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Partial purification and biochemical characterization of amylase from *Aeromonas caviae*NK1isolated from Industrial waste of India

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Abstract

A partial purification and biochemical characterization of theamylase from *Aeromonascaviae*NK1 were carried out in this study. The extracellular extract was

concentrated using ammonium sulfate precipitation and optimum operational conditions for the enzyme activity from the strain were evaluated. The optimum pH and temperature were observed 11.5 and37^{0}C respectively. Ca^{2+}, Mn^{2+}, Mg^{2+}, Zn^{2+}, Pb^{2+}, Co^{2+} were found to have effect on amylase activity.Furthermore, the analysis of kinetic showed that the enzyme has K_m of 2.4mg/mL and $V_{\max}$ of 21853.0μmol/min/mg for starch. The results indicate that the enzyme reflects their potentiality towards industrial utilization.

Keywords: Amylase, Partial purification, Km, V max, Zymogram

1. Introduction

The enzyme of amylase familyhas a great significance due to its wide area of potential application. Amylases can be of two types, endoamylases and exoamylases. Endoamylaseshydrolyzein a random manner in the interior of starch molecules and thus can produce linear and branched oligosaccharides of various chain lengths. On the other handexoamylaseshydrolyze the starch molecules from the non-reducing end successively which results in short end products (Reddy, Nimmagadda& Rao, 2004).

Amylases catalyze the hydrolysis of alpha-1,4-glycosidic linkages of polysaccharides to yield dextrin, oligosaccharides and maltose. Mainly three types of amylases are found differing in the glycoside bond they attack-α, β and γ amylases. Although they can be isolated from different source like animal, plant and microorganism, the microbial amylases are more thermostable and have higher yield(Burhan et al., 2003). The production of microbial amylases from bacteria is dependent on type of strain, composition of medium, method of cultivation, cell growth, nutrient requirements, metal ions, pH, temperature, time of incubation and thermo-stability (Haq, Javed, Hameed & Adan, 2004). Spectrum of application of amylase has widened in many sectors such as food, textile, baking and detergent industries. Besides its use in the saccharification or liquefaction of starch that converts starch into fructose and glucose, the enzyme is also used as a partial replacement for the expensive malt in the brewing industry to improve flour in the baking industry. They are used for the warp sizing of textile fibers, the clarification of haze formed in beer or fruit juices and for pre-treatment of animal feed to improve digestibility (Nusrat& Rahman, 2008). Amylases have been derived from several fungi, yeast, bacteria and actinomycetes but members of the genus Bacillus are heterogeneous and they are very versatile in their adaptability to the environment.Amylases have been purified earlier from various Bacillus species such as *Bacillusmegaterium*(Oyeleke, Auta&Egwim, 2010) from *Bacillussubtilis* (Riaz,Haq&Qadeer, 2003), from *Bacilluslicheniformis* SPT 27 (Aiyer, 2004), from *Bacillus licheniformis*, *Bacillus amyloliquifaciens* (Vidyalakshmi, Paranthaman&Indhumathi, 2009). Diverse fungal strains are able to produce amylase both intracellularly and extracellularly depending upon the fermentation process (Ellaiah,Adinarayana, Sunitha, &Bhavani Devi, 2003; Vahidi, Shafagi&Mirzabeigi, 2005; Chadha, Rubinder&Saini, 2005).

In the present study we report partial purification and characterization of wide pH tolerant,α–amylase produced by *Aeromonascaviae*NK1which was isolated from industrial waste of an industrial zone in West Bengal, India.

2. Materials and Metdods

2.1 Strain

A new strain of *Aeromonascaviae*NK1 was identified from the industrial waste. The strain was maintained in the nutrient agar slant at 4^0C. The bacteria were revived by inoculating in LB (0.1% v/v) and incubated at 37^0C in a shaker incubator for overnight.

