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Acid stable α-amylase from *Pseudomonas balearica* VITPS19—Production, purification and characterization

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ABSTRACT

In the present study, α-amylase from *Pseudomonas balearica* VITPS19 isolated from Kolathur, Tamil Nadu, India was studied. Initially, one factor at a time (OFAT) approach was used to optimize the medium parameters like pH, temperature, carbon and nitrogen sources and the presence of metal ions to enhance the amylase activity. After the optimization, 6.5-fold increase in the enzyme production was observed. Enzyme purification was carried out in three stages. The molecular weight of purified α-amylase was estimated to be 47 kDa.The optimum activity for the purified enzyme was observed at pH 6 in 0.1 M phosphate buffer at 25 ± 2 °C and the activity is enhanced in the presence of ions like Mn^{2+}, Mo^{6+}, Na^{+}, $Mg^{2+}$and Zn^{2+} and was inhibited in the presence of Hg^{2+} ions. Compounds such as Sodium dodecyl sulfate (SDS), Ethylenediaminetetraacetic acid (EDTA), urea and β- mercaptoethanol reduced the amylase activity. The K_m and V_{max} of the α-amylase was estimated to be 45.23 mM and 20.83 U/mL, respectively.

1. Introduction

Alpha-amylases are enzymes that cleaves the 1,4-α-D-glycosidic linkages between the adjacent glucose molecules in the linear polysaccharides such as starch, glycogen, and oligosaccharides [1]. Amylases are broadly classified into alpha, beta and gamma subtypes in which the alph and beta amylases have been extensively studied. α-Amylase is a faster-acting enzyme than β-amylase. Amylases are also called glycoside hydrolases as they act on α-1-4 glycosidic bonds. All α-amylases (EC 3.2.1.1) act on starch (polysaccharide) as the main substrate and yield small units of glucose and maltose [2]. Amylases constitutes about 30 % of the world's enzyme market and covers many industrial applications such as sugar, textile, production of starch syrups, distilling, baking industries, and detergent formulation [3–7]. Chemical methods of starch hydrolysis have been replaced by amylases and its use has dominated the market by 25 % [8].

Amylases are produced by plants, animals and microbes. Microbial amylases are used extensively for industrial applications due to their stability, availability and economic value [9]. For industrial production, microbial amylases are the most suitable due to their short growth period, cost effective production, safe handling of microbes, productivity and easy manipulation of bacterial genes. Bacteria and fungi tend to secrete extracellular amylases i.e. amylases secreted outside the cells to perform extra cellular digestion of starch into sugars. Increasing industrial demand for microbial amylases has been observed due to their reaction specificity, mild optimal conditions for enzyme-substrate reaction, and less energy consumption than the conventional non-enzymatic chemical methods [10].

In industry, the major amylase producing bacteria used are *Bacillus subtilis, Bacillusamyloloquefaciens, Bacillus cereus* and *Bacillus megaterium*and fungi such as *Penicillum, Aspergillus niger, Cephalosporium, Neurospora* and *Rhizopus*. Microbes such as actinomycetes, yeast and algae are also used in the production of amylase [11]. Starch-cleaving enzymes i.e. amylases, from *Pseudomonas, Pimelobacter* and *Thermus*, have been used for cosmetics, food production, and pharmaceuticals, and enzymes of the same type from *Rhizobium, Arthrobacter, Brevibacterium* and *Micrococcus*, from *Pyrococcus* and from *Sulfolobus* for starch liquefaction at elevated temperatures and strongly acidic reaction conditions, respectively. Amylases from *Bacillus* sp. have been put to use at alkaline pH. Amylases from various *Bacilli* sp. are suitable for use in detergents or cleaning agents owing to their low sensitivity to detergents [50].

Amylases can be produced intracellular as well as extracellular. For the extracellular amylase production, the composition and concentration of various components of media greatly influence the growth and production of amylase [12]. Being a metal-loenzyme, Ca^{2+} is required for its integrity, activity and structural

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stabilization [13]. So, in order to yield maximum amylase production, media components and environmental conditions should be optimized. Acid stable α-amylases have diverse industrial applications and therefore their demand is increasing [14]. Currently, acid stable α-amylases are used in starch processing industries for the liquefaction of starch [15]. Acid stable amylases in combination with acid stable proteases/lipases are used in the treatment of exocrine pancreas insufficiency and in fermentation process to yield ethanol [51,52].

The current study mainly focuses on the production, purification and characterization of amylase produced by *Pseudomonas balearica* VITPS19 isolated from the rhizosphere of the agricultural soils of Kolathur, India.

2. Materials and methods

2.1. Materials

All the chemicals, reagents and media components used in this study were purchased from HiMedia Laboratories, Mumbai, India. The chemicals used were of LR (Laboratory Reagent) grade.

