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Original Research Article

Production, Purification and Characterization of A-Amylase from Thermoalkalophilic Strain *Bacillus Aerius* GC6 [KJ775810]

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Abstract

In the present study, α-amylase was produced from newly isolated *Bacillus aerius* GC6 [KJ775810] strain from mushroom compost. The produced *α*-amylase was purified through various chromatographic techniques. *B. aerius* GC6 α-amylase activity of crude enzyme was 844.97 IU and 462.55 IU after ammonium sulphate precipitation with 1.38 purification fold and recovery of 54.74 %. Enzyme showed an increased respective enzyme activity of 382.44 IU with a purification fold of 15.16 and 45.26% recovery during gel exclusion chromatography. Anion exchange chromatography was then carried out, yielding 225.78 IU of α-amylase for *B. aerius* GC6 with a final purification fold of 17.86 and 26.72% recovery. Purified α-amylase appeared as a 43 kDa monomeric enzyme, as estimated by SDS-PAGE and Sephadex G-75 gel filtration. The *α*-amylase had an optimal temperature and pH of 50 ^{o}C and 9.0, respectively. The *α*-amylase enzyme showed great stability against the solvents like ethanol and acetone i.e. 99.55 and 96.35% respectively. Values of V_{max} and K_m for the purified enzyme were found 4.40 mMol/min and 1404 µmol/ mg/min. The spectrum of amylase application has widened in various fields such as clinical, medical, analytical chemistries, textile, food, fuel as well as detergent industries.

Keywords: Purification, characterization, *Bacillus aerius* GC6, α-amylase.

INTRODUCTION

Enzymes are substances present in the cells of living organisms in minute amounts which are capable of speeding up chemical reactions, without themselves being altered after the reaction. They accelerate the velocity of the reaction without necessarily initiating it. Compared with chemical catalysts, enzymes have many advantages such as a high specificity, a high catalytic efficiency, and an adjustable activity, which greatly promote the use of enzymes in pharmaceutical, chemical, and food industries [1]. Industrial enzymes are specific catalysts that catalyze and accelerate a chemical reaction that does not occur naturally. Due to these novel features, the demand for industrial enzymes has catapulted to the new heights which call for a constant research and development, to optimize their production and minimize resource costs [2]. Amylases are among the most widely used enzyme in starch processing industries. Based on their mode of action, they are further classified into three categories: *α*-amylases, *β*-amylases, and glucoamylases. All amylases are glycoside hydrolyses and act on *α*-1, 4 glycosidic bonds [3]. Among the types of amylases, industrially, *α*-amylase is the most widely used enzyme particularly in starch liquefaction, brewing, textile, pharmaceuticals, paper, detergents, drugs, toxic wastes removal, and oil drilling. To some extent amylases are also used as digestive aids to supplement the diastatic activity of flour and to improve digestibility of some of the animal feed ingredients .Since *α*-amylases are active over a broad pH (5–9) and temperature (35–105 ^{o}C) ranges, they are worldwide centre of attraction for researchers [4]. α-amylases (endo-1,4-*a*-D-glucan glucohydrolase, EC 3.2.1.1) are starch-degrading enzymes that catalyze the hydrolysis of internal α-1,4-*O*-glycosidic bonds in polysaccharides with the retention of α-anomeric configuration in the products [5]. Most of the α-amylases are metalloenzymes, which require calcium ions (Ca^{2+}) for their activity, structural integrity and stability.

