile of α-amylase from *B. sonorensis* GV2

Purification step	Total Activity (IU)	Total protein (mg/ml)	Specific activity (IU/mg)	Purification fold	%Recovery/ Yield
Crude enzyme	827.70	37.64	2.18	1	100
Ammonium sulphate precipitation	444.75	27.63	3.21	1.47	53.73
Dialysis	371.96	21.23	4.37	2.00	44.93
Gel exclusion chromatography	298.38	4.24	25.05	11.46	36.04
Anion exchange chromatography	213.52	3.37	31.67	14.52	25.79

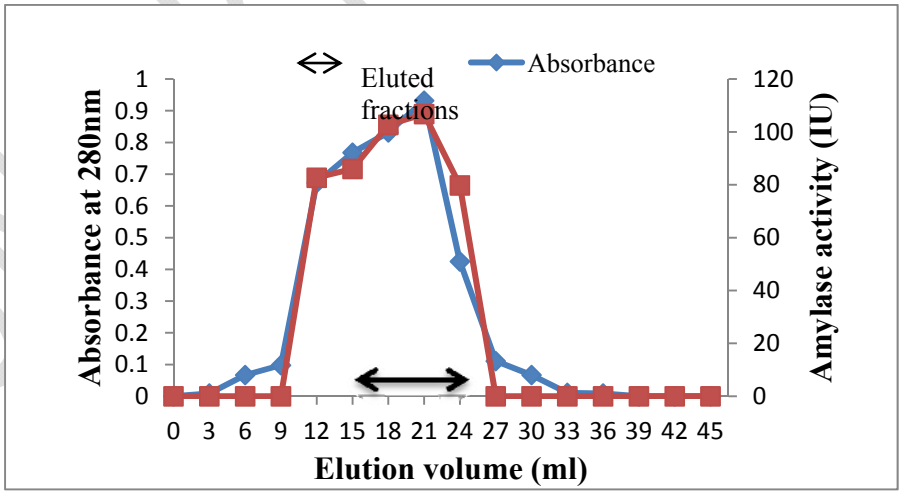
Ammonium sulphate salted out protein precipitates of *B. sonorensis* GV2 α-amylase were dialyzed against 20 mM Tris HCl (pH-8.0) buffer overnight at 4°C using dialysis membrane of cut-off 14 kDa dialyzed *B. sonorensis* GV2 α-amylase has a protein content of 21.23 mg protein and a specific activity of 4.37 IU/mg and 2 fold purification of α-amylase was achieved with 44.93% amylase yield (Table 1). The enzyme-rich samples obtained after dialysis were mounted onto Sephadex G-100 column pre-equilibrated with 20mM Tris HCl buffer (pH-8.0). 60 fractions of 2 ml volume each were collected with a flow rate of 2 ml/3.5min. Many protein peaks were observed and only one activity peak was detected (fractions 22-28) (Fig.1). Active fractions were pooled and lyophilized. The enzyme activity of pooled fractions was checked by quantitative titrimetric assays. Gel chromatographic separation resulted in 36.04% yield and 11.46 fold purification respectively. The amylase was purified using precipitation by ammonium sulphate (60%) and dialysis, the refined amylase had a maximum activity at pH 7, the amylase was stable with pH values ranging between (7 - 8) and in temperature 30 °C also amylase was stable in 30- 40 °C analyses of the amylase for molecular weight was carried out by SDS-PAGE electrophoresis which revealed 52 KDa [13].



188

189 **Fig1. Protein and enzyme activity profile of fractions of Sephadex G-75 column chromatography of the**
190 **dialyzed α-amylase of *B. sonorensis* GV2**

191 The pooled sample from Sephadex G-75 column was applied onto DEAE-cellulose anion exchange column (fig 2).
192 Fraction numbers 4-8 for *B. sonorensis* GV2 exhibiting highest α-amylase activity were pooled for further
193 characterization studies. *B. sonorensis* GV2 resulted in 25.79% yield (Table 1) and 14.52 fold purification with a
194 specific activity of 31.67 IU/mg. In a study, thermostable α-amylase was purified from soil isolate *Bacillus* sp.
195 strain B-10 to 48.21 fold purification by ion exchange chromatography with a specific activity of 62.44 U/mg [14].
196 In a study, in the first step of gel filtration the specific activity was the protein peak of (14.130 U/mg) with
197 purification fold (1.436), while in the second step of gel filtration the specific activity reached (16.360 U/mg) with
198 purification fold of 4.785 [13].