2.2. Isolation of rhizobacteria, molecular and morphological characterization

In the present study, *Pseudomonas balearica* VITPS19,a rhizobacteria isolated from the agricultural fields of Kolathur village, Tamil Nadu was studied. The strain was previously screened for amylase production and was reported earlier [16,17]. The isolate was initially designated as VITMS19. Molecular identification of the isolate was done by *16S* rRNA sequencing. The identified strain was designated as *Pseudomonas balearica* VITPS19. The sequence was then submitted to GenBank with an Accession number: MF164145 [18]. Phylogenetic analysis was done using MEGAX software. Scanning electron microscopy (SEM) analysis was carried out to investigate the morphology of the isolate. Physiochemical growth characteristics of the strain had been reported earlier [16].

2.3. Amylase production and enzyme assay

For the production of α-amylase, *Pseudomonas balearica* VITPS19 was initially grown in a media containing the following components in g/L - Soluble starch - 10, yeast extract - 2, peptone – 5, $MgSO_4$ – 0.5, KH_2PO_4 – 0.5, NaCl – 1.5 and $CaCl_2$ -0.5 (HiMedia Laboratories, Mumbai, India, LR grade) [19]. pH of the medium was adjusted to 7.3. The culture was incubated for 28 °C up to 12 days. After incubation, culture broth was centrifuged at 18,000xg for 15 min at 0 °C to remove the cell debris and cellular components. The supernatant was then collected and the amylase activity was estimated. The amylase activity was assayed as reported elsewhere [18]. The amount of glucose released was assayed by 3,5-Dinitrosalicylic acid (DNSA) method [20]. Bradford's method was used to estimate the protein content [21].

2.4. Optimization of amylase production by one factor at a time (OFAT) approach

OFAT method was carried out by changing parameters of factors in a sequential manner. The factors considered for optimization were pH of the media, Incubation temperature, Carbon and nitrogen sources and presence of metal ions. The pH of the media was adjusted between 4 and 10. The incubation temperature ranged between 4 °C and 45 °C. Additional carbon sources like glucose, fructose, sucrose, dextrose, inositol, mannitol, rhamnose, maltose, lactose, trehalose, sodium citrate, arabinose (HiMedia Laboratories, Mumbai, India, LR grade) was supplemented at 10 g/L along with Starch. The effect of nitrogen source on amylase production was evaluated by replacing peptone with malt extract, beef extract, yeast extract, peptone, casitone, corn steep liquor (HiMedia Laboratories, Mumbai, India, LR grade) and inorganic nitrogen sources such as ammonium sulphate, ammonium chloride, ammonium carbonate, potassium nitrate and sodium nitrate (HiMedia Laboratories, Mumbai, India, LR grade). Various metal ions such as Fe^{2+}, Cu^{2+}, Mg^{2+}, K^{+}, Ca^{2+}, Mn^{2+}, Ba^{2+}, Sn^{2+}, Zn^{2+}, Co^{2+}, Ni^{2+}at 0.5 g/L were supplemented in the media in the form of metal salts. All the experiments were performed in triplicates and the data is represented in Mean ± Standard deviation.

2.5. Purification of α–amylase

Followed by OFAT, Response ssurface methodology (RSM) was carried out to further optimize the amylase production and was reported elsewhere [18]. Crude amylase after optimization was purified by precipitating the enzyme with ammonium sulphate. The precipitate was then further purified by dialysis and separation by size exclusion chromatography. Ammonium sulphate was slowly added to the crude by varying the salt concentration (10 %–90 % saturation) with constant stirring (4 °C) and was kept at 4 °C overnight. The solution was centrifuged at 12300xg for 15 min at 4 °C and the precipitate was dissolved in 0.1 M phosphate buffer (pH 6.9). The precipitated enzyme was dialyzed against 0.1 M phosphate buffer for 18−24 h. The dialyzed samples were then applied on to a sephadex G50 column, pre-equilibrated with the same buffer. A constant flow rate of 0.72 mL/min was maintained and 2 mL fractions were collected. The collected fractions were assayed for amylase activity. To analyze the purity, the active fractions were pooled and applied to Reverse phase high-performance liquid chromatography (RP-HPLC) column.

2.6. Characterization of purified α–amylase

The purified fractions collected after size exclusion chromatography were used to characterize the enzyme for various parameters such pH, Temperature, effect of metal ions and complex compounds, molecular mass estimation and to study the enzyme kinetics. All the experiments were performed in triplicates and the data is represented in Mean ± Standard deviation.