Thermostable *α*-amylases are available from different sources and they have extensive commercial applications. The major advantage of using microorganisms for production of amylases are the ability to produce in bulk and ease at which it can be

manipulates for desired products. Each application of *α*-amylase requires unique properties with respect to specificity, stability, temperature and pH values dependence [6]. Thermostability is an important characteristic as enzymatic liquefaction and saccharification of starch are performed at high temperatures (100–110°C). Thermostable amylolytic enzymes are being investigated to improve industrial processes. Use of enzyme produced by thermophiles has the added advantage of reduced risk of contamination by mesophiles. Till today, a very few bacteria are reported that are able to hydrolyze high levels of raw starch The use of α- amylase in detergents formulations has also increased dramatically with growing awareness about environment protection and are environmentally safe and enhance the detergents ability to remove tough stains. They are biodegradable and work at milder conditions than chemical catalysts and hence preferred to the latter. Screening of microorganisms with higher *α*-amylase activities could therefore, facilitate the discovery of novel amylases suitable to new industrial applications [3]. Thermophilic fermentation is also considered quite useful for technical and environmental purposes. In the present study, production, purification, and physicochemical and biochemical characterizations of a new extracellular α-amylolytic enzyme produced by thermoalkalophilic isolate *Bacillus aerius* GC6 is reported and its potential to degrade raw starch has also been investigated. The properties of this enzyme, including its pH and temperature profiles, kinetic parameters, irreversible thermo-inactivation and raw starch digestibility revealed that it has significant potential for the starch industry.

MATERIALS AND METHODS

Microorganism and Culture Conditions

Bacillus aerius GC6 a novel thermoalkalophilic bacterium accession no. [KJ775810 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.

Production and purification of extracellular enzyme by microbial isolates

Inoculum Preparation

Bacillus aerius GC6 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.

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.

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 were 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.

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. Absorbance of reaction mixture was read at 670nm against a reagent blank.

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 separation of precipitates and supernatants. The precipitate obtained after centrifugation was then resuspended in minimum volume of Tris HCl buffer (20 mM, pH 8.0) separately and were refrigerated for further use. The enzyme solution was dialyzed against 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.

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).

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.

Characterization of Purified amylase

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.

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.

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 standard assay procedure.

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.

Effect of Different Concentrations of Substrate

To evaluate the effect of different concentrations of 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.

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%.

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). Activity was done under standard assay condition and the sample without organic solvent was considered as 100%.

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.

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:

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

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

Analysis of hydrolyzed products by thin layer chromatography (TLC)

Hydrolytic 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).

Determination of Kinetic Parameters

Determination of the kinetic parameters for the hydrolysis of *α*-amylase enzyme were calculated according to the method of Lineweaver-Burk plot by using the starch as 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.

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.

RESULTS AND DISCUSSION

Production and Purification of α-amylase

A newly isolated thermoalkalophilic α-amylase producing bacterial strain of *Bacillus aerius* GC6 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 aerius* GC6 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. The enzyme activity of crude enzyme was 844.97 IU/ml with specific activity of 1.98 IU/mg as well as protein of 42.56 mg. The crude enzyme of *Bacillus aerius* GC6 was subjected to partial purification with ammonium sulphate precipitation (30-80%) which exhibited 33.56 mg protein and a specific activity of 2.75 IU/mg (Table-1). 1.38 fold purification of enzyme was achieved with 54.74% amylase yield after 80% ammonium sulphate fractionation. Below 30% and above 80% salt saturation, amylase activity was found considerably reduced. Lily *et al.,* [11] also reported 80% saturation as best for α-amylase form *B. subtilis* MTCC 9447. *B. methylotrophicus* P11-2 α-amylase was efficiently precipitated at 80% concentration of ammonium sulphate with purification fold of 2.3 by Xie *et al.,* [12]. Fincan *et al.,* [13] have reported 70% saturation as optimum for precipitation of extracellular α-amylase form *B. subtilis*.

