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Research Article

Partial Purification and Characterization of a Heat Stable α -Amylase from a Thermophilic Actinobacteria, *Streptomyces* sp. MSC702

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A partial purification and biochemical characterization of the α -amylase from *Streptomyces* sp. MSC702 were carried out in this study. The optimum operational conditions for enzyme substrate reaction for amylolytic enzyme activity from the strain were evaluated. The optimum pH, temperature, and incubation period for assaying the enzyme were observed to be 5.0, 55°C, and 30 min, respectively. The extracellular extract was concentrated using ammonium sulfate precipitation. It was stable in the presence of metal ions (5 mM) such as K⁺, Co²⁺, and Mo²⁺, whereas Pb²⁺, Mn²⁺, Mg²⁺, Cu²⁺, Zn²⁺, Ba²⁺, Ca²⁺, Hg²⁺, Sn²⁺, Cr³⁺, Al³⁺, Ag⁺, and Fe²⁺ were found to have inhibitory effects. The enzyme activity was also unstable in the presence of 1% Triton X-100, 1% Tween 80, 5 mM sodium lauryl sulphate, 1% glycerol, 5 mM EDTA, and 5 mM denaturant urea. At temperature 60°C and pH 5.0, the enzyme stability was maximum. α -amylase retained 100% and 34.18% stability for 1 h and 4 h, respectively, at 60°C (pH 7.0). The enzyme exhibited a half-life of 195 min at 60°C temperature. The analysis of kinetic showed that the enzyme has K_m of 2.4 mg/mL and V_{max} of 21853.0 μ mol/min/mg for soluble potato starch. The results indicate that the enzyme reflects their potentiality towards industrial utilization.

1. Introduction

Microbial amylases are among the most important hydrolytic enzymes and have been studied extensively. This group of enzymes represents one of the three largest groups of industrial enzymes and accounts for approximately 25–33% of the world enzyme market, in second place after proteases [1]. They have numerous applications in the industrial processing of different items, namely, starch liquefaction process [2], improve flour in the baking industry, produce modified starch for paper industry [3] and as an ingredient in automatic dishwasher and laundry detergent formulations [4].

Various microorganisms in nature, mostly fungi and bacteria, have complex amylolytic enzyme systems that are associated with starch decomposition and are responsible for hydrolyzing starch into simple sugars. Recently, several members of group actinobacteria provided a remarkable alternative to these traditional groups [5–7]. Application of

thermophilic microorganisms to produce enzyme for industrial use is a general practice because they provide broader temperature range and higher thermostability compared to enzymes from mesophilic microorganisms. The utilization of thermophilic actinobacteria in the cellulolytic, laccase, and xylanase enzyme production was well categorized [8–10]. Moreover, no report was published for the characterization of thermostable α -amylase isolated by thermophilic actinobacteria.

The previous publications by us covered the screening of strain *Streptomyces* sp. MSC702 and the optimization of the fermentation medium [11, 12] for the production of α -amylase enzyme. α -Amylase production by *Streptomyces* sp. MSC702 is significant as it is a thermostable and Ca²⁺-ion independent and exhibits a high degree of raw starch digestibility [12]. The partial purification and characterization of the enzyme as well as some kinetic data from *Streptomyces* sp. MSC702 are presently reported.

2. Materials and Methods

2.1. Actinobacteria and Culture Conditions. The amylolytic *Streptomyces* sp. MSC702 isolated from the mushroom compost in India was used as biological material [11]. Strain MSC702 was isolated on M medium agar [13] for 45°C at pH 7.0. M medium was modified with 1% (v/v) trace metal salt solution [14]. The strain was maintained on modified M medium agar slants at 4°C. All the culture media were autoclaved at 121°C (15 lbs) for 20 min.

