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Research Article

PURIFICATION AND DETERMINATION OF PHYSICOCHEMICAL PROPERTIES OF α-AMYLASE SECRETED BY *PENICILLIUM CITRINUM* S1

BHISMA NARAYAN SWARGIARI[1,2]*, SARANGA RANJAN PATGIRI[1]

[1]Department of Botany, Cotton University, Guwahati, Assam, India. [2]Department of Botany, Kokrajhar University, Kokrajhar, Assam, India. *Corresponding author: Bhisma Narayan Swargiari; Email: bswargiari@gmail.com

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ABSTRACT

Objective: The α-amylase is a key industrial enzyme with widespread applications having about 30% share in the world enzyme market. The purpose of the study was to purify the α-amylase produced from *Penicillium citrinum* S1 and to evaluate the physicochemical and kinetic properties of the enzyme for its potentiality as an industrially valuable enzyme.

Methods: Production of extracellular α-amylase was done by using *P. citrinum* S1 in liquid medium and subjected to purification of the enzyme through ammonium sulfate precipitation, dialysis, followed by gel filtration chromatography.

Results: The enzyme was purified to 10.05 fold with a specific activity of 23.32 µg/mL. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis analysis of the enzyme revealed that the molecular weight of the enzyme is ≈43 kDa. From enzyme kinetic study, the Km value of α-amylase was found as 1.1 µg/mL, and the Vmax value was 37.31 U/mL. The optimum temperature showing the highest enzyme reaction is 50°C, and the most ideal pH range is 6.5–7.5. The purified enzyme remained stable at moderately high temperature (50–60°C) and up to 120 min and remained active in alkaline medium (0.1%) of commercial detergents such as Rin and Surf Excel.

Conclusion: These distinctive properties of this α-amylase purified from *P. citrinum* S1, such as low Km value, wide range of pH tolerance, stability of the enzyme at 50–60°C, compatibility with commercial detergents such as rin and surf, may be promising for application in starch processing, brewing, textile desizing, and detergent formulations.

Keywords: *Penicillium citrinum* S1, α-amylase, Purification, Characterization, Sodium dodecyl sulfate–polyacrylamide gel electrophoresis, Specific activity.

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INTRODUCTION

For a long time, enzymes from microbial sources have been utilized in various industries as catalyst to transform raw materials into desirable byproducts. The expanding necessity for microbial enzymes in a variety of industries, such as textiles, leather, paper, agriculture, biotechnology, pharmaceuticals, detergents, food processing, animal feed processing, and waste management has induced a search for superior microbes for extracellular enzyme biosynthesis [1,2].

The α-amylase has extensive application in manifold industries with a share of about 30% in the world enzyme market [3]. In 2023, the estimated market of α-amylase was USD 10.53 Billion and was expected to grow at 7.57% compound annual growth rate (CAGR) to turn into USD 17.54 billion till 2030 [4]. The bread and baking industries have the extensive use of α-amylase. Region-wise, North America has control over the market with 37% market share in 2023. The market is expanding in Asia Pacific due to the increasing population, where the demand baked products is raising. In a report by Grandview Research [5], it was mentioned that in India, the enzyme market size was USD 440.5 million in 2023 which is likely to grow with a CAGR of 7.8% till the end of 2030.

α- amylases (E.C. 3.2.1.1; 1,4-α-D-glucan glucanohydrolase) are members of GH-13 family of the glycoside hydrolase group of enzymes. These endo-acting enzymes irregularly break down α-1, 4-glycosidic bonds linking contiguous glucose units present in starch giving rise to a mixture of smaller sugars like maltose, maltotriose, maltodextrins, and glucose [6,7].

Due to the easy handling of fermentation parameters, less complicated downstream processes, cost-effectiveness, and environmental friendliness, microorganisms are considered as superior source of α-amylase in comparison to plants or animals sources [7]. Moreover, microorganisms have a fast growth rate, amiable to genetic modification, and can secrete extracellularly [2]. The bacterial and fungal enzymes in industry have more extensive use, with amylases finding several applications across a range of sectors [8].

The leading bacterial producers of α-amylases belong to *Bacillus* species, including *Bacillus subtilis, Bacillus amyloliquefaciens*, *Bacillus licheniformis*, *Bacillus stearothermophilus,* etc. [9,10]. Among fungi, species belonging to *Aspergillus oryzae* and *Aspergillus niger* are the common industrial sources of α-amylases and preferred for food processing applications as their extracellular enzymes exhibit higher activity under acidic to neutral pH [8,11].

Studies have shown that several Penicillium species can produce different industrially relevant enzymes, namely, amylases cellulases, lipases, and proteases. Many of them have also documented for the ability to produce α-amylases, for example, *Penicillium citrinum, Penicillium chrysogenum, Penicillium expansum, Penicillium janthinellum, Penicillium fellutanum, and Penicillium camemberti* [12-17].