Table-1: Purification profile of α-amylase from *B. aerius* GC6

Purification step	Total activity	Total protein	Specific activity	Purification fold	%Recovery/ Yield
Crude enzyme	844.97	42.56	1.98	1	100
Ammonium sulphate precipitation	462.55	33.56	2.75	1.38	54.74
Dialysis	413.84	31.24	3.31	1.67	48.97
Gel exclusion chromatography	382.44	12.47	10.22	15.16	45.26
Anion exchange chromatography	225.78	3.19	35.38	17.86	26.72

The ammonium sulphate (80%) fraction was applied to sephadex G-75 gel filtration column. Many protein peaks were observed and only one activity peak was detected (fractions 16-22) (Fig-1). Active fractions were pooled and lyophilized. The enzyme activity of pooled fractions was checked by quantitative enzyme assays. Gel chromatographic separation resulted in 45.26 and 36.04% yield for *B. aerius* GC6. The Sephadex G-75 active fractions were pooled and loaded onto an DEAE-cellulose anion exchange column. Fincan *et al.,* [14] purified α-amylase from a thermophile *Anoxybacillus flavithermus* to 5.2 fold homogeneity by applying DEAE- cellulose anion exchange chromatography. Amylase activity was recovered in the unbound material, and SDS-PAGE analysis of the purified fraction revealed a single band with single subunit (Fig-2), indicating that the amylase was purified to homogeneity and had a 43 kDa apparent molecular mass. Xie *et al.,* [12] utilized a four step regime to a purify α-amylase from *B. methylotrophicus* P11-2 to a purification fold of 13. *Nesterenkonia* sp. strain F produced a novel SDS stable α-amylase, which was purified to homogeneity by Shafiei *et al.,* [14] by a three step purification technique with a 10.8-fold increase in specific activity. 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 [15].

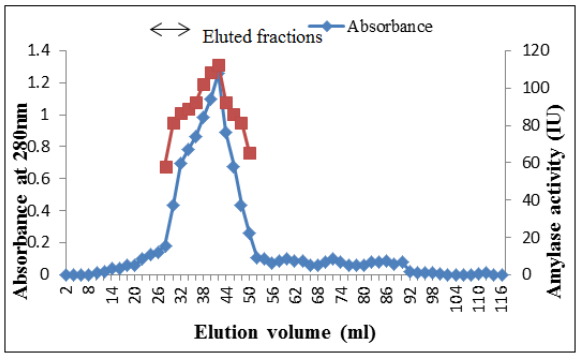

Fig-1: Protein and enzyme activity profile of fractions of Sephadex G-100 column chromatography of the dialyzed α-amylase of *B. aerius* GC6

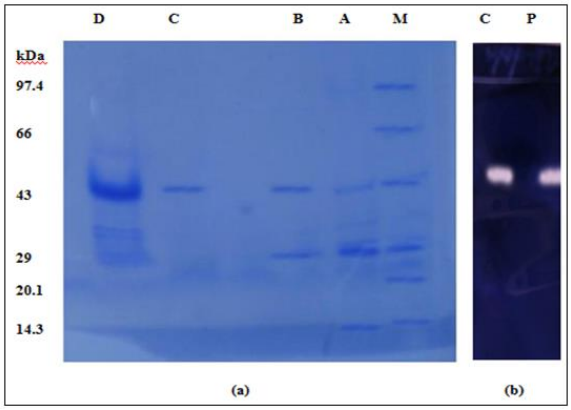

Fig-2: SDS-PAGE of *B. aerius* GC6 α- amylase at various stages of purification and (b) zymogram of crude and purified amylase

Lane A: Ammonium sulphate fractionated enzyme
Lane B: Sephadex G-100 column chromatography pooled fractions
Lane C: DEAE Cellulase anion exchange chromatography pooled fractions
Lane D: Crude enzyme

Zymogram Analysis

Zymoghraphy of α-amylase revealed white region in the dark background of non-denaturing gel corresponding to 43 kDa. 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].

Characterization of Purified α-amylase

Effect of pH on α-Amylase enzyme activity and stability

Enzymes being proteins are sensitive to changes in the environment in which they work. Any change in hydrogen ion concentration (pH) can profoundly affect the activity of an enzyme. This is very important in the industrial use of enzymes to be active at varied pH levels. The pH must be controlled and industrial processes usually try to get the maximum rates of activity by choosing the appropriate pH at which the enzyme is active but is not denatured.