199

200 **Fig2. Protein and enzyme activity profile of fractions of DEAE-cellulose anion exchange chromatography of**
201 **the gel chromatogram α-amylase of *B. sonorensis* GV2**

The purity and molecular weight of the purified α-amylase of *B. sonorensis* GV2 was determined on SDS-PAGE. Purified α-amylase of *B. sonorensis* GV2 exhibited single band with only one sub unit. The molecular weight of purified enzyme was calculated according to the RF values of the standards used in the protein molecular weight marker. The molecular mass of denatured α-amylase from *B. sonorensis* GV2 estimated from the relative mobility of proteins on SDS-PAGE was ~ 45 kDa (Fig. 3) respectively. Researchers examined the molecular weight of α-amylase from marine *Streptomyces gancidicus*-ASD_KT852565 and was found to be 44 KDa [15].

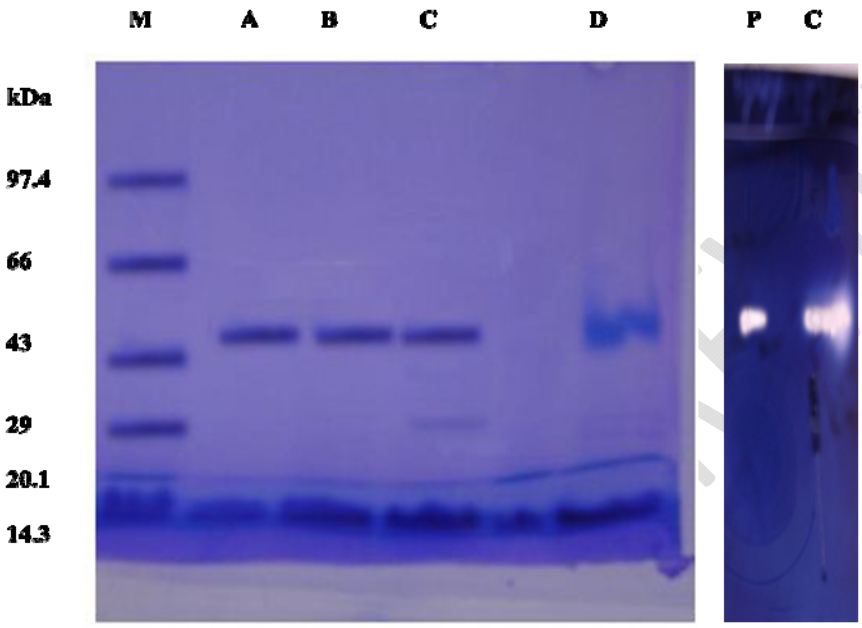


Fig. 3 (a) SDS-PAGE of *B. sonorensis* GV2 α- amylase at various stages of purification. (b) zymogram of purified and crude amylase Lane A: DEAE Cellulase anion exchange chromatographed enzyme; Lane B: Sephadex column chromatographed enzyme; Lane C: Ammonium sulphate fractionated enzyme; Lane D: Crude enzyme

3.2 Zymogram analysis

Zymogram of α-amylase showed a prominent activity band against blue background corresponding to 45 kDa for *B. sonorensis* GV2 (fig 4) α-amylase. The alternative method utilized for zymography i.e. the portion of gel cut and plated onto the starch agar plate also produced a clear zone of hydrolysis around the band confirming the presence of starch hydrolysing α-amylase. Iodine interacts strongly with the polysaccharide containing α (1-4) and α (1-6) linked reducing sugar units. The resulting dye-glucan complexes are intensely coloured making them very sensitive for detection of such polysaccharides. In a similar study, the gel obtained from electrophoresis was dipped in 1% starch solution for 20 min followed by the addition of few drops of Lugol solution to it (0.67% Potassium Iodide and 0.33% Iodine). The gel was observed for a yellow band [16].