Both SmF (Submerged Fermentation) and SSF (Solid State Fermentation) methods are employed to produce α-amylase. Nowadays, SSF is preferred for the ability to use different plant-based wastes materials for enzyme production contributing to sustainable development [8]. However, SmF is regularly used due to the easy handling of fermentation parameters.

In spite of the growing demand, there is limited research on the purification and thorough characterization of α-amylase from *Penicillium* species in comparison to the *Aspergillus*. To assess the industrial feasibility of these enzymes, it is crucial to characterize their biochemical properties, such as molecular weight, ideal pH, temperature stability, effect of metal ions, and kinetic parameters (Km and Vmax). Besides, the hunt for new fungal strains from distinct ecological niches might produce α-amylases that are more stable and active in harsh environments, increasing their industrial relevance.

Different industrial application requires α-amylases with unique properties relating to specificity, stability, temperature, and pH dependency. Exploration of microorganisms for bioactive compounds with novel properties is a never-ending process. For α-amylase enzyme, also search for new microorganism is a continuous process. Therefore, the present work focuses on the purification and characterization of α-amylase from P. citrinum S1, isolated from soil of forest ecosystem, Chakrashila Wildlife Sanctuary, Assam. It is aimed to elucidate the physicochemical and kinetic properties of the enzyme, providing insights into its potentiality as an industrially valuable enzyme.

METHODS

Microorganism

The microorganism *P. citrinum* S1 was isolated from the soil of Chakrashila Wildlife Sanctuary, Assam by the authors. The culture was maintained at 4°C on PDA (Potato Dextrose Agar) slants. For the α-amylase production, the culture was grown freshly, and 7-Days-old culture was used as inoculums.

α-amylase fermentation

Spores of *P. citrinum* S1 were harvested from 7-day-old slant as a suspension in sterile distilled water containing 0.01% Tween-80. Maintaining aseptic conditions, the spores were removed by using the sterile inoculation loop. The inoculum containing spore suspension was diluted to appropriate concentration (5×10^7 spores/ml) [16]. A culture medium containing (g/l) KH_2PO_4-1.5, $MgSO_4.7H_2O$-0.5, $FeSO_4$-0.01, Yeast extract-3, Peptone- 5, Starch-20 in a 250 mL Erlenmeyer flask was used for submerged fermentation. Fermentation was carried out in 50 mL of sterile culture medium by adding the inoculum. Inoculated flasks were incubated in a static BOD incubator at 30°C for 70 h. After fermentation, flasks were agitated for 15 min, and the broth was filtered through Whatman No. 1 filter paper. The filtrate as crude α-amylases source was used for further analysis.

Enzyme assay

The α-amylase assay was done by Dinitrosalicylic Acid (DNS) method [18]. The reaction mixture consisting of 1 mL of enzyme extract and 1 mL of substrate (i.e. 1% soluble starch solution) was taken in test tube and incubated for 30 min at 40/50°C. The reaction in test tube was stopped by the addition of 3 mL of DNS reagent and boiled for 10 min. The reaction mixture was cooled to room temperature, and absorbance was measured at 540 nm by a spectrophotometer (ultraviolet-visible double beam spectrophotometer-2201, Systronics). A standard graph was prepared with 0–100 µg maltose, and amylase activity of the test samples measured from the graph. One unit (IU) α-amylase activity is defined as the amount of enzyme that releases 1 µg of reducing sugar per minute under the standard reaction conditions.

Protein estimation by Lowry's method

The Lowry's [19] method was followed for protein estimation. A standard curve was prepared using bovine serum albumin (BSA). For this, different concentrations of BSA (e.g., 10–50 µg/mL) were prepared in distilled water. To each 1 mL of standard or enzyme sample, 5 mL of freshly prepared alkaline copper reagent (prepared by mixing 2% sodium carbonate in 0.1 N NaOH with 0.5% copper sulfate and 1% potassium sodium tartrate) was added. The mixture was incubated at room temperature for 10 min. Then, 0.5 mL of diluted Folin–Ciocalteu reagent (1 N Folin–Ciocalteu reagent diluted 1:1 with distilled water just before use) was added rapidly with mixing. The reaction mixture was incubated at room temperature in the dark for 30 min. The absorbance was measured at 660 nm against a reagent blank. A standard curve was plotted between BSA concentration and absorbance, and the protein concentration of the enzyme sample was calculated from this curve.