On pH profile, the purified enzyme had a preference to work over a range of pH (7-10), where an optimum pH plateau was observed at pH 9 (Fig-3). More than 85% of the maximum observed activity was achieved at pH 9.0 and 10.0, proving alkaline nature of α-amylase. Below pH 7.0 and above 10.0, amylase activity decreased considerably. Most bacterial amylases are active at slightly acidic to near neutral pH.

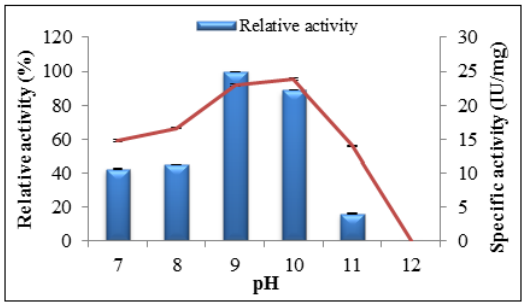

Fig-3: Effect of pH on purified α-amylase of *B. aerius* GC6

Hmidet *et al.*, [17] reported α-amylase from *B. licheniformis* NH1 which was highly active in the wide pH range of 5.0-10.0, with maximum activity at pH 9.0. In a report *Bacillus gibsonii* S213 showing best amylase activity from neutral to alkaline pH (7-9.5) Kohli *et al.*, [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 [15]. Many industrial processes involving enzymatic processes do not have an adequate pH control and thus the pH usually fluctuates around the required value. Thus it is important to determine the pH activity curve in quantitative detail for a particular substrate enzyme system under consideration. In such conditions, an enzyme with a wider pH stability range is sought after.

Effect of temperature on activity of purified α-amylase and its thermostability

The effects of temperature on the activity of an enzyme are complex and can be considered as two forces acting simultaneously but in opposite directions. As the temperature is raised, the rate increases, but at the same time there is a progressive inactivation (denaturation) of the enzyme protein. This becomes more pronounced as the temperature increases, so that an apparent temperature optimum is observed. In the present study, for estimation of the optimum temperature of the purified *α*-amylase, the activity was determined at different temperatures from 35-121°C (Fig-4). The optimum temperature for enzyme activity was found to be ranged between 35°C to 60°C. Highest enzyme activity was observed at 50°C for *Bacillus aerius* GC6. Optimum α-amylase production at 50°C is a characteristic of moderately thermostable microorganisms [19] and has been reported in literature by several workers. Demirkan *et al.*, [20] reported an amylase from *B. amyloliquefaciens* with 70% relative activity at 50°C. α-amylase from *Aspergillus oryzae* S2 has been shown to exhibit maximum activity at 50°C with a retention of 70% activity between 40-55°C [21]. *B. subtilis* α-amylase purified by Sani *et al.*, [22] exhibited maximum activity at 55°C.

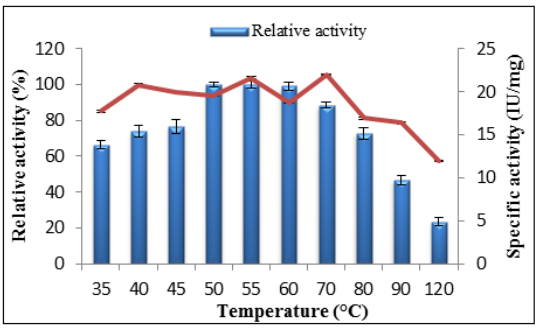

Fig-4: Effect of temperature on purified α-amylase of *B. aerius* GC6

The irreversible thermoinactivation of alpha-amylase was studied at a temperature range 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. As shown in Fig-5, purified *B. aerius* GC6 α-amylase was stable at a range spanning 50-70°C with retention of approximately 50% of the maximum activity for 60 min; however at 80°C, 46% 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. Fincan *et al.*, [13] studied 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.