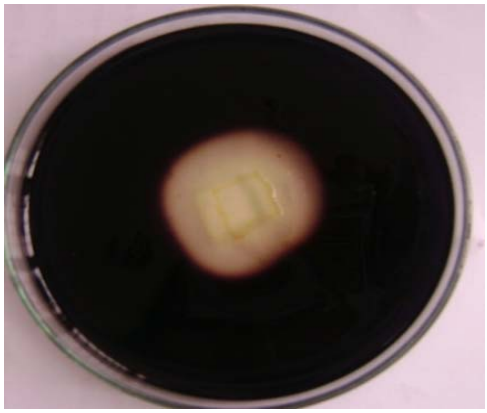


Fig. 4 Zymogram analysis of purified α-amylase of *B. sonorensis* GV2 on starch agar plates with gel cut from Native PAGE showing zone of hydrolysis

3.3 Characterization of purified α-amylase

3.3.1 Effect of pH on α-Amylase enzyme activity and stability

The purified enzyme showed the optimal pH ranged from pH 7.0-11.0, with maximum activity at pH 10.0 (fig. 5). More than 75% of the maximum observed activity was achieved at pH 8.0, 9.0 and 10.0. At higher pH of 11.0, 42.36% of the original activity was retained. The wide pH stability range for purified α-amylase spanning 7.0-11.0 makes it a promising candidate for paper, detergent, textile and biofuel industries as per their requirement of activity to be in between neutral to alkaline pH. Amylases having alkaline pH, maintain the necessary stability under detergent conditions and the oxidative stability of amylases is one of the most important criteria for their use in detergents where the washing environment is very oxidizing. Removal of starch from surfaces is also important in providing a whiteness benefit, since starch can be an attractant for many types of particulate soils. It has been documented that 90% of all liquid detergents contain α-amylases. In a report *B. megaterium* Strain KAN1 showing elevated amylase activity at pH 11.0 [17]. In a report *Bacillus gibsonii* S213 showing best amylase activity from neutral to alkaline pH (7-9.5) [18]. In a study, the rise in the activity of amylase purified from *E. coli* with rise in the pH until reach to greatest activity (0.297U/ml) at pH 7 [13].

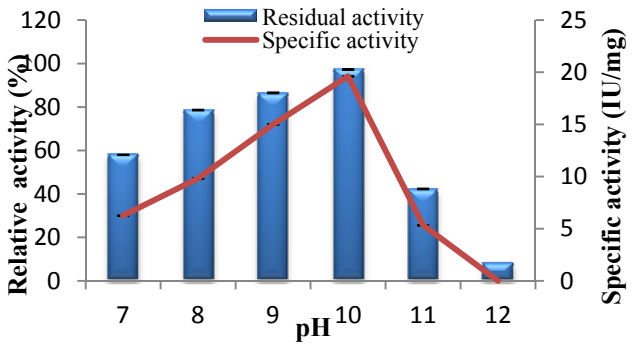


Fig5. Effect of pH on purified α-amylase of *B. sonorensis* GV2

3.3.2 Effect of temperature on activity of purified α-amylase

Behaviour of purified α-amylase from *B. sonorensis* GV2 at a temperature regime varying from 35-121°C was explored and its highest enzyme activity was observed at 50°C (fig. 6). Optimum α-amylase production at 50°C is a characteristic of moderately thermostable microorganisms and has been reported in literature by several workers. α-amylase from *B. sonorensis* GV2 has shown a significant retention of activity up to 90°C. Thermostability of purified α-amylase from the isolate is thus a striking feature which turns this enzyme a potential candidate to be used in saccharification industry because there will not be any denaturation of proteins molecules of enzyme at raised high temperatures of reaction. The enzyme was found active to be an extent at as high temperature as 121°C with a relative activity of approximately 20%. *B. subtilis* α-amylase purified which exhibited maximum activity at 55°C. α-amylase from *B. sonorensis* GV2 showed a wide range of temperature tolerance with 92% activity at 60°C respectively and 63% respective activity at a temperature of 70°C [19].