Ammonium sulfate precipitation

For a 60 mL enzyme solution, ammonium sulfate precipitation was carried out stepwise at ~4°C. First, 15.3 g of solid $(NH_4)_2SO_4$ was added with gentle stirring to reach 40% saturation, the mixture was equilibrated for 20–30 min, and centrifuged at 10,000–15,000 ×g for 15–20 min at 4°C (Cooling Centrifuge, Sigma 318 KS). To increase the saturation from 40% to 60%, an additional 7.6 g was added (making a total of 22.9 g for 60%), mixed for 20–30 min, and centrifuged again. To further increase from 60% to 80%, an additional 10.4 g was added (total 33.3 g for 80%), followed by mixing and centrifugation under the same conditions. At each step, either the pellet (precipitated proteins) or the supernatant (proteins remaining in solution) was collected depending on the desired fraction, and the pellets were redissolved in minimal buffer and dialyzed to remove ammonium sulfate.

Purification by gel filtration chromatography

Gel filtration chromatography was carried out using silica gel as the matrix for further purification of the enzyme. The column was packed with pre-equilibrated silica gel using 0.05 M phosphate buffer (pH 7.0). The enzyme solution obtained after ammonium sulfate precipitation and dialysis was carefully loaded onto the top of the column without disturbing the gel bed. The column was then eluted with the same buffer at a constant flow rate, and fractions of 2 mL each were collected. The absorbance of each fraction was measured at 280 nm to monitor protein elution, while enzyme activity was determined separately by the standard assay. Fractions showing both high protein content and enzyme activity were pooled together, concentrated if necessary, and stored at 4°C for further characterization.

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) analysis of extracted protein

The protein samples were analyzed by SDS-PAGE (Mini-Protean Tetra Cells, Bio-Rad) to determine the type of proteins and their respective molecular weight. The protein samples were loaded on the SDS-PAGE gel composed of 12% separating gel and 5% stacking gel. The gel solutions were prepared according the given composition and poured in the gel casting tray and allowed to solidify. The protein pellet was dissolved in 100 µL sample buffer (gel loading dye) and incubated at 95°C for 5 min. After incubation, the sample (20 µL) was loaded into separate wells on the stacking gel. The samples were allowed to run alongside the protein marker till the end of the separating gel. Then the gel was stained with 0.1% Coomassie Brilliant Blue R250 dye, followed by de-staining to visualize the protein bands.

Effect of different parameters on α-amylase activity

Effect of pH

The enzyme activity was evaluated at various pH values (3.5, 5.5, 6.5, 7.5, and 8.5) using 50 mM phosphate buffer. Reaction mixtures containing 0.5 mL of 1% starch solution, 0.5 mL of buffer (at the desired pH), and 1.0 mL of enzyme solution were incubated, and activity was determined (Digital pH meter-335, Systronics).

Effect of temperature

The reaction was carried out at various temperatures (30°C, 40°C, 60°C, 70°C, and 90°C). Each reaction mixture (1 mL enzyme, 0.5 mL starch solution, and 0.5 mL buffer at pH 7.0) was incubated for 15 min before DNS assay.

Thermal stability

Enzyme solutions were incubated at different temperatures (30°C, 50°C, 60°C, 70°C, and 80°C) for 120 min. Aliquots were taken at 30 min intervals to assess residual enzyme activity. After the final time point,

samples were centrifuged (10,000 rpm, 10 min), and the supernatant was subjected to acetone precipitation.

Effect of substrate concentration

To determine the influence of substrate concentration, the enzyme was incubated with starch at various concentrations (0.3%, 0.6%, 0.9%, 1.5%, and 2.0%, w/v) under optimal assay conditions, and amylase activity was measured.

Effect of commercial detergents

The enzyme was incubated with different detergents and surfactants – Rin, Surf Excel, SDS, Sodium lauryl sulfate (SLS), and Tween-20 (each at 0.1% w/v, final enzyme concentration 1 mg/mL) – for 30 min at 30°C. The residual enzyme activity was determined post-incubation.

Effect of metal ions

To assess the effect of metal ions (K^+, Zn^{2+}, Mn^{2+}, Mg^{2+}, and Cu^{2+}), 1 mM of each ion was added to separate enzyme samples. The mixtures were incubated at 50°C for 60 min, followed by activity determination.

Effect of enzyme inhibitors

The effect of various inhibitors (5.0 mM) EDTA (Ethylenediamine tetraacetic acid), N-ethylmaleimide, Phenylmethylsulfonyl fluoride (PMSF), Glutaraldehyde, and ascorbic acid on enzyme activity was studied under standard assay conditions.

Enzyme kinetics study

The kinetic parameters Km and Vmax of purified α-amylase were determined by measuring enzyme activity at varying substrate (starch) concentrations ranging from 0.3% to 2.0% (w/v). Reaction mixtures containing 0.5 mL starch solution, 0.5 mL of 0.1 M phosphate buffer (pH 7.0), and 1.0 mL of enzyme solution were incubated at 50°C for 10 min. The reaction was terminated by adding 1.0 mL of DNS reagent, followed by boiling for 5 min. Absorbance was measured at 540 nm, and reducing sugar concentration was determined using a glucose standard curve. Initial reaction velocities (v) were calculated in μmol/min and plotted against substrate concentrations ([S]). Kinetic parameters were estimated using the Lineweaver–Burk plot (double reciprocal plot of 1/v vs. 1/[S]. Km and Vmax were calculated from the slope and y-intercept of the linear regression line, respectively.