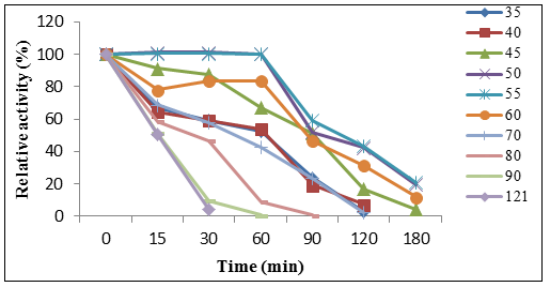

Fig-5: Thermostability profile of purified α-amylase of *B. aerius* GC6

Thermostable *a*-amylases are generally preferred in industry as their application minimizes contamination risk and reduces reaction time, thus providing considerable energy saving [23]. The major utility of α-amylases is in the starch industry for the liquefaction process that converts starch into fructose and glucose syrups. This process requires the use of a highly thermostable α-amylase which can act at temperatures around 70-100^{o}C.

Effect of metal ions on the enzyme activity

The activity of various enzymes is influenced by the presence of metal ions either by directly involving in their catalysis or by structural modifications. Some enzymes require certain metal ions as cofactors and thus called metalloproteins while others are inhibited by their presence in the reaction mixture. The effects of various divalent metal ions at 5mM on the *α*-amylase enzyme activity were assessed (Table-2). α-amylase activity of *B. aerius* GC6 was strongly influenced by the presence of divalent ions. An enhanced activity of 152.89% and 136.66% respectively was observed with Ca^{2+} and Mg^{2+} suggesting the enzyme to be a metalloprotein needing a cofactor for its maximum activity. Similar increase in enzyme activity has been shown by Bano *et al.*, [24]. A relative activity of 111 and 117% has been reported in the presence of Ca^{2+} and Mg^{2+} respectively.

Table-2: Effect of divalent ions on the activity of purified *B. aerius* GC6 α-amylase

Divalent ions	Relative activity (%)
	***B. aerius* GC6**
Ca^{2+}	**152.89±0.15^{a}**
Mg^{2+}	**136.66±0.11^{b}**
Cu^{2+}	91.02±0.23^{c}
Ba^{2+}	11.32±0.39^{d}
Fe^{2+}	58.89±0.17^{e}
Mn^{2+}	81.93±0.14^{f}
Co^{2+}	0±0.00^{g}
Zn^{2+}	2.78±0.16^{g}
Ni^{2+}	91.02±0.13^{c}
Control	100.00±0.09^{h}
SE(m)	**0.16**

Effect of Substrate specificity

Substrate specificity of *B. aerius* GC6 α-amylase was investigated using various substrates viz. corn starch, amylase, amylopectin glycogen, dextran and cellulose. For *B. aerius* GC6 α-amylase, results revealed that corn starch and amylopectin were better substrates for purified amylase than glycogen, dextran and cellulose yielding 56.26% and 48.30% activities respectively. This indicated that soluble starch, corn starch and amylopectin were the physiological substrates for the enzyme (Table-3). Azad *et al.*, [25] isolated and purified amylase which showed maximum activity with soluble starch as substrate. Ashwini *et al.*, [26] reported starch soluble as a substrate of choice for amylase isolated and purified from *B. marini.*

Table-3: Substrate specificity of purified *B. aerius* GC6 α-amylase

Substrate	Relative activity (%)
	***B. aerius* GC6**
Soluble starch (control)	100.00
Corn starch	56.26
Amylose	-
Amylopectin	48.30
Dextran	-
Cellulose	-
Glycogen	-

Effect of surfactants and inhibitors

Substances that reduce the activity of an enzyme catalyzed reaction are known as inhibitors. They act by either directly or indirectly influencing the catalytic properties of the active site. Inhibitors can be foreign to the cell or natural components of it. In the latter instance, they can represent an important element of the regulation of cell metabolism. For application in detergent industries, α-amylase must be stable in various detergent ingredients such as surfactants and chelators. Various surfactants were tested for their effect on purified *B. aerius* GC6 α-amylase and presence of anionic surfactant SDS was able to retain 94.14% of the original activity of *B. aerius* GC6 α-amylase. Reduction or complete inhibition of enzyme activity in the presence of SDS has also been reported by several workers [27-29]. Non-ionic surfactants like Tween 20, Tween 80 and Triton X 100 also stabilized enzyme with a relative activity of 101.31, 101.31 and 99.56% respectively for *B. aerius* GC6 α-amylase (Table-4).