2.2 Extraction and Partial Purification of Amylase Enzyme from the Isolated Bacteria

The isolated bacteria were cultured in 100 ml starch containing mineral medium for 30h in shaker incubator at 120 rpm and 37^0C. The extracellular enzyme was collected by centrifugation at 3000 X g for 20 min using cooling centrifuge (REMI C30 plus). The supernatant was collected as crude extract of enzyme. The enzyme was purified by ammonium sulphate precipitation up to 80% saturation. The precipitates were collected from different concentration of ammonium sulphate. Then the precipitates were re-suspended in 10 ml of 100mM Tris buffer of pH 10 and dialyzed overnight.

2.3 Protein Content Estimation

For protein content estimation standardLowrey method of protein estimation was used (Lowry, Rosebrough, Farr & Randall, 1951). Different dilutions from 0.2 mg/ml to 0.8 mg/ml of Bovine Serum Albumin (BSA) solutions were prepared by mixing of stock BSA solution (1 mg/ ml) and distilled water in test tubes to final volume of 5ml. 0.2 mlproteinsolutionsfrom each were pipetted in fresh test tubes and 2 ml of alkaline copper sulphate reagent was added to each. The solutions were mixed well and incubated at room temperature for 10 min. 0.2 ml of FolinCiocalteau solution was then added to each tube and incubated for 30 min. The absorbance of each mixture was then measured using spectrophotometer (Bio Spectrometer, Eppendorf) at 600 nm. The absorbance was plotted against protein concentration to get a standard calibration curve. The concentration of the unknown samples was determined from the plotted standard curve.

2.4 Molecular weight determination of the purified amylase by SDS- PAGE

The molecular weight of the partially purified amylase was determined by using SDS-PAGE as per description of Laemmli(Laemmli, 1970).The concentrations of staking gel and resolving gel in this experiment were 4.5% and 12.5% respectively. The purified amylase was loaded into the well parallel to standard protein marker. The protein band was detected by staining with 0.2% Coomassie brilliant Blue R250 and destining by methanol-acetic acid-water solution (4:1:5).

2.5 Zymogram of Amylase Activity

For determination of Zymogram, enzyme was subjected to native gel electrophoresis with the use of 10% acrylamide gel. 0.2% soluble starch was incorporated into the separating gel. After the electrophoresis,washing the gel at room temperature in solutions containing 50 mM Na_2HPO_4, 50 mM NaH_2PO_4 (pH 7.2 for 1 h for activity of enzyme.Gel was stained in a solution of iodine (Iodine 5 g/l, KI 50 g/l) (Lee,Morikawa, TakagiandImanakawa, 1994; Lin,

Chyau and Hsu1998).

2.6 Enzyme Assay

Amylase activity was determined by measuring the release of reducing sugar from soluble starch. The reaction mixture contained 0.5 ml of crude enzyme and 1 ml of sodium phosphate buffer (pH 7.0). 1% soluble starch was then added and incubated at 25℃ for 10 min. The amount of reducing sugar released in the mixture was determined by the addition of 2 ml of 3,5-dinitrosalicylic acid (Miller, 1959) followed by boiling for 10 min to develop color. The absorbance of the mixture was measured at 540 nm, and D-glucose was used to create a standard curve. One unit of enzyme activity was defined as the amount of enzyme releasing reducing sugar equivalent to 1 µmol glucose per minute under the assay condition. The effect of temperature, pH and substrate concentration on amylase activity was studied and Km and Vmax values of the enzyme were calculated from Lineweaver-Burk (double-reciprocal) plot (Mishra &Behera, 2008; Najafi, Deobagkar&Deobagkar, 2005; Sudharhsan, Senthilkumar&Ranjith, 2007).

2.7 Optimization of Enzyme Activity

Amylase activity was optimized in respect of pH, temperature, substrate concentration and metal ions.

2.7.1 Effect of pH for on the activity and stability of amylase

Enzyme activity at the pH range from 4 to 12 were determined by 3,5- dinitrosalicylic acid reagent method as described earlier in 2.6.