Statistical analysis

All experimental results represent values of three independent observations. The samples from each observation were analyzed to characterize the enzyme. Each result represents the average of three parallel replicates, along with standard deviation represented by error bars. To assess the differences between the groups, one-way analysis of variance (ANOVA) along with Tukey's Honestly Significant Difference (HSD) test was performed using IBM-SPSS 20 was used.

RESULTS AND DISCUSSION

It is indispensable to characterize the physicochemical properties of enzymes, such as molecular weight, ideal pH, temperature stability, effect of metal ions, and kinetic parameters (Km and Vmax) for industrial viability. In addition, the search for novel fungal strains from different ecological niches may yield α-amylases that are more robust and active in challenging conditions, enhancing their industrial significance. Notably, enzymes with specific pH and thermostability are required in the production of pulp, paper, and textiles. Enzymes having optimal activity at low pH and temperature are typically desirable in dairy, beverage, and food manufacturing processes as well as detergent compositions.

Selection of strain

The *Penicillium* species used in this study was isolated previously from soil of forest floor of Chakrashila Wildlife Sanctuary, Assam, and was screened for the amylase biosynthesis and found to be positive in terms of amylase secretion. The identification of this fungal strain was based on morphological and microscopical observations supported by manuals such as A Manual of Soil Fungi [20] and Handbook of Soil Fungi [21]. The *Penicillium* culture was identified as *P. citrinum* and designated as *P. citrinum* S1 by the authors.

Purification of the enzyme

The crude enzyme filtrate was subjected to ammonium sulfate precipitation (40–80%), and fractions were collected. The fractions were dialyzed, and silica gel chromatography was done. The crude extract contained a total protein of 850.50 μg, which showed the total activity of 1977.12 units with a specific activity of 2.32 U/μg proteins. The yield was considered to be 100%. After ammonium sulfate fractionation, the specific activity was increased to 4.82 U/μg with 62.68% yield. The specific activity was increased to 23.32 U/μg, and the fold purification is 10.05 after gel filtration chromatography on silica gel. Purified enzyme has been observed as a single band in SDS-PAGE (Fig. 1). The molecular weight revealed from the SDS-PAGE of the purified enzyme is ≈43kDa. The purification profile of the enzyme is presented in Table 1.

Gupta *et al*. [23] reported that the molecular mass of α-amylases derived from filamentous fungi usually ranges between 41.5 and 76 kDa. Several other studies have shown that the molecular weight of α-amylases from *P. janthinellum* is 42.7 kDa [16], *P. camemberti* PL21 – 60.5 kDa [17], *B.*

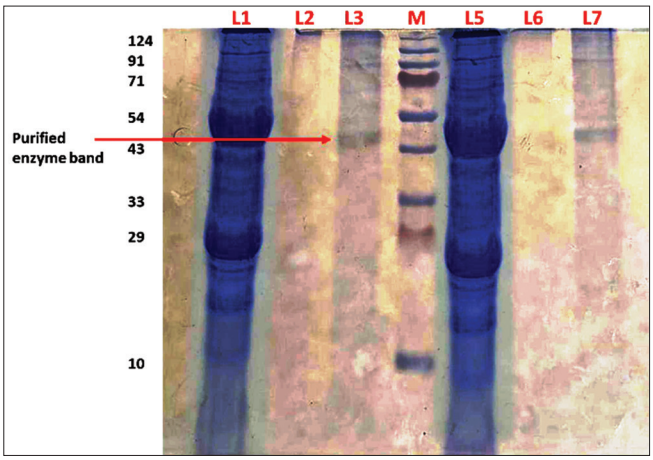

Fig. 1: Sodium dodecyl sulfate–polyacrylamide gel electrophoresis gel after detaining showing the protein bands for the respective samples of α-amylase (*Penicillium citrinum* S1) where Lane L1-crude protein extracted from isolate; Lane L3-purified protein showing protein band at ≈43 kDa; Lane M-Protein ladder (size in kDa); Lane L5-crude protein extracted from isolate; Lane L7-purified protein showing protein band at ≈ 43kDa

Table 1: Purification profile of α-amylase from *Penicillium citrinum* S1

Purification steps	Total activity (U)	Total protein (μg)	Specific activity (U/μg)	Purification fold	Yield (%)
Crude	1977.12	850.50	2.32	1.00	100.00
Ammonium sulfate precipitation (40–80%)	1239.40	257.10	4.82	2.08	62.68
Gel filtration chromatography	324.72	13.92	23.32	10.05	16.42

V: Volume, OD: Optical density

subtilis KIBGE HAS – 56 kDa [23], *Bacillus cereus* GL2 - 58 kDa [7]. These reports are also consistent with the findings of this study showing the molecular weight of α-amylase to be ≈43 kDa.