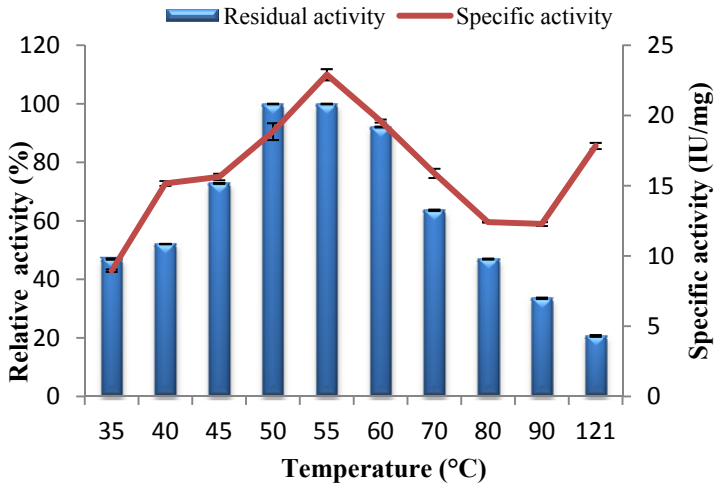


Fig 6. Effect of temperature on purified α-amylase of *B. sonorensis* GV2

3.3.3Thermal inactivation of Purified α-amylase

Thermostability studies were carried out by pre incubating purified α-amylase for 180 min at various temperatures ranging from 35°C to 121°C. Thermal stability of the enzyme was determined by studying the time dependent inactivation of enzyme at temperatures it was subjected to. Purified α-amylase showed 50% retention in amylase activity from 50–70°C (fig. 7) for 60 min. At 80°C, 50% relative activity for 30 min was observed. Activity retention of upto 50% was observed at higher temperatures of 90 and 121°C for 15 min. The purified α-amylase has shown stability at comparatively wider range of temperatures are mandatory, thereby proving its applicability in industrial processes where reactions to be carried out at higher temperatures. The thermostability of the thermophilic enzyme is mainly due to additional salt bridges involving a few specific lysine residues (Lys-385 and Lys-88 and/or Lys-

253). Purified *B. licheniformis* AI20 α-amylase and amylase retained 75, 50 and 20% of its activity at 70, 75, and 80°C [20]. Thermal stability of α-amylase purified from *Anoxybacillus flavithermus* and found that the enzyme was stable from 40-45°C for 120 min however, at 50°C; up to 45 min a reduction in activity up to 50% was observed [12].

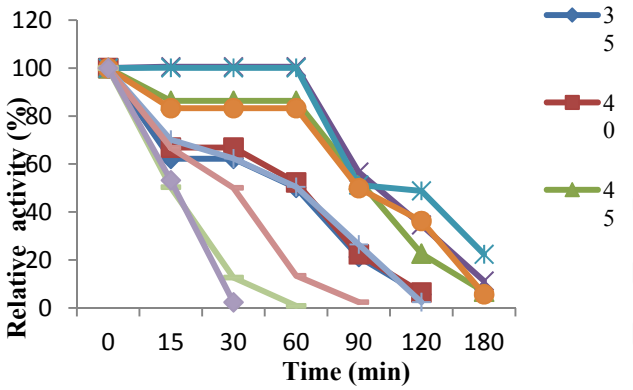


Fig7. Thermostability profile of purified α-amylase of *B. sonorensis* GV2

3.3.4 Effect of metal ions

To investigate the effect of metal ions, enzyme activity was assayed in the presence of various divalent ions at a concentration of 5 mM. An increased activity of 146.48%, 138.67% was shown in the presence of Ca^{2+} and Mg^{2+} respectively (Table 2) suggesting the enzyme to be a metalloprotein needing a cofactor for its maximum activity. A few divalent ions viz. Co^{2+}, Zn^{2+}, Cu^{2+} and Ba^{2+} were inhibitory for purified α-amylase. Inhibitory activity found with various ions on amylase activity may be due to competition between the exogenous cations and the protein-associated cations, resulting in decreased metalloenzyme activity. Similar increase in enzyme activity has been recorded [21]. Where a relative activity (%) of 111 and 117 had been reported in the presence of Ca^{2+} and Mg^{2+} respectively. An increase of 12% and 19% in enzyme activity was observed in *Bacillus persicus* α-amylase in the presence of Ca^{2+} and Mg^{2+} respectively [22].