Table-4: Effect of surfactants on the activity of purified *B. aerius* GC6 α-amylase

Surfactant	Relative activity (%)
	***B. aerius* GC6**
SDS	94.14±0.13[a]
EDTA	9.84±0.24[b]
CTAB	17.9±0.17[b]
Tween 20	101.31±0.22[c]
Tween 80	101.31±0.27[c]
Triton X 100	99.56±0.19[c]
Glycerol	87.09±0.14[d]
Control	100.00±0.11[c]
SE(m)	**0.19**

Surfactant stability of α-amylase is comparatively a rare feature and is very important at the same time considering the applicability of amylase in industry. The surface active agents in the medium increase the turn over number of the α-amylase thereby increasing its efficiency by increasing the contact frequency between the active site of the enzyme and the substrate by lowering the surface tension of the reaction mixture [30]. Variable behaviour of the enzyme towards these chemical reagents arose from the chemical nature of the enzyme, its origin and the constituent amino acids. The inhibitory and stimulatory effect of these additives however may be an important factor in the commercial exploitation of the enzyme where stability and activity of the enzyme are paramount [31].

Effect of Different Solvents on *α*- Amylase Enzyme Activity

The purified *α*-amylase enzyme was tested against different solvents. *B. aerius* GC6 α-amylase exhibited high tolerance to ethanol and acetone i.e. 99.55 and 96.35% respectively (Table-5). Thermostable enzymes are known to be resistant to organic solvents [30]. However, the presence of benzene and chloroform resulted in decreased activity to 56.21% and 76.78%.

Table-5: Effect of organic solvents on the activity of purified *B. aerius* GC6 and *B. sonorensis* GV2 α-amylase

Organic solvetnts	Relative activity (%)
	***B. aerius* GC6**
Acetone	96.35±0.28[a]
Methanol	90.41±0.37[b]
Ethanol	99.55±0.18[a]
Benzene	56.21±0.16[c]
Chloroform	76.78±0.47[d]
Xylene	11.09±0.11[e]
Control	100±0.07[a]
SE (m)	**0.22**

Shelf stability of Purified amylase

Shelf stability of the enzyme was studied at 4°C and at room temperature for 70 days. *B. aerius* GC6 α-amylase was found to be quite stable with a relative activity of 73% after 70 days (Fig-6) at 4°C. Kiran and Chandra [32] studied shelf life of amylase from *Bacillus* sp. TSCVKK for 2 months and found it stable at 4^{o}C, however a loss of 15% activity was observed after 48 h when it was incubated at 30^{o}C.