2.6.1. Effect of pH & temperature

Optimum pH for enzyme activity was studied by incubating the enzyme with substrate (i.e. 1% starch) in various pH buffers such as Glycine-HCl buffer (pH 3), acetate buffer (pH 4 & pH 5), phosphate buffer (pH 6,7 & 8) and Glycine-NaOH buffer (pH 9 & pH 10). Optimum temperature was estimated by incubating the enzyme-substrate mixture at temperatures ranging between 4 °C and 100 °C. All the chemicals used to prepare buffers were purchased from HiMedia Laboratories, Mumbai, India, LR grade.

2.6.2. Effect of metal ions and complex compounds

Effect of metal ions on amylase activity was assayed by incorporating 1 mM concentration of various metal ions such as Fe^{2+}, Cu^{2+}, Mg^{2+}, Ca^{2+}, Mn^{2+}, Hg^{2+}, Mo^{6+}, Zn^{2+}, Ag^{+} and Na^{+} in the reaction mixture. Amylase activity was also estimated in the presence of 1 mM concentration of compounds such as Sodium dodecyl sulfate (SDS), Ethylenediaminetetraacetic acid (EDTA), β-mercaptoethanol and urea in the reaction mixture. The reaction mixture containing the purified enzyme without the addition of any metal ions/ complex compounds was used as a control. The enzyme used in the control was purified after dialyzing it in the phosphate buffer in the presence of 1 mM EDTA to remove any residual metal ions. All the chemicals used in the assay were purchased from HiMedia Laboratories, Mumbai, India, LR grade.

2.6.3. Enzyme kinetics

In the reaction mixture, the soluble starch concentration were varied ranging from 0.5 to 1000 mM and was assayed at optimal assay condition. Kinetic data obtained were plotted as Lineweaver–Burk (LB) plot. From the LB plot, the K_m and V_{max} were determined.

2.6.4. Molecular mass estimation

Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) was used to estimate the molecular mass of the purified enzyme. The samples were run at a constant voltage of 50 V and then, the gel was stained with Coomassie brilliant blue, and the resultant bands were compared with standard protein markers to estimate the molecular weight [22].

3. Results and discussion

3.1. Isolation and production of α-amylase

*Pseudomonas balearica*VITPS19 was isolated from the agricultural soils of Kolathur, India. Among the 30 isolates, it showed the maximum amylolytic activity when screened on starch agar plate. Phylogenetic analysis revealed that the isolate is closer to *Pseudomonas balearica* (Suppl. Fig. 1). Morphology analyzed using SEM showed that the isolates were roughly spherical in shape and the size was less than 1 μm (Fig. 1). The initial media for the production of α-amylase from *Pseudomonas balearica* VITPS19 contained 10 g/L starch to induce the production of amylase. 5 g/L peptone as the nitrogen source, 2 g/L yeast extract provided the required vitamins and growth factors. Ions such as $MgSO_4$, KH_2PO4, NaCl. to support the growth of the organism and 0.5 g/L $CaCl_2$ was provided as it is required for the activity of amylase. Initial pH was maintained at 7.3. The flasks were incubated at 28 °C±2 °C till 12 days at 150 rpm. The peak enzyme activity was seen on seventh day. The initial amylase (specific) activity was estimated to be 175 U/mg.

3.2. Optimization by OFAT

3.2.1. Optimization of carbon and nitrogen source by OFAT

Carbon sources are important in any media formulation. The utilization of a particular carbohydrate is unique to a particular strain and the pertaining environmental condition [23]. Among the media supplemented with various carbon sources, the production medium supplemented with maltose along with soluble starch gave the maximum amylase production of about 240 U/mg. This was then followed by rhamnose (160U/mg). Addition of trehalose and sodium citrate in the media resulted in poor amylase activity (data not shown). Earlier studies on *Pseudomonas aeruginosa* showed results in which addition of maltose as carbon source enhanced the amylase production [11]. In case of *Pseudomonas stutzeri ISLB5*, addition of starch increased the amylase production [24].Glucose enhanced the enzyme production in *Pseudomonas florescens*and *Pseudomonas mendocina* [25,26].

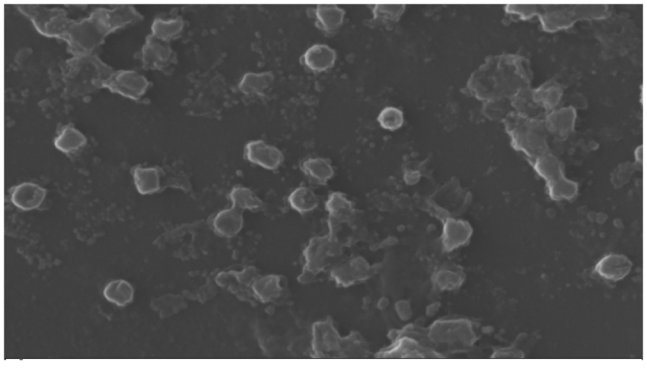

Fig. 1. The morphology of *Pseudomonas balearica*VITPS19 strain as viewed by SEM at 20kx.