2.2. Improvement of α -Amylase Production. α -Amylase production in submerged fermentation (SmF) was carried out in 250 mL Erlenmeyer flask using basal medium containing 1.0% rice bran, 2.0% wheat bran, 0.1% K_2HPO_4 , 0.1% $(NH_4)_2SO_4$, 0.1% NaCl, and 0.1% $MgSO_4 \cdot 7H_2O$ at pH 7.0. Cotton plugged flask was autoclaved at 121°C for 20 min and cooled. The medium was inoculated with 1% inoculum and incubated at 50°C for 48 h. Samples were harvested by filtering through Whatman filter papers 1 (qualitative circles, 125 mm diameter) and centrifuged at 5,000 g for 20 min at 4°C; the cell-free supernatant (crude enzyme) was used for α -amylase assay.

2.3. Amylase Assay and Protein Determination. α -Amylase activity was estimated by analyses of reducing sugar released during hydrolysis of 1.0% (w/v) starch in 0.1 M phosphate buffer (pH 7.0) by enzyme (cell-free supernatant) incubated at 50°C for 10 min. The amount of reducing sugar level released in the mixture was determined by the dinitrosalicylic acid (DNS) method [15]. Absorbance at 550 nm was recorded by using UV-visible spectrophotometer (UV-1700 Pharmaspec Shimadzu) and activity was calculated from a standard curve using maltose as the standard. One unit (U) of enzyme activity was defined as the amount of enzyme required for the liberation of 1 μ mol reducing sugar as maltose per minute under standard assay conditions. Total protein was estimated using BSA (bovine serum albumin) as standard, as described by Lowry et al. [16]. All experiments were carried out in triplicate and the data presented are average values.

2.4. Amylase Purification. The various steps of enzyme purification were carried out at 4°C unless otherwise mentioned. The crude enzyme was treated with solid ammonium sulphate with continuous overnight stirring and separation into the following saturation ranges: 0–20%, 20–40%, 40–60%, and 60–80%. The precipitates collected by centrifugation (10,000 g for 15 min) were dissolved in 0.1 M phosphate buffer, pH 7.0. The enzyme solution was dialysed against the same buffer for 12 h with several changes to remove the salt and assayed by the method described by Roe [17].

2.5. Estimation of Optimum Operational Conditions for Amylolytic Enzyme Activity. The optimum incubation temperature was examined by carrying the enzyme-substrate reaction for 10 min at different temperatures (50–90°C) keeping constant pH 7.0 (0.1 M phosphate buffer). Further optimum reaction time was determined by carrying the enzyme-substrate reaction at optimum temperature (55°C) and constant pH 7.0 (0.1 M phosphate buffer). Enzyme activity was checked

for 65 min at 5 min interval and was expressed as percentage relative activity.

The pH optima of the α -amylase were estimated by preparing the reaction mixture with various pH buffers and assayed for 10 min at 55°C. Three buffers (0.1 M) were used for different pH, that is, phosphate-citrate buffer for pH 3.0, 4.0 and 5.0, phosphate buffer for pH 6.0, 7.0 and 8.0, and glycine-NaOH buffer for pH 9.0, 9.8 and 10.6. Enzyme activity was expressed as percentage relative activity.

2.6. Characterization of α -Amylase

2.6.1. Effect of Temperature and pH on Enzyme Stability. To estimate thermostability, crude enzyme was preincubated for 30 min, at different temperatures (50–85°C) before enzyme assay, and promptly cooled on ice and residual activity was determined under standard assay conditions. The half-life of α -amylase was determined by incubating the crude enzyme at 60°C and residual activity was measured after every 15 min for 240 min (4 h) under standard assay conditions.

Effect of various pH buffers (3–10.6) on enzyme stability was studied by incubating the enzyme with various pH buffers, as stated above, for 30 min at 60°C before enzyme assay and the residual activity was determined under standard assay conditions. Effect of pH on enzyme thermostability was also determined at 60°C by measuring the residual activity after every 15 min for 240 min (4 h) under standard assay conditions.