Table 2. Effect of divalent ions on the activity of α-amylase

Divalent ions	**Relative activity (%)**
	***B. sonorensis* GV2**
Ca^{2+}	**146.48±0.08[a]**
Mg^{2+}	**138.67±0.19[b]**
Cu^{2+}	93.27±0.20[c]
Ba^{2+}	20.11±0.11[d]
Fe^{2+}	62.36±0.32[e]
Mn^{2+}	86.90±0.12[f]
Co^{2+}	0±0.00[g]

Zn^{2+}	11.87±0.32[h]
Ni^{2+}	96.24±0.29[i]
Control	100.00±0.10[j]
SE(m)	**0.23**

283

284 **3.3.5 Effect of Surfactants**

285 For application in detergent industries, α-amylase has to be stable in various detergent ingredients such as
286 surfactants and chelators. Different surfactants were tested for their effect on α-amylase and results exhibited a good
287 stability in the enzyme activity (Table 3). The purified α-amylase in presence of anionic surfactant SDS was able to
288 retain 99.52% of the original activity. Non-ionic surfactants like Tween 20, Tween 80 and Triton X 100 also
289 stabilized enzyme with a relative activity of 102.36, 102.93 and 101.59% These results are in consonance with the
290 reports in which an increased enzyme activity i.e. 126%, 120% and 80% in the presence of Tween 20, Tween 80 and
291 Triton X 100 [23]. In a study, the retention of 80% of the original activity in the presence of SDS for *Bacillus* sp.
292 strain TSCVKK was reported [24].

293 **Table 3. Effect of surfactants on the activity of purified *B. sonorensis* GV2 α-amylase**

Surfactant	**Relative activity (%)**
	***B. sonorensis* GV2**
SDS	99.52±0.21[a]
EDTA	7.619±0.09[b]
CTAB	13.24±0.12[b]
Tween 20	102.36±0.31[a]
Tween 80	102.93±0.23[a]
Triton X 100	101.59±0.33
Glycerol	89.38±0.15
Control	100±0.09
SE(m)	**0.24**

294

295 **3.3.6 Effect of organic solvents**

296 Thermostable enzymes are known to be resistant to organic solvents. Therefore the purified *α*-amylase enzyme was
297 evaluated against different solvents for its activity. The enzyme exhibited high activity with ethanol, methanol and
298 acetone i.e. 95.48%, 90.84% and 95.68% respectively. The enzyme however showed a lesser tolerance towards
299 benzene and chloroform (Table 4). This trend of purified α-amylase towards organic solvents reflect it as desired
300 preparation for the industries using organic solvents in medium. Scientists studied the effect of different solvents on
301 purified α-amylase from *Bacillus licheniformis* Isolate AI20 and enzyme showed a great stability against tested
302 solvents up to 10% (ethanol, methanol and isopropanol) [20]. The effect of different solvents like ethanol,

methanol, isopropanol, acetone and n-butanol on purified amylase from *Thermotoga petrophila* was studied and showed stability against these solvents but no considerable effects were observed [25].