Nitrogen plays an important role in the growth, aminoacid synthesis,and also act as secondary energy sources. Previous literature suggests that the concentration and the nature of the nitrogen source have a major influence on the production of enzyme [23]. In this study, maximum amylase production was observed in the medium supplemented with malt extract (400 U/mg) at a concentration 0.5 % followed by corn steep liquor (370 U/mg), casitone (310 U/mg) and yeast extract (290 U/mg). Addition of peptone (10 U/mg) and beef extract (20U/mg) showed very less production of amylase. All the inorganic nitrogen sources used in this study showed very less the amylase production (data not shown). However, the results are contrast to the previous studies, where peptone was reported to be the best organic nitrogen source for high amylase production in *Pseudomonas stutzeri* ISLB5 [24]. Addition of casein and beef extract yielded maximum amylase from *Aspergillus oryzae*and *Brevibacillus borstelensis* R1, respectively [27,28]. In a previous study, NH_4Cl gave maximum production of α-amylase from *Bacillus amyloliquefaciens* [29]. In *Marinobacter* sp. *EMB825*, casein enzyme hydrolysate was observed to enhance α-amylase production [30]. Very few published literature reports the use of Malt extract being used as nitrogen source and enhancing the production of amylase.

3.2.2. Optimization of pH and temperature by OFAT

All the enzymes are pH sensitive and pH influences stability of the enzyme. [31]. Optimization of pH was carried out by changing the pH of the production media between 4 and 10. Maximum amylase production was observed at pH of 6.5 with the enzyme activity of about 480 U/mgThe production of amylase was lower when the pH of the medium was either lesser than 6.5 or towards the basic pH. It is to be noted that that, optimum pH for the amylase production was 7 for *Pseudomonas aeruginosa*, *Pseudomonas florescens* and *Pseudomonas mendocina* and 7.5 for *Pseudomonas stutzeri*ISLB5, which were higher than the present study [11,24–26].

The production media along with the strain were incubated at various temperatures ranging between 4° and 45 °C. Maximum production (460 U/mg) of α-amylase from *Pseudomonas balearica* VITPS19 was observed at 28 °C of incubation temperature. Around 380U/mg of activity was observed at 37 °C. Approximately 50 % loss in production was observed at incubation temperature of 45 °C. For many of the *Pseudomonas* species, such as *Pseudomonas florescens* (35 °C), *Pseudomonas aeruginosa* (40 °C), *Pseudomonas stutzeri* ISLB5 (40 °C), *Pseudomonas aeruginosa* JCM5962 (40 °C) and *Pseudomonas mendocina* (40 °C), the optimum production temperatures were greater than 35 °C [11,24–26,32].

3.2.3. Optimization of metal ions

Metal ions are the key regulators of enzyme production and the effects of metal ions on production and activity on several amylases from bacteria and fungi. Metal ions such as Fe^{2+}, Cu^{2+}, Mg^{2+}, K^+, Ca^{2+}, Mn^{2+}, Ba^{2+}, Sn^{2+},Zn^{2+}, Co^{2+} and Ni^{2+} were added at 0.5 g/L in the production media and the effect of amylase production was evaluated. The study showed the highest enzyme production in the presence of calcium ion (1150 U/mg). This was about 6.5-fold increase in the amylase production when compared to the initial production of 175 U/mg. Following Ca^{2+}; Ba^{2+} (1020 U/mg), Mn^{2+}(980 U/mg), Fe^{2+} (980 U/mg) showed higher amylase production. Enzyme production was reduced in the presence of Mg^{2+}, K^+, Sn^{2+}, Zn^{2+} and Co^{2+}ions(data not shown).The enhanced amylase production was reported in presence of metal ions such Ca^{2+}, K^+

(KCl) and Mg^{2+} for *Pseudomonas stutzeri* ISLB5, *Pseudomonas florescens* and *Pseudomonas mendocina* respectively [24–26].

3.3. Purification of α-amylase

After optimization by OFAT method, statistical optimization was carried out by RSM [16]. α-amylase from the optimized media was purified and was further characterized. The crude broth yielded about 1.15 μg/mL of enzyme. The specific activity of the crude enzyme was 2173U/mg. Ammonium sulphate precipitation was then performed to precipitate the enzyme. Ammonium sulphate was added to the crude enzyme and the concentration of the salt was gradually increased from 10 % to 90 %. Precipitation at 30 % saturation yielded the maximum enzyme and maximum specific activity of about 2390U/mg was observed. The precipitate was then dissolved in phosphate buffer and was stored at 4 °C till further use. This was then followed by dialysis to remove the residual ammonium sulphate, the solution was dialyzed against 0.1 M phosphate buffer at 4 °C for about 18−24 h. Post dialysis the enzyme showed a maximum activityof about 3235U/mg. For further purification, the dialysate was applied on a sephadex G50 chromatography column. 0.1 M phosphate buffer (pH6.9) was used as the mobile phase. The flow rate was maintained at 0.72 mL/min. 2 mL fractions were collected at equal intervals of time and each fraction was assayed for amylase activity. The fractions showing the maximum activity were pooled. The fractions F2, F3, F4 and F5 showed amylase activity. The active fractions were pooled together and the activity was 14040 U/mg and this activity was about 6.46 times more than the crude enzyme (Table 1).