2.6.2. Effect of Various Reagents on Enzyme Activity. Effect of various additives such as salts of 16 metal ions (5 mM) (K^+ , Ag^+ , Pb^{2+} , Mn^{2+} , Mg^{2+} , Fe^{2+} , Co^{2+} , Cu^{2+} , Zn^{2+} , Ba^{2+} , Mo^{2+} , Ca^{2+} , Hg^{2+} , Sn^{2+} , Cr^{3+} , and Al^{3+}), 4 surfactants {Triton X-100 (1%), Tween 80 (1%), sodium lauryl sulphate (5 mM), and glycerol (1%)}, chelating agent EDTA (5 mM), and denaturant urea (5 mM) on enzyme activity was tested by incorporating 1 mL solution of each additive in enzyme-substrate reaction mixture. The reaction was carried out for 30 min. Enzyme activity was measured under standard assay conditions. Enzyme activity was determined as percentage relative activity of control (without additives) considered as having 100%.

2.6.3. Steady State Kinetics Measurement. Kinetic parameters for α -amylase were determined by incubating the crude enzyme with various concentrations (0.5–8.0 mg/mL) of soluble potato starch under standard assay conditions. The Michaelis-Menten constant (K_m) and maximum velocity (V_{max}) values were determined from Lineweaver-Burk plots. The K_m and V_{max} values were calculated from the kinetic data using the “GraphPad Prism” software.

3. Results and Discussion

3.1. Optimum Operational Conditions. The optimum temperature for the α -amylase activity from *Streptomyces* sp. MSC702 was in a wide range of 50–75°C (retained >74% relative activity at the temperature upto 75°C) with maximum activity at 55°C (Figure 1). However, at temperatures 85°C and 90°C, the retained relative activity of α -amylase was

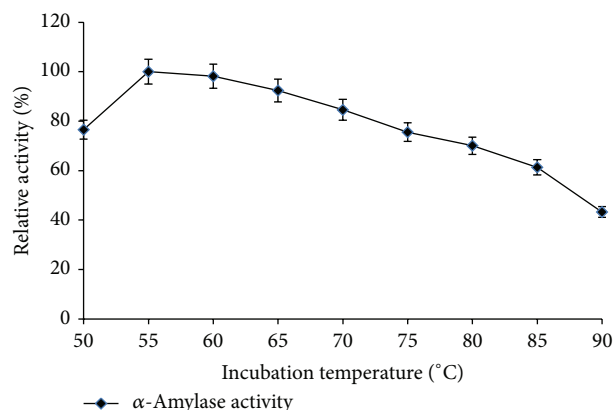


FIGURE 1: Effect of different incubation temperatures on enzyme activity (10 min incubation).

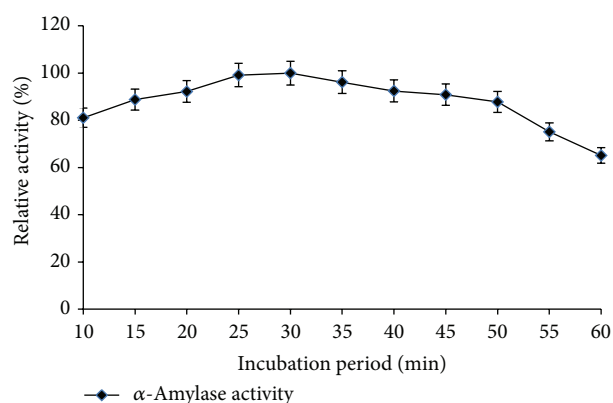


FIGURE 2: Effect of different incubation periods on enzyme activity (at 55°C for alpha-amylase).

61.33% and 43.26%, respectively. Enzyme-substrate reaction was maximally active in the range of 10 min to 50 min (>80% relative activity) with maximum alpha-amylase activity achieved in 30 min at 55°C (Figure 2). There was a remarkable decrease in alpha-amylase activity after 50 min incubation. The increase in incubation period might induce conformational changes in 3D structure of the enzyme affecting its substrate affinity. Chakraborty et al. [18] reported a drastic decrease in alpha-amylase activity at 90°C with maximum activity at 50°C from *Streptomyces* sp. D1. Syed et al. [19] reported optimal activity at 45°C for alpha-amylase from *S. gulbargensis*. Results from present study provide lines of evidence that alpha-amylase from *Streptomyces* sp. MSC702 could be a good candidate for the efficient liquefaction of gelatinized starch.