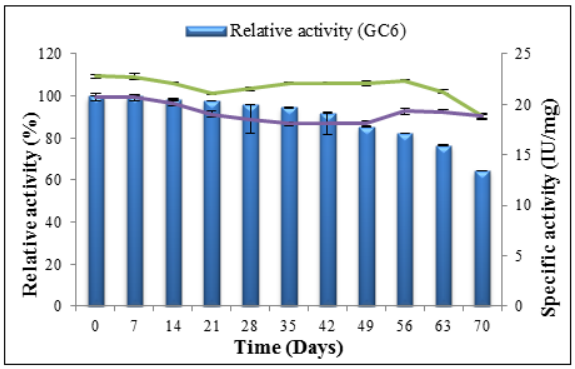

Fig-6: Shelf stability of purified *B. aerius* GC6 α-amylase at 4°C

Raw starch Adsorption and Hydrolysis

There has been a considerable interest in the potential use of raw starch degrading enzymes for industrial applications in food industry, especially those capable of hydrolysing starch molecules in large raw starch granules. Conventionally, conversion of starch to glucose and other oligosaccharides required a two-step process namely liquefaction and saccharification before addition of α-amylase and glucoamylase [33]. As a replacement of these steps, efforts are being made to exploit microorganisms to produce raw starch degrading enzymes. Studies on the affinities of the enzyme from *B. aerius* GC6 showed 94.29% adsorbability to raw potato starch and 68.46% adsorbability towards raw corn starch granules (Table-6) thus indicating an additional property of the enzyme which makes it very useful in efficient hydrolysis of raw starch granules. This is a highly cherished property in industry as the enzymes capable of digesting raw starches are economically attractive for they can increase the range of starch sources for direct saccharification. α-amylase from *Alicyclobacillus* sp. showed a respective hydrolysis efficiency of 58% and 52% for raw potato and corn starches, as reported by Bai *et al.*, [29]. This is a highly cherished property in industry as the enzymes capable of digesting raw starches are economically attractive for they can increase the range of starch sources for direct saccharification.

Table-6: Raw starch hydrolysis efficiency of purified *B. aerius* GC6

Raw starch	**% Hydrolysis**
	***B. aeiurs* GC6**
Corn starch	68.46±0.23[a]
Potato starch	94.29±0.31[b]
SE(m)	**0.21**

End products of starch hydrolysis

The end products of hydrolysis produced by *Bacillus aerius* GC6 were analyzed by thin layer chromatography that confirmed maltooligosaccharides as end products of starch hydrolysis confirming that the enzyme is α-amylase. Glucose, maltose and maltooligosaccharides were used as standards. The RF values of the samples and standards were determined and it was observed that the main hydrolysis products for enzyme were maltooligosaccharides The hydrolysis patterns presented by *B. aeiurs* GC6 purified amylase showed that the enzymes are typical endo-acting α-amylases. Kiran and Chandra [34] also reported the production of maltooligosaccharides as the hydrolysis products by purified *Bacillus* sp. TSCVKK α-amylase.

Determination of kinetic parameters

K_m is a useful and fundamental characteristic for an enzyme and a particular substrate. It can be viewed as an index of how easily the enzyme can be saturated by the substrate (i.e. the affinity) under defined conditions of temperature and pH. The smaller the value of K_m the more readily the enzyme may be saturated with substrate. Kinetic parameters were also determined by the incubation of the purified enzyme solution in the presence of different concentrations of soluble starch. The Michaelis-Menten constant (Km) and Vmax values estimated to be 1404 µmol/ mg/min and 4.40 mMol/min from the Michaelis-Menten and Lineweaver-Burk plots (Fig-7). Rasiah and Rehm [35] studied the kinetics of purified α-amylase from *B. licheniformis* and V_{max} and K_m values were found to be 506 U/mg and 5µmol respectively. Perusal of these reports revealed that purified *B. aerius* GC6 α-amylase have shown very good kinetic characteristics thereby making them efficient enzymes in terms of quality and quantity.

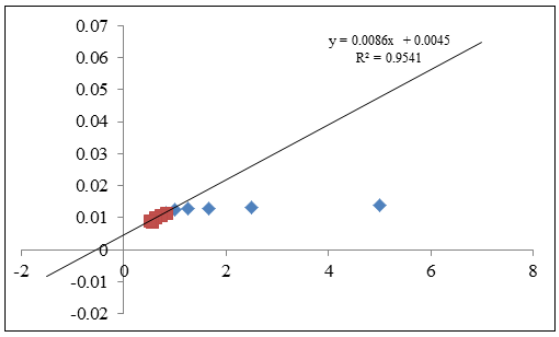

Fig-7: Lineweaver Burk Plot for enzyme kinetics of *B. aerius* GC6

Internal Sequence analysis by MALDI-TOF MS

An amino acid sequence from the digested protein was analyzed by MALDI-TOF mass spectrum to identify the purified enzyme. The MALDI-TOF mass spectrum (peptide mass fingerprinting) analysis of the reduced and carboxymethylated amylase digested with chymotrypsin (Table-7). The results of Mascot Search Engine from Matrix-Science search indicated that this partial amino acid sequence was little homologous by a score of 5 with UDP N-acetylglucosamine transferase. Amino acid sequence of amylase UDP N-acetylglucosamine transferase from *Geobacillus thermodenitrificans* NG80-2 and the matched peptides are shown in bold. The protein sequence coverage had been found only up to 5%. The peptide sequence obtained is: Ser-Gly-Ser-Ser-Asp-Pro-Leu-Gln-Thr-Leu-Leu-Ala-Leu-Ile-Gln-Asn-Thr-Ala-Arg.