3.4. HPLC analysis

After the size exclusion chromatography, the active fractions were pooled together and were checked for purity by HPLC. Purified α-amylase from malt (HiMedia) was used as a standard. Profile obtained from the standard shows two peaks with a retention time of 2.36 min and 5.71 min. When purified α-amylase sample (pooled after Sephadex G50 fractionation) was injected, the profile showed two peaks with retention time of 3.31 min and 6.18 min confirming that α- amylase has been purified to homogeneity (Fig. 2). The slight variation in the retention time may be due to the property of the enzyme from different sources.

3.5. Characterization of purified α-amylase

3.5.1. Effect of pH

The effect of pH on the α-amylase activity was studied by using starch as a substrate at various pH values at 25 °C. The purified enzyme was active in a pH range from 4.0 to 6.0, with an optimum around pH 6.0. The relative activities at pH 7.0, 8.0, 9.0, and 10.0 were about 38 %, 22 %, 21 %, and 19 %, respectively, of that at pH 6.0. The optimum pH for the purified amylase was around pH 6 (Fig. 3). Since the enzyme activity was stable over the acidic range (pH 3–6), the purified amylase is assumed to be acid stable. Acid stable enzymes find a huge application in starch liquefaction industries. Amylase from *Pseudomonas luteola* and *Pseudomonas* sp. *2* showed an optimum pH at pH 5 and 5.5, respectively [34,35]. Kobayashi et al. reported that the G4-amylasesA had an optimum pH of about 6.3 [36]. Amylases from *Pseudomonas aeruginosa* JCM5962, *Pseudomonas stutzeri* ISLB5 and *Pseudomonas* sp. MS300 had an optimal activity at between 7 and 7.8 [24,32,36].

Starch is primarily used in the food industry, dairy products, beverages, sugar syrups, processed meat and snacks [37]. The pH of the native starch is in the acidic range and there is a requirement for amylases which are stable at a lower pH (i.e. between 3.2–4.5). In the starch processing industries, α-amylases that are currently employed are active at 95 °C, pH 6.8 and stabilized by Ca^{2+} ions. In the starch liquefaction process, the pH of the starch slurry is increased from 3.2 to 4.5 to 5.8–6.2, in order to be compatible with the optimum activity of the enzyme used. Ca^{2+} is further added to enhance the activity of the enzyme. Another pH adjustment to pH 4.2–4.5 is done during the saccharification step. Both the steps i.e., pH adjustment and removal of salts is to preferably omitted as these steps are time consuming and increases the product cost. Therefore, the need for acid stable amylases has increased in order to perform the steps in the pH close to that of the native starch [33].

3.5.2. Effect of temperature

The effect of temperature on the α-amylase activity was determined by assaying enzyme activity at different temperatures. The optimum temperature for enzyme activity was at 25 °C±2 °C, and decreased sharply when the temperature was above 37 °C. The enzyme showed 79, 60 and 57 % of inhibition in the enzyme activity at temperatures 37 °C, 45 °C and 60 °C respectively (Fig. 4). At temperatures more than 80 °C,a total loss in the amylase activity was observed. Similar findings have been reported for the G4-amylase F-2 from *Pseudomonas stutzeri* NRRL B-3389 where the enzyme completely lost its activity at temperatures greater than 55 °C [38]. Optimal temperature for amylase produced by *Pseudomonas sp*. 2 was approximately 30 °C [34]. But, *Pseudomonas aeruginosa* JCM5962 and *Pseudomonas luteola* amylases showed maximum activity at 40 °C and 60 °C respectively [32,35].

3.5.3. Effect of metal ions and complex compounds

The activity of the extracellular was assayed in the presence of various metal ions (1 mM). An increased amylase activity was observed in in the presence of Ca^{2+} ions by 185 % when compared with the control (no metal ions added). In the presence of Mn^{2+} (145 %), Mo^{6+} (140 %), Na^{+}(120 %), Mg^{2+}(105 %) and Zn^{2+} (100.5 %) ions, increased enzyme activity was observed. Whereas, the amylase activity dropped to about 75 %, 60 %, and 50 % of its initial activity (compared with the control) when the enzyme was incubated with Ag^{+}, Fe^{2+} and Cu^{2+} ions, respectively. However, presence of Hg^{2+} ions completely inhibited the amylase activity. It has been reported that calcium ions are required for activity or stability in most α-amylases [5]. The present study corroborates

Table 1
Table showing the protein purification at various stages of purification. All the experiments were performed in triplicates and the mean data is represented.