Table 4. Effect of organic solvents on the activity of purified *B. sonorensis* GV2 α-amylase

Organic solvetnts	**Relative activity (%)**
	***B. sonorensis* GV2**
Acetone	95.68±0.11[a]
Methanol	90.84±0.23[b]
Ethanol	95.48±0.17[a]
Benzene	57.32±0.21[c]
Chloroform	82.77±0.13[d]
Xylene	0±0.00[e]
Control	100±0.09[f]
SE (m)	**0.17**

3.3.7 Substrate specificity

The various substrates viz. corn starch, amylase, amylopectin glycogen, dextran and cellulose were used for substrate specificity. The results revealed that corn starch and amylopectin were better substrates for purified amylase than glycogen, dextran and cellulose with 116.53% relative activity for corn starch considering its activity 100% with soluble starch (Table 5). This is a rare feature as most of the amylases reported till date showed maximum activity towards soluble starch which is easily hydrolysable. A 43.96% of the original activity was observed for amylose, indicating corn starch, soluble starch and amylose as the physiological substrates for *B. sonorensis* GV2 purified α-amylase

Table 5. Substrate specificity of purified *B. sonorensis* GV2 α-amylase

Substrate	**Relative activity (%)**
	***B. sonorensis* GV2**
Soluble starch (control)	100.00
Corn starch	116.53
Amylose	43.96
Amylopectin	-
Dextran	-

Cellulose	-
Glycogen	-
S.E. (m)	0.34

3.3.8 Shelf stability of Purified amylase

The purified enzyme was studied for shelf stability at 4°C and room temperature for 70 days. Purified α-amylase showed 64% relative activity at 4°C for 70 days (fig. 8). But at room temperature, a rapid loss in activity was observed, thus recommending the storage of this enzyme at refrigerated temperature. A respective relative activity of 25% of the original was retained after 49 days of incubation at room temperature by *B. sonorensis* GV2 (Fig.9). Shelf life of amylase from *Bacillus* sp. TSCVKK for 2 months was studied and found it stable at 4°C, however a loss of 15% activity was observed after 48 h when it was incubated at 30°C [24].