The optimum pH for alpha-amylase activity from *Streptomyces* sp. MSC702 ranged from pH 3.0 to 7.0 (retained >91% activity) with a maximum activity at pH 5.0 (Figure 3). Although a decline in enzyme activity was observed between pH 8.0 and pH 9.0, the enzyme was still active at pH 8.0 and 9.0, retaining its 52.71 and 34.78% activity. A complete loss in the enzyme activity was observed above pH 9.8. Activity of alpha-amylase at low pH range is very important for industrial applications [20]. The application of liquefying amylases that are active and stable around the saccharification pH is

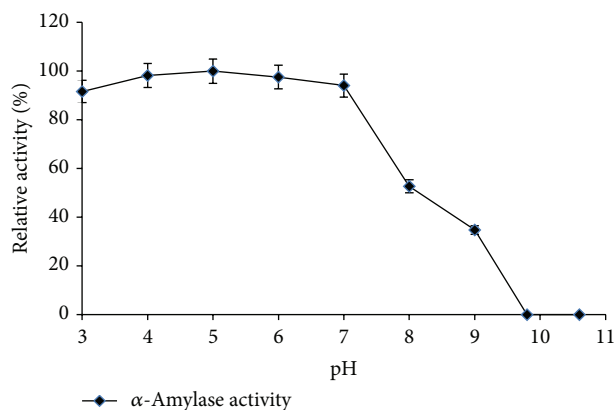


FIGURE 3: Effect of different pH on enzyme activity with 10 min incubation (at 55°C for alpha-amylase).

attractive to avoid or reduce the use of acid to lower the pH from liquefying to saccharifying range and also to simplify the procedures during downstream processing. Further, the use of alpha-amylases that operate at lower pH values reduces the formation of some by-products, such as maltulose, which is usually produced at higher operation pH [21]. Ammar et al. [22] reported optimum pH 6.0-7.0 for *Streptomyces* sp. alpha-amylase. In contrast, Chakraborty et al. [18] and Syed et al. [19] reported optimum activity at pH 9.0 for *Streptomyces* sp. D1 and *S. gulbargensis* alpha-amylases, respectively.

3.2. Effect of Metal Ions and Surfactants on alpha-Amylase Activity.

The variety of ways by which metal ions affect enzyme catalysis that is, by modifying the electron flow in the enzyme substrate reaction or by changing the orientation of the substrate with reference to the functional group at active site. Metal ions accept or donate electrons and act as electrophiles, mask nucleophiles to prevent unwanted side reactions, bind enzyme and substrate by coordinate bonds, hold the reacting groups in the required 3D orientation, and simply stabilize a catalytically active conformation of the enzyme [23]. Effect of metal ions and other additives on the activity of alpha-amylase by *Streptomyces* sp. MSC702 and its comparison with the earlier reports are presented in Table 1.

Among the various metal salts and chemical reagents tested, it was found that the alpha-amylase activity was almost completely inhibited by (5 mM) Pb^{2+} , Mn^{2+} , Mg^{2+} , Cu^{2+} , Zn^{2+} , Ba^{2+} , Ca^{2+} , Hg^{2+} , Sn^{2+} , Cr^{3+} , and Al^{3+} metal ions. Ag^{+} and Fe^{2+} inhibited alpha-amylase activity up to 40.27% and 50.96%, respectively. Metal ions such as K^{+} (154.32% relative activity), Co^{2+} (391.82% relative activity), and Mo^{2+} (154.81% relative activity) strongly stimulated alpha-amylase activity. The effect of Co^{2+} ions on alpha-amylase activity varies drastically with strain to strain of *Streptomyces*. Chakraborty et al. [18] reported stimulation while Syed et al. [19] reported inhibition of alpha-amylase activity in *Streptomyces* sp. D1 and *S. gulbargensis*, respectively, in the presence of Co^{2+} ions. The unusual behavior of the enzymes for Co^{2+} ions might be related to its special structure and the mechanism of action behind this is subject to further research. Metal ions such as

TABLE I: Comparative analysis of the effect of different additives on enzyme stability.