2.7.2 Effect of temperature on the activity and stability of amylase

The enzyme activity (U) were measured by 3,5- dinitrosalicylic acid reagent at the temperature of 20^0C, 30^0C, 37^0C, 40^0C, 50^0C at the optimum pH,thatwas noted earlier, for the determination of activity and the stability of the enzyme.

2.7.3 Effect of substrate concentration on the activity and stability of Amylase

For the determination of the optimum substrate concentration,2, 4, 6, 8, 10 mg/ml of starch solutions were prepared. Amylase activity was measured at varying concentrations of starch under optimal conditions on the basis of the information obtained in previous experiment (optimum pH, optimum temperature).

2.7.4 Effect of cations and heavy metal ions on the activity and stability amylase

The effect of cations on enzyme activity was studies by using Calcium (0.4-2.5 mM), Magnesium (1-7 mM), Manganese (0.4-2.8 mM), and Zinc (0.1-1mM) ions. They have positive effect on amylase production. We used Lead (0.1 -1mM) and Cobalt (0.1-1mM) to study the effect of heavy metal ions on enzyme activity.

3. Results and Discussions

3.1 Molecular Weight of the Purified Amylase

The molecular weight of the purified amylase on the basis of SDS-PAGE is ~55kDa as shown in Figure 1. The figure shows that the amylase is partially purified.

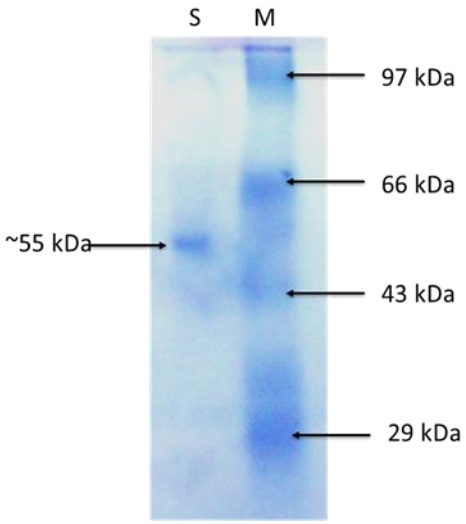

Figure 1. Molecular weight of the crude enzyme determined by SDS-PAGE. S stands for partially purified sample, M stands for molecular weight marker

3.2 Zymogram

The enzyme activity was observed in native PAGE gel containing 0.2% agar. A hallow zone of digested starch was detected in gelindicating activity of amylase enzyme (Figure 2).

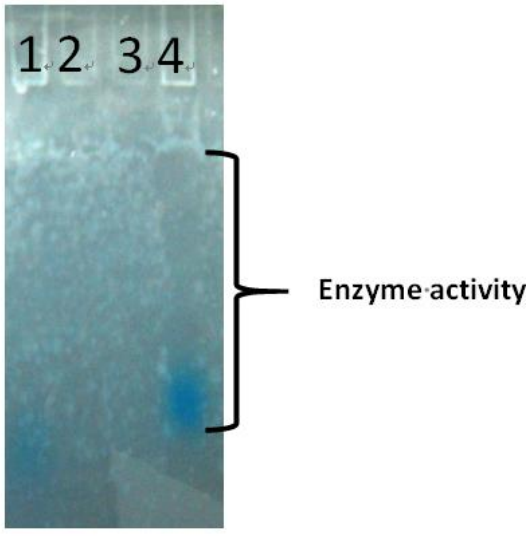

Figure 2. Zymogram of amylase activity. Proteins were loaded in Lane 1 & 4. Lanes 2 and 3 were empty lanes. 20µg of BSA was loaded in lane 1 and 20 µg of partially purified amylase was loaded in Lane 4. Amylase breakdown the starch and clear zone is appeared in Lane 4

3.3 Enzyme Kinetics

The partially purified α-amylase enzyme was incubated with various concentrations (0.5–8.0mg/mL) and time of soluble potato starch under 37 ^{0}C temperature and pH 7 to determine the kinetic parameter (Roy et al., 2014). The Michaelis-Menten constant (Km) and maximum velocity (Vmax) values were determined from Lineweaver-Burk plots. The Km and Vmax values were calculated from the kinetic data. The enzyme has K_m of 2.4 mg/mL and V_{max} of 21853.0μmol/min/mg for starch (Figure 3).