Effect of temperature

To observe the effect of temperature on α-amylase, starch hydrolyzing activity was assayed at 30–90°C (Fig. 2). The purified enzyme showed optimum temperature of enzyme reaction is 50°C. The α-amylase showed enzyme activity in a wide range of temperature between 30°C and 90°C. There is a gradual rise in enzyme reaction from 30°C, and the highest activity is found at 50°C (p≤0.05, one-way ANOVA). However, the enzyme activity gradually declined after optimum temperature, and it has exhibited activity at 90°C as well (Fig. 3). In general, the optimum temperature for amylases extracted from fungal and yeast sources was reported to be in the range of 30 and 70°C [11,15,17,22,23]. In several researches on *Penicillium* species, the ideal temperature range for α-amylase activity was revealed to be between 30°C and 60°C. [13,16,17]. These outcomes are showing similarity to our research. In contrast, in our experiment, it was noticed that even at 90°C the enzyme was found to be working, which indicates the higher temperature tolerability of the enzyme.

Effect of pH

The response of α-amylase at different pH level ranging from 3.5 to 9.5 was determined, and results are presented in Fig. 2. The optimum pH for α-amylase activity was recorded at pH 7.5 (94.44 U/ml, p 0.05, one-way ANOVA). The enzyme was active at both acidic and basic pH through a broad range from 3.5 to 9.5 (p≤0.05 by Tukey's HSD test). However, in most of the cases, the optimum pH values for α-amylases from bacteria and fungi were reported to have in the acidic to neutral range [22]. α-amylases often exhibit catalytic activity across a wide pH range, from 2.0 to 12.0 [24]. Metin *et al.* [15] reported that the amylase from *P. citrinum* HBF62 was active through within the pH 3.5–8.5 at 40°C for 24 h and retaining about 71% of its activity. Our results are supported by these findings. The results from the experiment also suggest that the enzyme was less sensitive to change in pH. Therefore, the enzyme could be applicable in many industrial fields.

Thermostability of the enzyme

Thermal stability of the enzyme was evaluated by incubating the enzyme for 120 min at 30–80°C. The results depicted in Fig. 4 show that at 50°C the enzyme has the highest stability from 30 min up to 120 min (p≤0.01, two-way ANOVA). Besides, at 60°C, the enzyme exhibited considerable stability. At 30°C and 40°C, the enzyme is less stable. However, at 80°C, the enzyme was least stable. The enzyme showed almost 100% stability up to 90 min at 50°C. After 90 min, the residual activity declined to about 90%. A similar trend was reported by Sindhu *et al.* [16] by the α-amylase isolated from *P. janthinellum* (NCIM 4960). The enzyme is also showing considerable stability at 60°C retaining 92% of the original activity. This outcome demonstrates the enzyme's suitability for use in procedures requiring a moderately high temperature regime, as in the same line with Sethi *et al.* [11].

Effect of substrate concentration

During the examination, the effect of starch concentration (0.30–2.0%) as substrate on enzyme activity showed it performed best at 2% substrate concentration (Fig. 5). From the results in Fig. 5, it is evident that the enzyme activity improved with the raise of starch concentration and reaching peak at 2% concentration and declined drastically beyond this concentration. However, the enzyme showed better activity within 0.6–2.0% of the substrate concentration. Sethi *et al.* [11] obtained the best enzyme activity at 1.25% of starch concentration with *Aspergillus terreus* NCFT 4269. In another study by Nouadri *et al.* [17] on *P. camemberti* PL21 reported, 1% starch showed the highest enzyme activity.

Effect of commercial detergents

To examine the stability of amylase in detergents, various commercial detergents were added to the purified enzyme solution at a concentration of 1 mg/mL [11]. The enzyme was shown to be compatible with all detergents, with Surf Excel exhibiting better