Additives	α -Amylase relative activity (%)					
	A ₁ [#]	A ₂ [#]	A ₃ [#]	A ₄ [#]	A ₅ [#]	A _x [#]
Metal ions	—	100	—	108	—	154.32
KCl (5 mM)	—	100	—	108	—	154.32
AgCl (5 mM)	—	—	—	—	—	69.23
Pb(NO ₃) ₂ (5 mM)	—	—	40	—	—	8.65
MnSO ₄ ·H ₂ O (5 mM)	84.37	110	68	143	—	0
MgSO ₄ ·7H ₂ O (5 mM)	87.38	109.87	46	113	—	7.21
FeSO ₄ ·7H ₂ O (5 mM)	—	56.67	43	107	0	49.04
CoCl ₂ (5 mM)	44.78	104.50	—	138	76.46	391.82
CuSO ₄ (5 mM)	34.61	108.79	50	142	38.95	0
ZnSO ₄ (5 mM)	84.78	—	45	106	0	0
BaCl ₂ (5 mM)	—	—	—	—	58.82	0
(NH ₄) ₆ Mo ₇ O ₂₄ (5 mM)	—	—	—	—	—	154.81
CaCl ₂ (5 mM)	100	125	87.50	115	185	14.90
HgCl ₂ (5 mM)	31.45	64.23	29	72	36.46	0
SnCl ₂ (5 mM)	—	—	—	—	—	0
CrO ₃ (5 mM)	—	—	—	—	—	0
AlCl ₃ (5 mM)	—	—	—	—	—	0
Surfactants						
Triton X-100 (1%)	—	—	—	—	—	40.92
Tween 80 (1%)	—	—	—	—	—	47.37
SLS (5 mM)	—	—	—	—	—	34.21
Glycerol (1%)	—	—	—	—	—	36.52
Chelating agent						
EDTA (5 mM)	95.23	2.13	—	85	—	0
Denaturant						
Urea (5 mM)	16.45	9.45	—	—	—	23.93
* Control	100	100	100	100	100	100
References	[26]	[34]	[25]	[24]	[35]	Current study

* Enzyme without any additive; A₁[#] = *Streptomyces* strain A3; A₂[#] = *Saccharopolyspora* sp. A9; A₃[#] = *Bacillus subtilis*; A₄[#] = *Nocardiopsis* sp. 7326; A₅[#] = *Bacillus cereus* GA6; A_x[#] = *Streptomyces* sp. MSC702 (enzyme preincubated at 60°C with 30 min); —: Not tested.

K⁺ strongly stimulated the α -amylase activity, similar to the report of Zhang and Zeng [24].

Most of the amylases reported till date are metal ion dependent enzymes and Ca²⁺ ions known to be a stabilizer for amylases isolated by various microorganisms. In case of the strain MSC702, the role of Ca²⁺ ions is inhibitory, showing its applicability in the confectionary industry, particularly in the making of fructose syrups. When the strain MSC702 α -amylase was incubated with Pb²⁺, Mg²⁺, Cu²⁺, Zn²⁺, Ba²⁺, Ca²⁺, Hg²⁺, and Fe²⁺, the activity reduced drastically, similar to the report of Uyar et al. [25]. The inhibition of Hg²⁺ indicates the presence of indole amino acid residues in enzyme [26]. In the previous reports of Chakraborty et al. [18] and Syed et al. [19], α -amylases from *Streptomyces* sp. D1 and *Streptomyces gulbargensis* were also strongly inhibited by Hg²⁺ ion. The inhibition due to Cu²⁺ ions reflected the competition between the exogenous and protein associated cations. Inhibition with Zn²⁺ determines the thermostable nature of enzyme [27].