	Enzyme Activity (U/mL)	Protein (μg)	Specific Activity (U/mg)	Fold Purification (%)	% Yield
Crude	2.50	1.15	2173	1	100
30 % $(NH_4)_2SO_4$ Precipitation	1.97	0.824	2390	1.099	78.8
Dialysis	1.87	0.5781	3235	1.488	4.8
Chromatography Sephadex G-50	1.755	0.125	14040	6.46	70.2

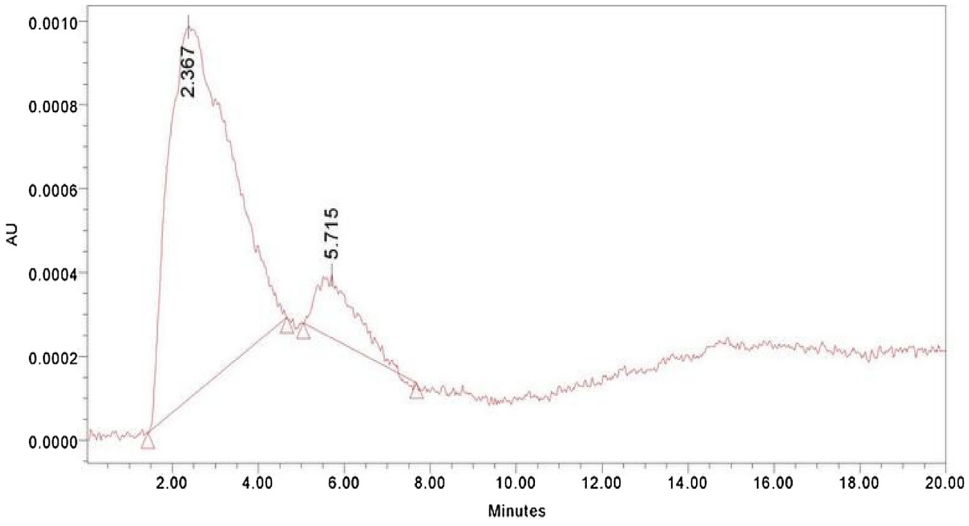

Fig. 2. HPLC profile of the pooled fractions of purified amylase after gel filtration chromatography.

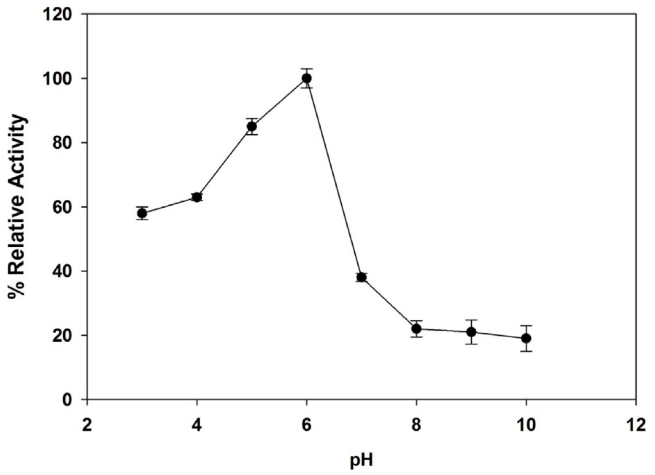

Fig. 3. Characterization of amylase activity of purified amylase at various pH, where the maximum activity was observed at pH 6.0. All the experiments were performed in triplicates and the data is represented in Mean ± Standard deviation.