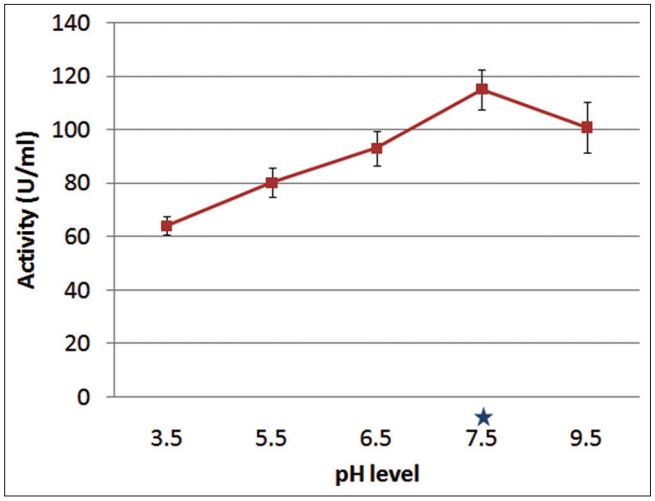

Fig. 2: Effect of pH on α-amylase activity from *Penicillium citrinum* S1 (Error bars indicate standard deviation of three independent observations, *p<0.05)

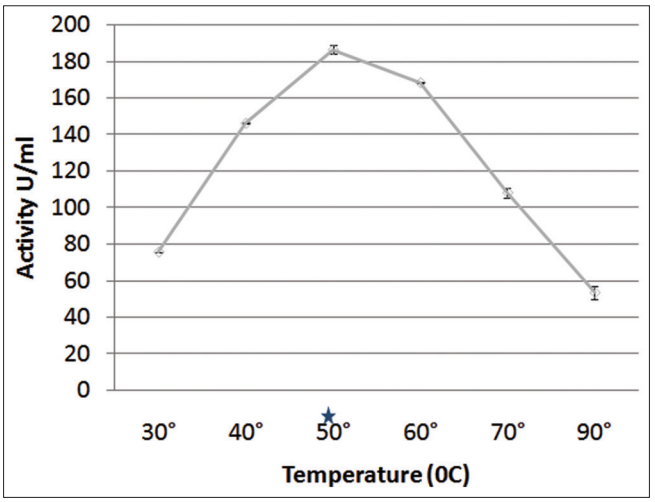

Fig. 3: Effect of different temperatures on α-amylase activity from *Penicillium citrinum* S1 (Error bars indicate standard deviation of three independent observations, *p<0.05)

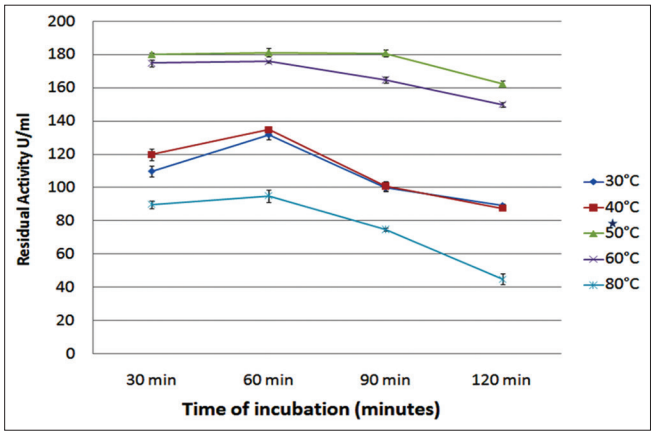

Fig. 4: Thermostability of α-amylase from *Penicillium citrinum* S1 (Error bars indicate standard deviation of three independent observations, **p<0.01)

activity, followed by Rin after 30 min of incubation. As seen in Fig. 6, SDS, SLS, and Tween 20 exhibited decreased amylase activity. In this study, effect of surfactants on enzyme activity, such as SDS, SLS, and Tween-20 can be compared with the commercial detergents like Rin, Surf Excel. α-amylases present in liquid detergents eliminate starchy trace or residues from clothes. A key role in industrial applications is implied by an enzyme's durability when combined with metal salts present in the detergent's formulation. The result shows the stability of α-amylase in the presence of commercial detergents such as Surf Excel. Similar results were observed by Sethi *et al.* [11] from α-amylase synthesized by *A. terreus* NCFT4269.10. pH optimum (7.5) obtained in the study also supports the compatibility commercial detergents with the purified α-amylase produced in this study.

Effect of metal ions

Presence of metal ions may show influence on the enzyme activity positively or negatively. In some cases, enzymes require metal ions as cofactors in the presence of which the performance of the enzyme is enhanced. Action of different metal ions on α-amylase activity was examined by incubating purified amylase with 1mM metal ion at a temperature of 50°C for 60 min duration. Ca^{2+} ion favors the enzyme activity as reported in other studies [11,15,17,25]. In the case of Mg^{2+}, the enzyme activity was reduced retaining only 47% of the total activity. Other ions such as Cu^{2+}, $Mn^{2+}$and K^{+} ions (Fig. 7) almost displayed inhibitory effect on enzyme activity.