With respect to the effect of surfactants, α -amylase showed 40.92%, 47.37%, 34.21%, and 36.52% relative activity

in presence of Triton X-100 (1% v/v), Tween 80 (1% v/v), SLS (5 mM), and glycerol (1% v/v), respectively. In order to have applications in detergent industries, amylase must be stable to various detergent ingredients, such as surfactants. The obtained results show a reasonably good stability in the enzyme activity using different detergents at 1% v/v, suggesting that the enzyme has a potential in starch liquefaction and detergent industry. Chelating agent EDTA completely inhibited α -amylase activity, similar to the previous report of Chakraborty et al. [18]. The inhibitory effect of EDTA provides evidence that the enzyme's activity is metal dependent. The denaturation of the original α -amylase activity with urea (23.93% relative activity) in the present study concluded that the enzyme consists of hydrophobic amino acid composition [28].

3.3. Thermal and pH Stability. Thermal stability is a very important aspect of industrial enzymatic bioreactors. Profiles of thermal stability of *Streptomyces* sp. MSC702 α -amylase between 50 and 90°C are presented in Figures 4 and 5. Temperature stability profile showed that α -amylase had

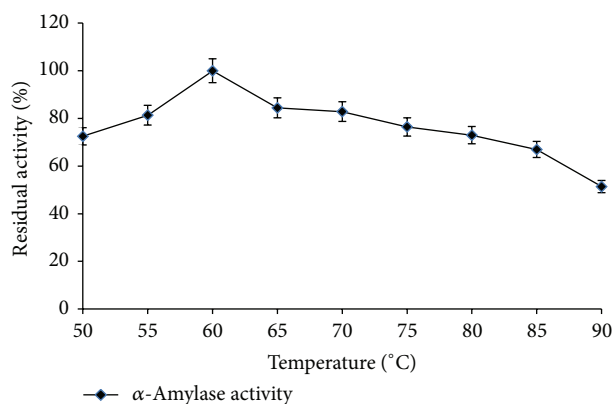


FIGURE 4: Effect of different temperatures on enzyme stability (preincubation for 30 min, pH 7.0).

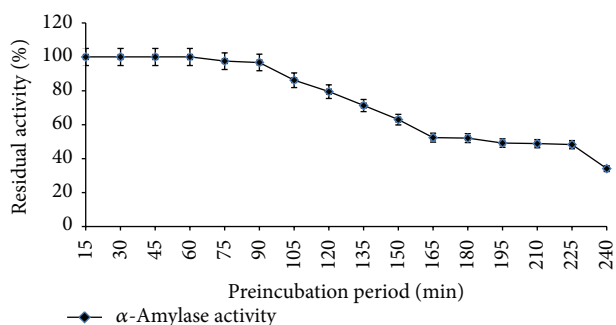


FIGURE 5: Effect of optimum temperature (60°C) on enzyme stability with varying preincubation period (pH 7.0).

maximum stability at 60°C and retained 51.38% activity at 90°C (Figure 4). α -Amylase was 100% stable for 1 h at 60°C and retained 34.18% activity after 4 h of preincubation (Figure 5). Half-life ($t_{1/2}$) of α -amylase was recorded 195 min at 60°C. From these results, the enzyme seemed to have considerable thermostability, which can be favorable in industrial operations for traditional brewing and food processing [29]. In contrast, Syed et al. [19] and Ammar et al. [22] reported instability at higher temperature (>60°C) for *Streptomyces* spp. α -amylases.