Table-7: The MALDI TOF mass spectrum (peptide mass fingerprinting) analysis of the reduced and carboxymethylated *B. aerius* GC6 α-amylase digested with chymotrypsin

m/z	Resolution	Intensity	Area
770.092	7233	4362	92
995	6380	735	53
1013.017	6486	749	55
1157.021	7205	836	02
1175.018	7356	836	11
1319.022	7710	893	61
1337.025	8560	983	79
1481.032	8153	1034	68
1499.040	8711	1108	84
1607.040	7749	746	66
1643.045	8691	1126	63
1661.045	9508	1332	46
1805.057	8921	1114	29
1823.057	9522	1430	03
1967.062	8859	1058	57
1985.067	9580	1447	87
2129.069	9100	1005	57

2147.077	9349	1333	89
2291.074	8803	861	10
2309.083	9397	1208	69
2453.087	8630	765	72
2471.088	9150	1048	18
2615.093	8631	678	25
2633.098	9207	906	34
2777.103	8349	583	64
2795.101	8826	776	76
2957.114	8890	651	65
3119.121	8240	543	97

*range from 700-3200m/z

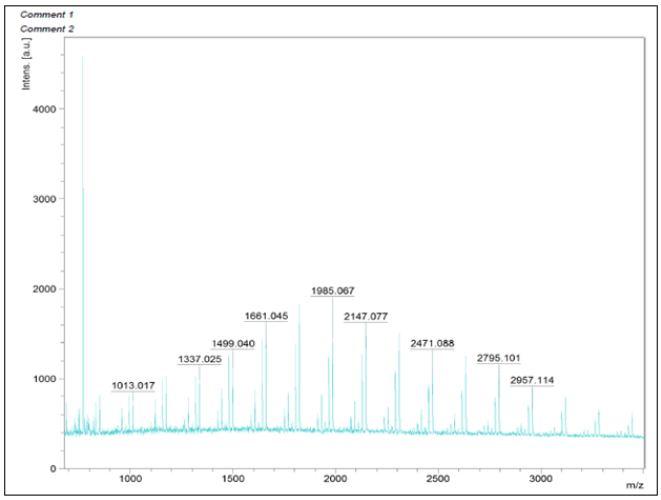

Fig-8: MALDI-TOF mass spectrum of purified *α*-amylase from *B. aerius* GC6. The mass spectrum shows a series of protonated peptide molecular ions

CONCLUSION

Amylases are important in many industrial processes. In conclusion, the present study described the purification and characterization of α-amylase from a novel thermoalkaophilic isolate *B. aerius* GC6. Nowadays, thermostable raw-starch degrading α-amylase has been attending a great interest for the starch hydrolyzing. Results of *Bacillus aerius* GC6 α-amylase characterization showed that this enzyme was stable at the wide ranges of temperatures from 35 to 60◦C and showed good activity at alkaline pH. Moreover, raw starch digesting effect by *Bacillus aerius* GC6 α-amylase was considerably observed on the high concentrations of raw corn starch. Due to these excellent properties, Stability at high temperature and broad pH range on alkaline side mark the possible application of *Bacillus aerius* GC6 α-amylase as a good candidate in detergent and starch saccharification industry. There are several processes in the medicinal and clinical areas that involve the application of amylases for the Ciba Corning Express clinical chemistry system and detection of higher oligosaccharides.

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Conflict of Interest

The authors declare that there is no conflict of interest.

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