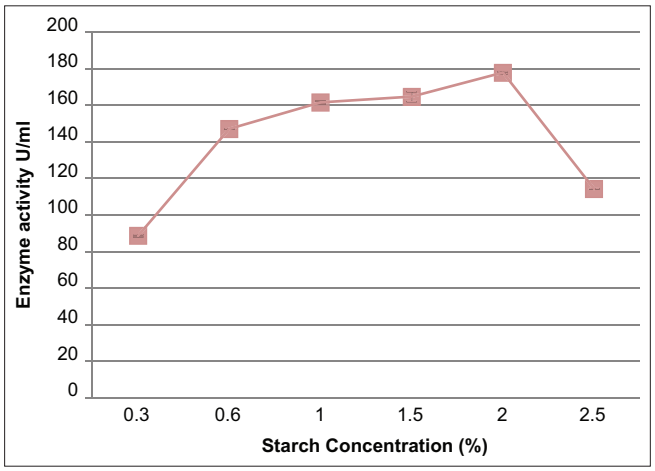

Fig. 5: Effect of substrate concentration (starch) on α-amylase activity of *Penicillium citrinum* S1 (Error bars indicate standard deviation of three independent observations)

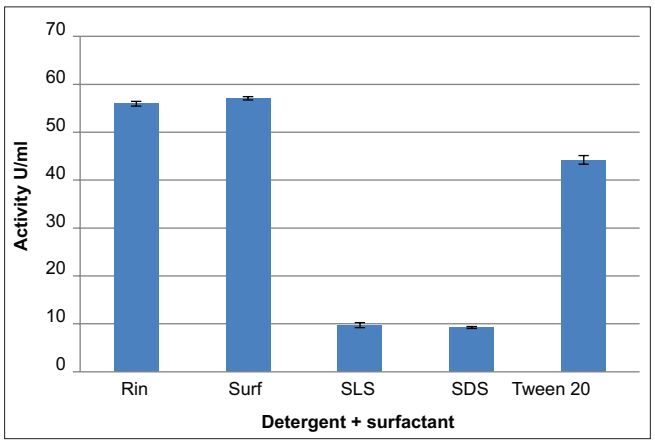

Fig. 6: Effect of commercial detergents on α-amylase activity from *Penicillium citrinum* S1 (Error bars indicate standard deviation of three independent observations)

Effect of inhibitors

The results presented in Fig. 8 have reflected that the activity of the purified enzyme was inhibited by inhibitors such as glutaraldehyde, EDTA, PMSF, N-ethylmaleimide, and ascorbic acid. Inhibitors such as Glutaraldehyde and N-ethylmaleimide act as irreversible inhibitors by cross-linking and modifying amino acid residues in the enzyme's active site, which ultimately repressed the α-amylase activity [11,15]. EDTA is a metal chelator, and the inhibitory effect of this chelating agent verified the ion requirement for α-amylase activity, which is consistent in line with a hypothesized metalloprotein that has a bound divalent cation. Conventionally, there is a calcium ions dependency of α-amylase for the enzyme stability and activity [11,16]. The PMSF inhibition of the activity indicated that serine and tryptophan residues, as well as carboxyl groups, were necessary for the enzyme active sites. Such amino acid residues are crucial to the activity, most likely because they aid in substrate binding. Related outcomes have also been described for α-amylases from *Thermomyces lanuginosus* [26] and *P. citrinum* HBF62 [15]. Inhibition α-amylase by ascorbic acid, a natural antioxidant, is due to the formation of hydrogen bonds with enzyme residues via its hydroxyl groups, thereby preventing starch binding and hydrolysis.

Kinetic parameters

Data represented in Fig. 9 for α-amylase produced by *P. citrinum* S1 illustrate Michaelis-Menten type of enzyme kinetics using soluble starch as the substrate. The observable Km and Vmax values for the enzyme, as determined by Lineweaver-Burk plot, were 1.1 mg/mL and 37.31 U/mg protein, respectively. Enzymes with a low Km value have higher affinity for its substrate, which implies that even at low substrate concentrations, they can bind and catalyze the reaction efficiently.

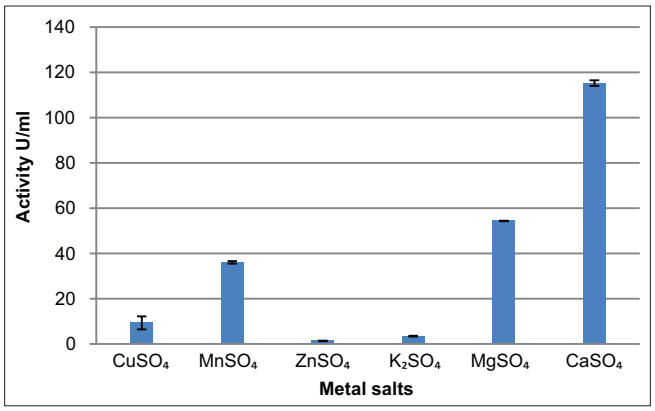

Fig. 7: Effect of metal ions on α-amylase activity from *Penicillium citrinum* S1 (Error bars indicate standard deviation of three independent observations)