Profiles of pH stability of *Streptomyces* sp. MSC702 α -amylase at 60°C are presented in Figures 6 and 7. α -Amylase showed broad pH stability (pH 3.0–8.0) profile with maximum stability at pH 5.0 (Figure 6). The pH stability profile showed that enzyme was highly stable at acidic pH range. However, the enzyme was 87.93% and 37.98% stable at pH 8.0 and 9.0, respectively; after this, a remarkable loss in stability occurred. α -Amylase was completely stable at 60°C with pH 5.0 after 150 min preincubation. However, α -amylase retained 72.19% stability after 4 h preincubation (Figure 7). Similar to the present study, Ammar et al. [22] observed the maximum enzyme stability at pH 5.0–7.0 for *Streptomyces* sp. α -amylase. In contrast, Chakraborty et al. [18] and Syed et al. [19] reported stability of enzyme at alkaline pH range (7.0–11.0) for *Streptomyces* sp. D1 and *S. gulgargensis* α -amylases,

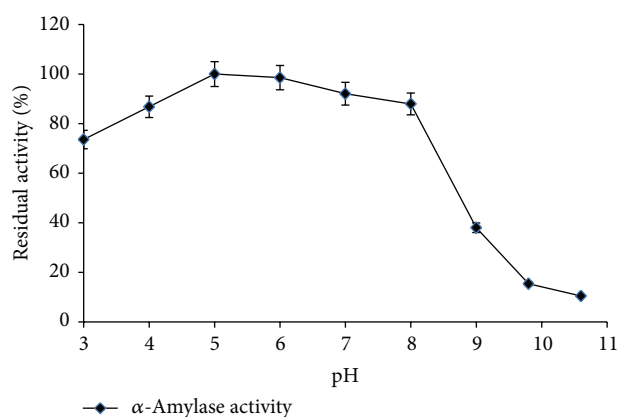


FIGURE 6: Effect of different pHs on enzyme stability at 60°C (preincubation for 30 min).

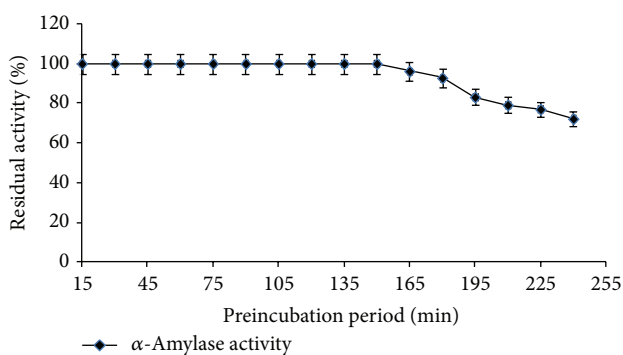


FIGURE 7: Effect of pH (pH 5.0 for α -amylase) on enzyme stability at 60°C with varying preincubation period.

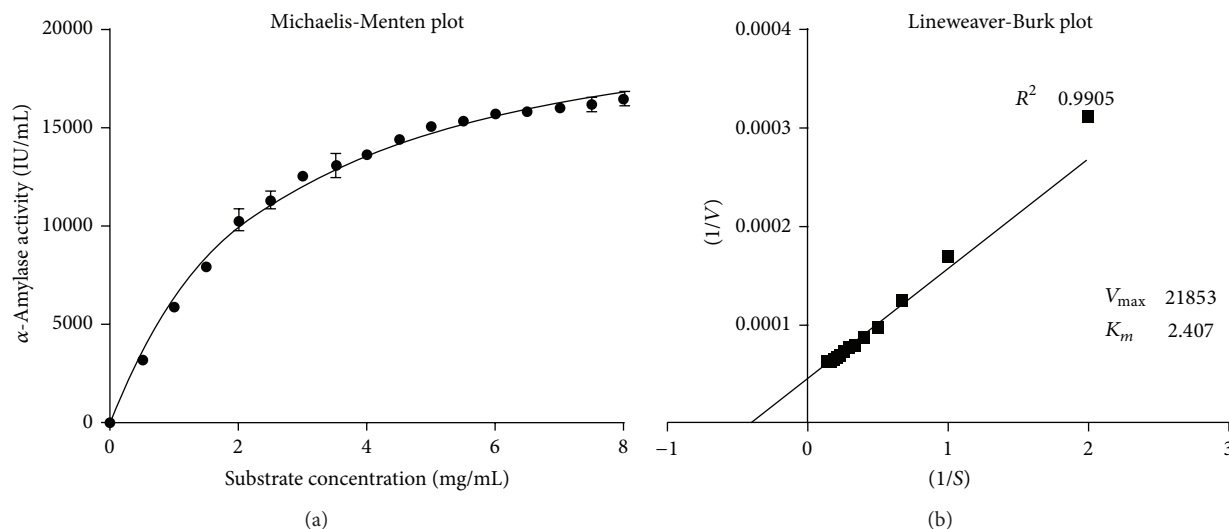
respectively. Chakraborty et al. [18] found stability of α -amylase for 6–48 h at pH 7.0–11.0.