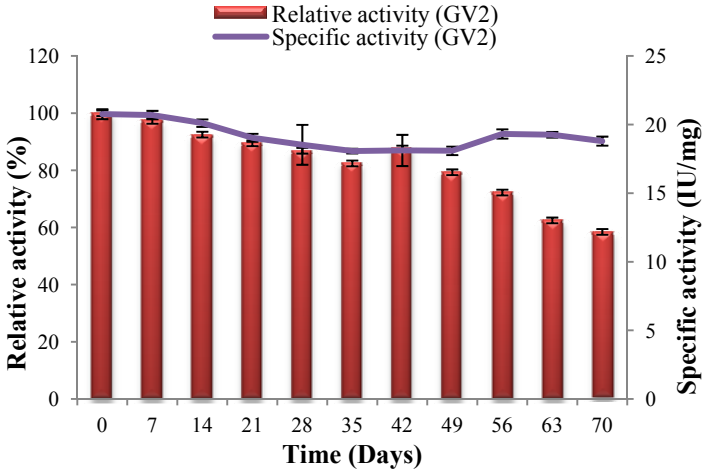


Fig 8. Shelf stability of purified α-amylase at 4°C

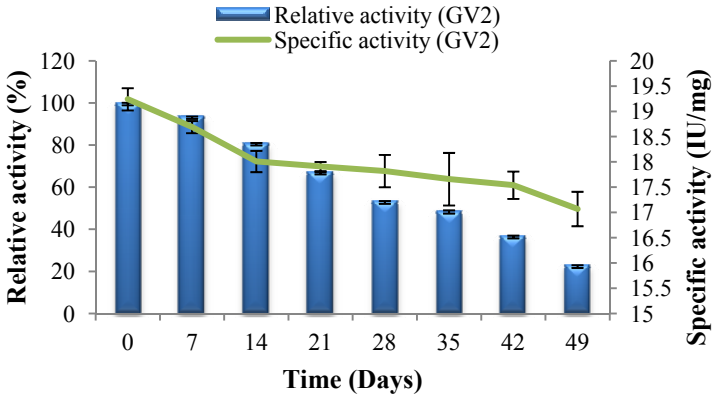


Fig 9. Shelf stability of purified α-amylase at room temperature

3.3.9 Raw starch adsorption and hydrolysis

Enzymes capable of hydrolyzing raw corn starch and potato starch form an important component of starch saccharification. The application of hydrolysis by the enzyme purified in the study was investigated by estimating the extent of raw starch hydrolysis. The purified α-amylase showed 46.23% adsorbability towards corn starch, indicating an additional property of the enzyme which makes it very useful in efficient hydrolysis of raw starch granules but no adsorption has been shown for potato starch (Table 6). The hydrolytic activity of amylases involves the breaking of glycosidic bonds, is very important in corn starch because it can replace chemical processing of starch in different industries due to cost effectiveness and technical advantages [14]. α-amylase from *Aspergillus carbonarius* SCSR-0002 showed a hydrolysis efficiency of 63.63% for corn starch [26]. α-amylase from *Alicyclobacillus* sp. showed a respective hydrolysis efficiency of 58% and 52% for raw potato and corn starches [27].

Table 6. Raw starch hydrolysis efficiency of purified α-amylase

Raw starch	**% Hydrolysis**
	***B. sonorensis* GV2**
Corn starch	46.23±0.21[a]
Potato starch	-
SE(m)	**0.21**

3.3.10. End products of starch hydrolysis

The end products of hydrolysis produced were analyzed by thin layer chromatography. Glucose, maltose and maltooligosaccharides were used as standards. The RF values of the samples and standards were determined and confirmed maltooligosaccharides as end products of starch hydrolysis confirming that the enzyme is α-amylase. The hydrolysis patterns presented by *B. sonorensis* GV2 purified amylase showed that the enzymes are typical endo-acting α-amylases. In conformity to our results, researchers have purified an endo-acting α-amylase from *B. licheniformis* NH1 yielding similar hydrolysis pattern [28]. In a study the end products of hydrolysis produced by *Bacillus gibsonii* S213 by thin layer chromatography, that confirmed maltose and maltotriose as end products of starch hydrolysis confirming that the enzyme is α-amylase [18].

3.3.11. Determination of Enzyme Kinetics

The kinetic parameters for hydrolysis of soluble starch were determined by plotting the initial velocities towards different concentrations of soluble starch as substrate, incubated with the fixed amount of enzyme. *B. sonorensis* GV2 α-amylase followed the Michaelis Menton kinetics of catalysis. The V_{max} and K_m values of *B. sonorensis* GV2 α-amylase were found to be 1347 μmol/mg/min and 3.46 mMol/ml. Low values of K_m indicate high affinity of the enzyme for substrate (Table 7 and fig 10). Shafiei *et al.* reported V_{max} and K_m values of 1.18mg/ml/min and 4.5

mg/ml respectively [29]. Hadipour *et al.*, studied the kinetics of purified α-amylase from *B. persicus* and V_{max} and K_m values were found to be 356 µM/min and 1.053 mg/ml respectively [22].

Table 7. Kinetic parameters of purified *B. sonorensis* GV2 α-amylase

Isolate	K_m	V_{max}	K_{cat}	K_{cat}/K_m	$t_{1/2}$ (45-60°C)
***B. sonorensis* GV2**	3.46 mMol/min	1374µmol/mg/min	136.56/min	38.88	90 min