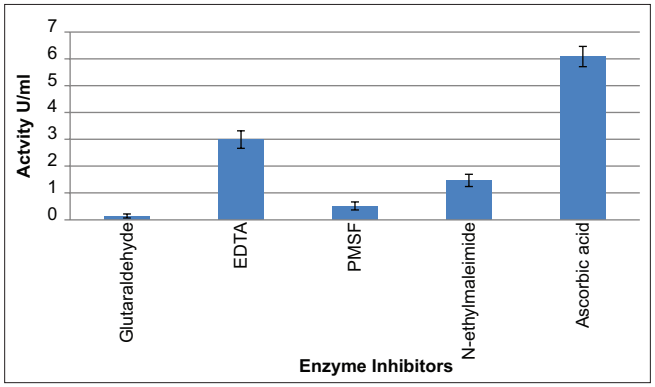

Fig. 8: Effect of inhibitors on α-amylase activity from *Penicillium citrinum* S1 (Error bars indicate standard deviation of three independent observations)

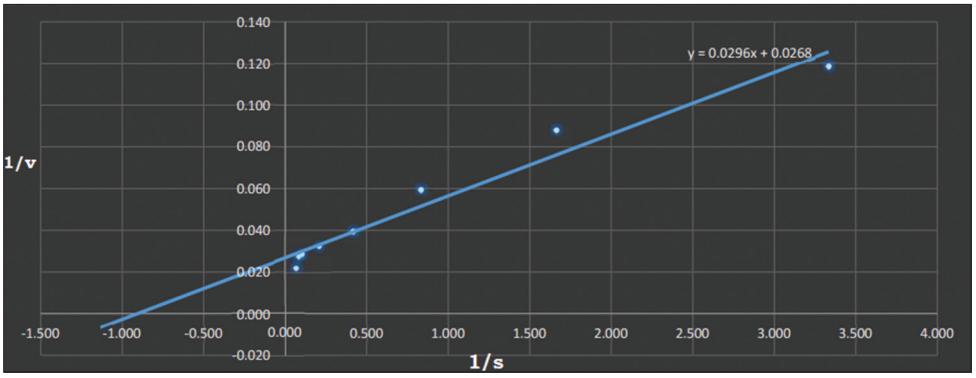

Fig. 9: Lineweaver-Burk plot (α-amylase by *Penicillium citrinum* S1)

The Km value for *P. camemberti* PL21 [17] is almost similar with this α-amylase. The Km of α-amylase of *P. griseofulvum* [27] and *B. subtilis* KIBGE HAS [23] is 9.1 mg/ml and 2.68 mg/ml, respectively, which are higher than the present study. The Km values of α-amylases from fungi and yeast have been described in between 0.13 and 5 mg/mL [15,22]. The finding of this study indicates that the enzyme employed produced a higher catalytic activity in comparison to α-amylases from other sources for starch. Whereas lower Km values of enzymes let for rapid and easier reactions in industrial processes, it can be assumed that the industrial potentiality of our α-amylase could be high.

CONCLUSION

It is important to bioprospect hyper α-amylase-producing microorganism for industrial biosynthesis with desirable properties [28]. In the present study, *P. citrinum* S1 was found to be a vital amylolytic fungus with reference to some recent studies conducted by Rengasamy and Thangaprakasam [29], Niharika *et al.* [30] and Kumar *et al.* [31]. The specific activity of the purified protein was 23.32 U/μg with a 10.05 fold of purification. The molecular weight revealed from the SDS-PAGE of the purified enzyme is ≈43kDa. The Vmax value of the enzyme is 37.314 (U/mL) and the Km value is 1.1 (U/mL). A lower Km value indicates a higher affinity of the enzyme for the substrate indicating the efficiency of the enzyme. The optimum temperature for enzyme reaction is 50°C. The best pH range is 6.5–7.5, however, can tolerate acidic pH as low as 3.5. It remains stable and up to 120 min at temperatures between 50 and 60°C. The optimum substrate range is 1.5–2% of starch and best result obtained at 2%. It may remain active in alkaline medium (0.1%) of commercial detergents such as Rin and Surf. Its activity is drastically affected by metal ions but enhanced by the presence of Calcium ion in the medium. Inhibitors like EDTA, PMSF etc. also inhibited the enzyme activity. These distinctive properties of this α-amylase are – low Km value, wide range of pH tolerance from 3.5 to 7.5, stability of the enzyme at 50–60°C, compatibility with commercial detergents such as rin and surf. Therefore, the P. citrinum S1, producing α-amylase with moderately thermostable and wide pH-tolerance may be promising for application in starch processing, brewing, textile desizing, and detergent formulations.

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