3.4. Partial Purification of α -Amylase by Ammonium Sulphate Precipitation. Several industrial processes are carried out using whole cells as the source of enzymes but the efficiency can be improved using isolated and purified enzymes. However, the criteria for selection of a particular method of isolation and purification depend on its end use. Various steps of ammonium sulphate precipitation followed by dialysis for partial purification of α -amylase from *Streptomyces* sp. MSC702 are summarized in Table 2.

In the present study, the ammonium sulphate precipitation (40–60% saturation) followed by dialysis of crude α -amylase yielded 56.58% of the enzyme with 2.98-fold purification. Yang and Liu [30] recovered 55% α -amylase of *Thermobifida fusca* NTU22 with 1.3-fold purification by ammonium sulphate precipitation. Mollania et al. [31] purified 1.3-fold α -amylase of *Geobacillus* LH8 strain with 4.29% yield by ammonium sulphate precipitation. Kikani and Singh [32] yielded 34.29% α -amylase of *Bacillus amyloliquifaciens* TSWK1-1 with 4.29-fold purification. In the present work, a good yield and purification of α -amylase was achieved compared to previous reports.

TABLE 2: Partial purification of α -amylase by ammonium sulphate precipitation followed by dialysis.

Fraction	Total protein (mg)	Total activity (IU)	Specific activity (IU/mg)	Purification (Fold)	Yield (%)
Raw extract	4821.3	450000	93.33	1	100
F (0–20%)	801.5	64285.7	80.2	0.86	14.29
F (20–40%)	561.05	46250.0	82.43	0.88	10.27
F (40–60%)	914.96	254651.2	278.32	2.98	56.58
F (60–80%)	136.63	12500.9	91.49	0.98	2.7

FIGURE 8: (a) Michaelis-Menten plot and (b) Lineweaver-Burk plot for K_m and V_{max} values of the α -amylase in the presence of different concentrations of soluble starch.

3.5. Kinetic Determinations. From an industrial viewpoint, it is desirable to choose an enzyme which will have the fastest reaction rate per unit amount of enzyme as this indicates the maximum effect for the minimum amount of added catalyst. The Michaelis-Menten plot and Lineweaver-Burk plot for α -amylase by *Streptomyces* sp. MSC702 are depicted in Figures 8(a) and 8(b).

Present results showed that the Michaelis-Menten constant (K_m) and V_{max} value for α -amylase were 2.407 mg/mL and 21853.0 μ mol/min/mg, respectively, (Figures 8(a) and 8(b)). It is difficult to compare the kinetic values of α -amylase obtained by other researchers in view of the usage of different starch concentrations or different assay conditions. Shafiei et al. [33] found K_m and V_{max} values for α -amylase by *Nesterenkonia* sp. strain F to be 4.5 mg/mL and 1.18 mg/mL/min, respectively. Kikani and Singh [32] reported K_m and V_{max} values for α -amylase by *Bacillus amyloliquifaciens* TSWK1-1 as 0.6 mg/mL and 2632 mol/mL/min, respectively.

4. Conclusions

For a successful biotechnological application, purification and characterization are important prerequisites. α -Amylase enzyme related to starch degradation was purified and characterized from a novel thermophilic actinobacteria strain, *Streptomyces* sp. MSC702. The strain had a broad range of

temperature stability showing its suitability in the industrial applications where temperature plays a critical role. A plausible assay mechanism of starch degradation by the enzymes was also suggested.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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