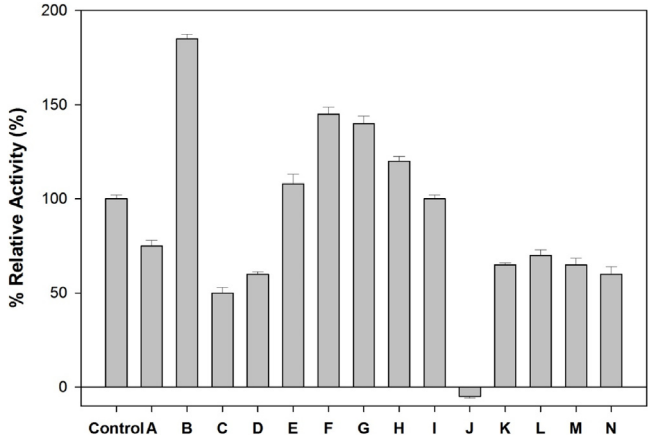

Fig. 5. Characterization of amylase activity of purified amylase with metal ions and complex compounds(A-Ag^{+}, B-Ca^{2+}, C-Cu^{2+}, D-Fe^{2+}, E-Mg^{2+}, F-Mn^{2+}, G-Mo^{6+}, H-Na^{+}, I-Zn^{2+}, J-Hg^{2+}, K-EDTA, L-Mercaptoethanol, M-Urea, N-SDS), where the presence of Ca^{2+}increased the activity by 185 % and Hg^{2+}inhibited the enzyme activity. All the experiments were performed in triplicates and the data is represented in Mean ± Standard deviation.

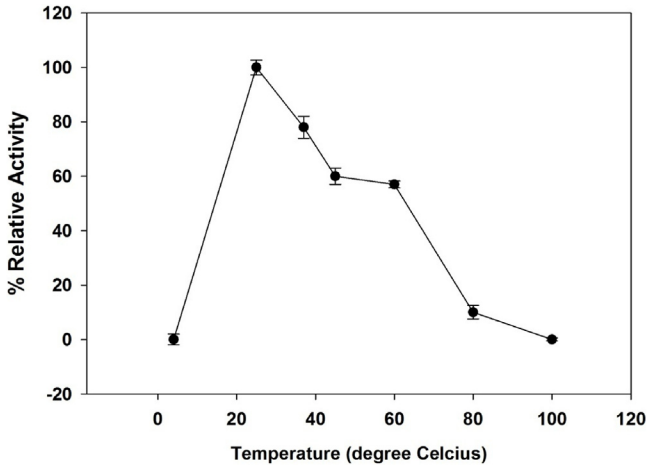

Fig. 4. Characterization of amylase activity of purified amylase at various temperatures,where the maximum activity was observed at 25 °C. All the experiments were performed in triplicates and the data is represented in Mean ± Standard deviation.

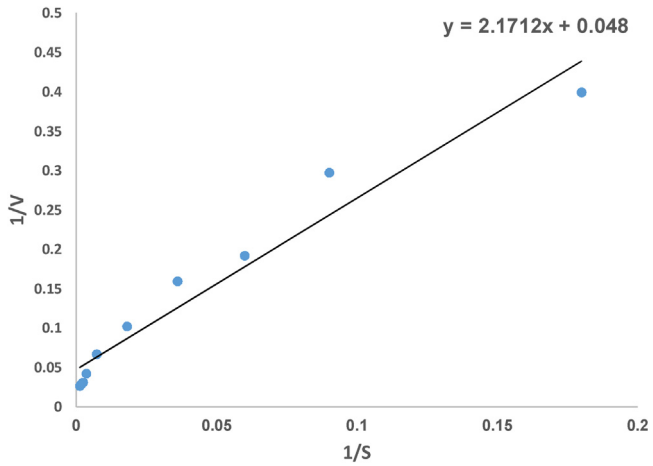

Fig. 6. Lineweaver–Burk plot of α-amylase from *Pseudomonas balearica*VITPS19.