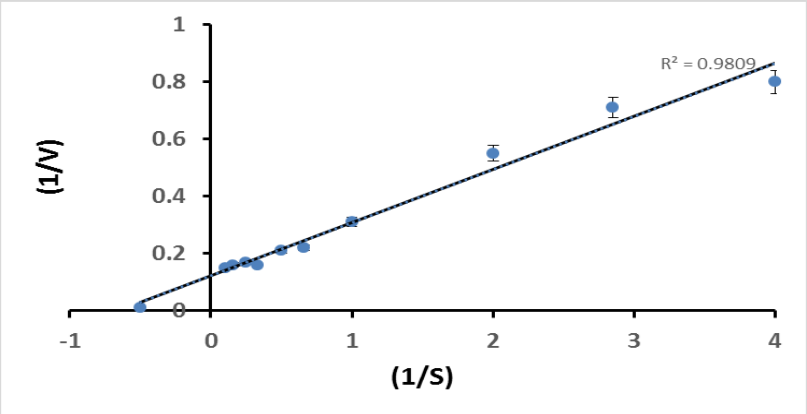

Figure 3. Lineweaver-Burk plot for $Km$and Vmax values of the α-amylase in the presence of different concentrations of soluble starch.

3.4 Optimum Condition and Stability of the Enzyme

The enzyme showed 100% activity at pH 11.5 in Figure 4a and temperature 37^{0}C in Figure 4b. The enzyme is also 70% to 100% active at the pH range from 7 to 12 and temperature 20^{0}C to 40^0 C. It was also noted that with increasing the substrate concentration up to 1 mg/ml the activity of the enzyme increases and showed 100% efficiency. With respected to the further increase in substrate concentration there is no change in the enzyme activity. The enzyme showed wide range of pH activity. It may eliminate the requirement of pH adjustment in its industrial application which is an important factor for its industrial application.