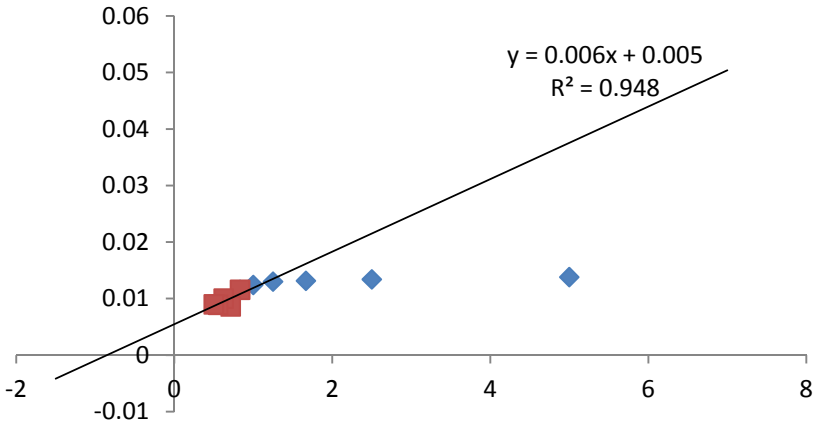


Fig 10. Lineweaver Burk Plot for enzyme kinetics of *B. sonorensis* GV2

3.4 Mass Spectrometry-Internal Sequence analysis by MALDI-TOF MS

The MALDI-TOF masss spectrum (peptide mass fingerprinting) analysis of the reduced and carboxymethylated amylase digested with chymotrypsin. MALDI-TOF mass spectrum of a tryptic protonated peptide molecular ions map (Fig. 11). The results of Mascot Search Engine from Matrix-Science search indicated that this partial amino acid sequence was partially homologous by a score of 6 with UDP transferalyase. Amino acid sequence of amylase from *Anoxybacillus flavithermus* WK1 has matched the peptides. The protein sequence coverage in this case is only 6%. The peptide sequence is Ala-Val-Ala-Ile-Asn-Glu-Leu-Thr-Asp-Arg-Asn-Ile-Thr-Ile-Asn-Glu-Ala-Lys. Michelin *et al.*, studied peptide sequencing by MALDI-TOF MS of purified α-amylase of *Paecilomyces variotii* and found 100% homology with *Bacillus* sp. amylase [30]. On the other hand Kolawole *et al.*, reported purified amylase from finger millet which had no homology with the reported amylases available in the literature and they concluded

that their enzyme had unique amino acid sequence [31]. In harmony to this findings, it can be concluded that minimal similarity (5 to 6%) between the purified α-amylases from *B. sonorensis* GV2 and their respective counterparts in terms of their amino acid sequencing this purified amylase as novel and unique.

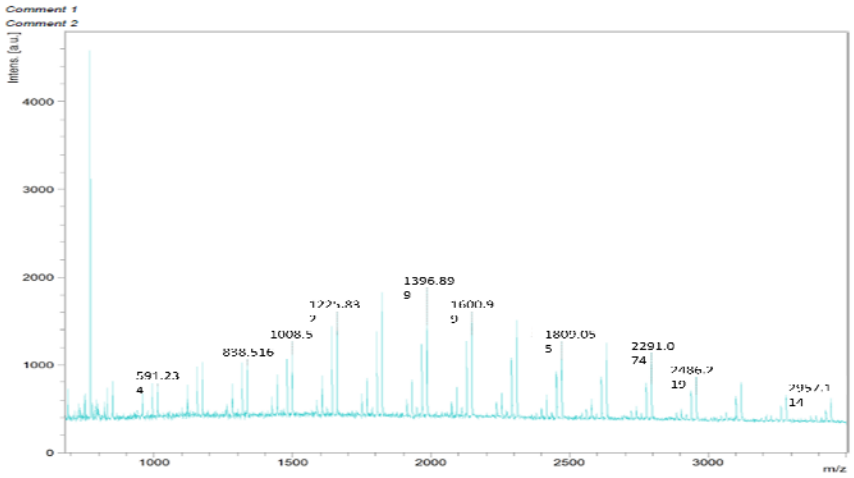


Fig. 11 MALDI-TOF mass spectrum of purified α-amylase from *B.sonorensis* GV2. The mass spectrum shows a series of protonated peptide molecular ions

Conclusion

In conclusion, the current findings describe the purification and characterization of α-amylase from a novel thermoalkaophilic isolate *B.sonorensis* GV2. Nowadays, thermostable raw-starch degrading α-amylase are being used as potential candidate for hydrolysis of starch. The results of our findings during characterization revealed the thermostable and alkaline nature of enzyme. Moreover, raw corn starch digesting effect was also observed with considerable concentrations from *B. sonorensis* GV2. Due to these stupendous attributes, like thermostability and high alkalinity makes the future application of *B. sonorensis* GV2 α-amylase as an excellent candidate in near future for starch and saccharification sector. Since thermostability is considered a useful and important feature of amylases for industrial application, attempts should be made to develop enzymes from thermophilic and extremely thermophilic microorganisms. It is hoped that amylases will continue to provide new opportunities in biotechnology as biocatalysts and that new applications will emerge in the biopharmaceutical sector.

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