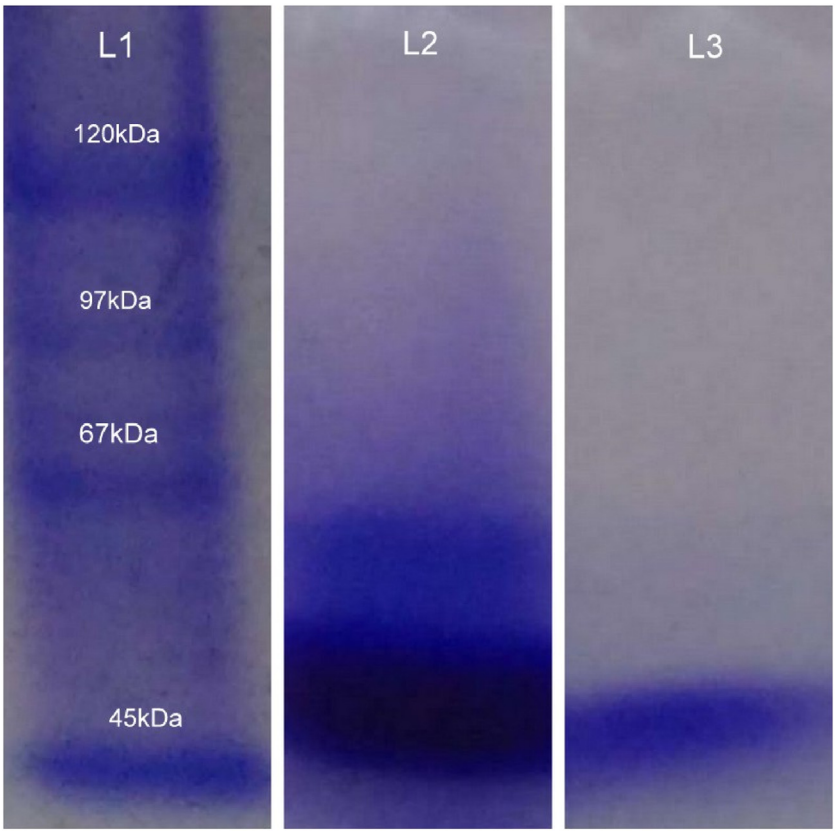

Fig. 7. SDS-PAGE of standard and purified α-amylase from *Pseudomonas balearica* VITPS19(L2 and L3) respectively and L1 – Protein molecular weight markers.

the fact that amylase show an increased activity in the presence of calcium ions.

Previous literature had reported that thermostability of some amylases are stabilized in the presence of Ca^{2+} ions [39]. There are few reports that demonstrate Ca^{2+}independent α-amylases [40–42]. However, it has been also reported that higher concentration of calcium ions might inhibit the enzyme catalysis [43]. This could be due to the fact that, when Ca^{2+} ions are at higher concentrations, Ca^{2+} ions bind to all the Ca^{2+} binding sites and these sites overlap with the catalytic site. Due to this the enzyme further cannot act on the substrate and as a result the enzyme activity decreases. Studies have also suggested the possibility of ions (other than Ca^{2+}) to increase amylase activity, such as Sr^{2+} for *Bacillus caldolyticus* amylase,and Ba^{2+} for *Pseudomonas variotia*α-amylase [44,45].

It was observed that 35 % of its original activity was lost in the presence of EDTA. SDS caused 40 % inhibition on the activity of the enzyme whereas urea caused 35 % inhibition. β-mercaptoethanol caused 30 % inhibition in the activity of the enzyme (Fig. 5). The enzyme activity was hindered in the presence of EDTA which indicates that purified amylase is a metalloenzyme. This result is in agreement with previous reports which that indicate amylases are often inhibited by chelating reagents [5]. The loss of activity in the presence of β-mercaptoethanol, Urea and SDS, might be due to change the conformational structure of the enzyme and leads to a slight lowering of the enzyme activity. Thus, suggesting that the conformation of the enzyme is possibly maintained by disulfide linkages.

3.5.4. Michealis Menton kinetics

Enzyme kinetics was analyzed using starch as the substrate. It was observed that enzyme activity increased with increase in starch concentration and reached maximum at 555.6 mM starch concentration and after which no increase in activity was observed. Lineweaver–Burk plot was used to determine the Michaelis–Menten constant (K_m) and the maximum velocity, V_{max} (Fig. 6). The K_m value of α-amylase was 45.23 mM and the V_{max} was found to be 20.83 U/mL.

3.6. PAGE analysis

The molecular weight (MW) of extracellular α-amylase from *Pseudomonas balearica* VITPS19 was 47 kDa (Fig. 7), which quite close to the MW of α-amylase isolated from *Bacillus subtilis* PKTH 10 which is about 55 kDa and *Streptomyces gulbargensis* around 57 kDa [46,47]. However, MW is higher than that of α-amylase isolated from *B. subtilis* DM-03(42.8 kDa) and *Bacillus* sp. TS-23(42 kDa) [48,49].

4. Conclusion

In this study, Optimization of α-amylase production by OFAT method was carried out. This is the first report of amylase production by *Pseudomonas balearica*. Optimization studies revealed that for enhanced amylase production Maltose as carbon source, malt extract as nitrogen source with supplementation of Ca^{2+} ions was required; pH should be maintained at 6.5 at an incubation temperature of 28 °C for about seven days. The amylase was purified by Ammonium sulphate precipitation followed by dialysis and size exclusion. The characteristics of the purified enzyme were studied. The maximum amylase activity was observed at pH6, temperature 25 °C in the presence of Ca^{2+} ions. It is to be also noted that the amylase was quite stable at acidic pH.

It is also to be noted that the amylase retained more than 50 % of its original activity in the presence of various detergents and chemical compounds. In the starch saccharification industries, selection of microbes for the production of α-amylase is an important criterion. Bacterial strains are the main choice of interest over fungal species due to their characteristics and kinetic properties. Activity of the current amylase at room temperature implies that no additional requirements such as heating and cooling equipments are required for carrying out the saccharification process, which generally takes place around 95 °C. These characteristics make the *Pseudomonas balearica* amylase an ideal candidate for several applications in starch processing industries and other allied industries.

Declaration of Competing Interest

The authors report no declarations of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.btre.2021.e00603.

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