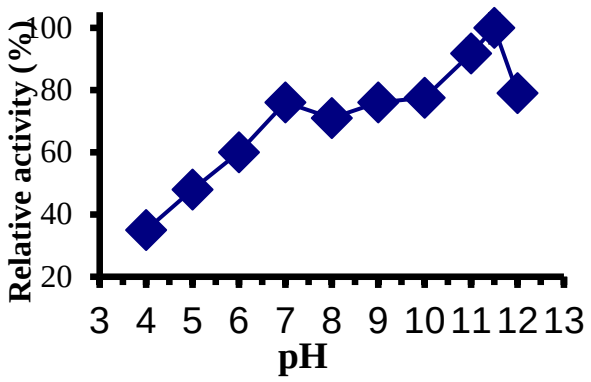

Figure 4a. Effect of pH on the activity of purified enzyme

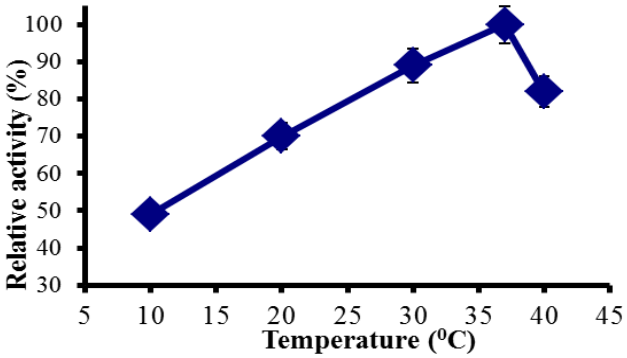

Figure 4b. Effect of Temperature on the activity of purified enzyme

3.5 Effect of Cationsand Heavy Metal Ions on Amylase Production

The data of effect of ions on enzyme activity indicate that the relative activity is 100% in presence of 0.8 mM, 1.5mM, 0.75mM, 5mMof $ZnCl_2$, calcium chloride, Manganese chloride, Magnesium chloride, and respectively (Fig 5 A, B,C, D). So calcium chloride and magnesium sulfate stimulated the amylase activity. On the other hand, in presence of leadion, cobalt ions (4mM and 0.1mM respectively), relative activity of enzyme is 100 % (Fig 5 E, F). Then after increasing of cobalt ion the activity gradually decreased. So cobalt ion has an inhibitory effect of heavy metal on amylase activity.

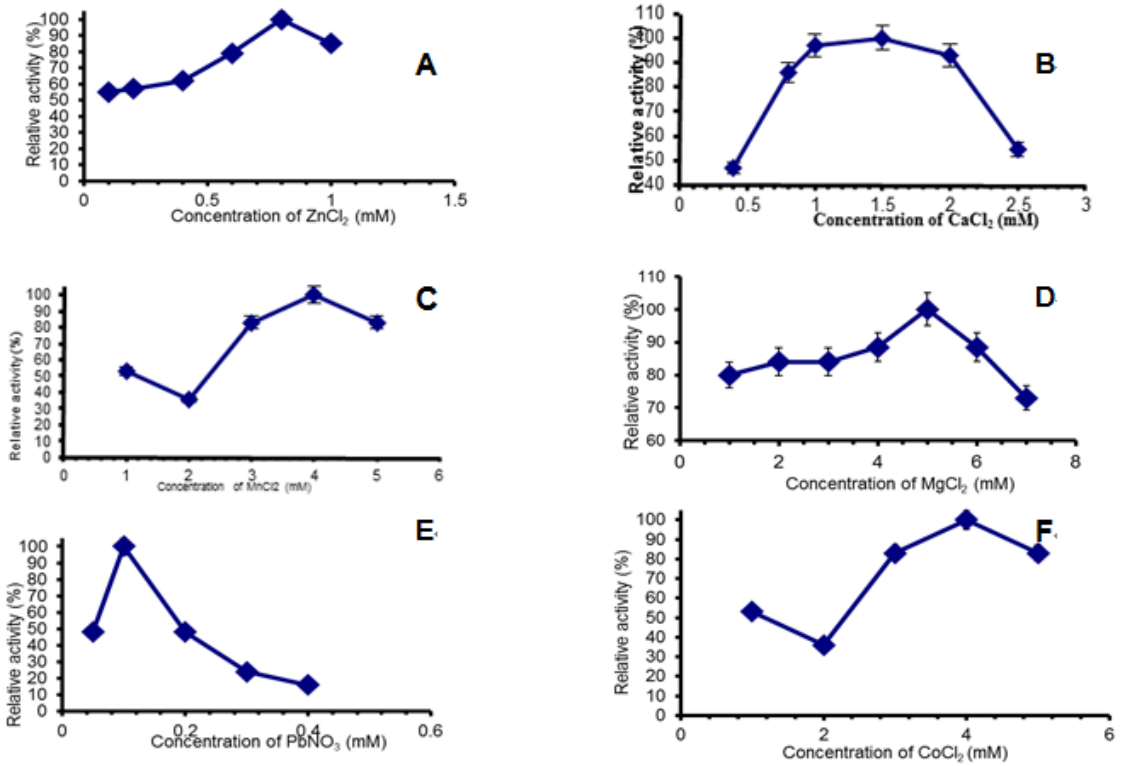

Figure 5. Effect of cations and heavy metals on the activity of purified enzyme

4. Conclusion

Current results indicate that the ~55kDa amylase enzyme is active at wide range of pH and temperature. This study also revealed that amylolytic activity was affected by cation like calcium and magnesium concentration. In presence of calcium and magnesium ion in a specific range the enzyme activity increased. Lead (Pb) ion has an inhibitory effect of heavy metal on amylase activity.

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