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PARTIAL PURIFICATION AND CHARACTERIZATION OF AMYLASE: AN IMPERATIVE CARBOHYDRASE FOR STARCH-BASED INDUSTRIES

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ABSTRACT

In the present study, amylase was produced using submerged fermentation technology from *Bacillus* ARF. The enzyme was purified to 5.9 folds as compared to crude enzyme extract by using 40% ammonium sulfate. The temperature and pH at which amylase showed maximum activity were found to be 50 ˚C and 7.0, respectively. The time course for enzyme substrate reaction was 20 minutes. The kinetic studies of amylase revealed the Km and Vmax values as 5.73 mg/mL and 2437 U/mL/min respectively. Different metal ions were tested for their stimulatory or inhibitory effects on amylase activity, among which, K^+ was found to be the activator of enzyme at 1mM concentration, whereas the enzyme was inhibited in the presence of Hg^{2+}, Mg^{2+}, Cd^{2+}, Ba^{2+} and Na^+. Enzyme activity was found almost unchanged in the presence of Ca^{2+}.

Key words: Carbohydrases, Amylase, Bacillus, Fermentation, Purification, Characterization

INTRODUCTION

Carbohydrases refer to the family of enzymes which are involved in degradation and synthesis of carbohydrates including amylase, invertase, β-galactosidase, glucosyltransferase etc. (Contesini *et al.,* 2013). Each member of this family is equally important in different starch based industrial processes especially in food industry. In the present study, we are concerning with amylase that is involved in the hydrolysis of α-1,4-glycosidic bonds in starch and other polysaccharides. Amylase has occupied about 25 % of the world market of enzymes that is expected to increase at a rate of 3.3 % yearly (Sachdev *et al.,* 2016). Currently, amylase is utilized in different industrial processes including: modification of pulp for paper making, quality dough making for bakery items (Saini *et al.,* 2017), production of sugar syrups to be utilized in different food items such as jams, jellies, candies, soft drinks, modification of starch during textile processing and dyeing of fabric, production of environment friendly bio-detergent for the removal of starchy stains (Sundarram and Murthy, 2014; Saini *et al.,* 2017), production of bioethanol from agricultural crops along with other carbohydrases like cellulases and production of digestive aid preparations. The significant production rate of amylase in order to meet the industrial demand is achieved by microbial fermentation including bacteria, fungi, algae and actinomycetes (Souza, 2010; Sivakumar *et al.,* 2102). In industrial processes, microbial source of enzyme has imposed significant advantages as compare to plant and animal sources including high production yield and low production cost of enzymes (Deb *et al.,* 2013). Due to the extensive utilization of amylase in different industrial sectors, the present study was intended on partial purification and characterization of amylase obtained from *Bacillus* ARF.

MATERIALS AND METHODS

Microorganism and Production of Amylase

A bacterial strain named as *Bacillus* ARF was used for the production of amylase in this study. Rapidly growing overnight culture of *Bacillus* ARF was inoculated in 10 mL inoculum tube and incubated at 37˚C for 24 h. After 24 h, the inoculum was transferred into 90 mL broth medium containing (g %): Starch1.0, Peptone 0.5, Yeast 0.1, Magnesium sulfate 0.05, Sodium chloride 0.05, Calcium chloride 0.02 and incubated again at 37˚C for 24 hours. The fermented broth was centrifuged at 10,000✕g for 10 minutes at 0˚C using cold centrifuge machine to obtain cell free filtrate (crude enzyme) as a supernatant.

Partial purification of amylase

Partial purification of amylase was performed using gradient precipitation technique according to the method of Englard and Seifter (1990). In order to concentrate exclusively the protein of interest, ammonium sulfate was added gradually in varying concentrations (40% and 50% saturations) to the crude extract with continuous shaking with a magnetic stirrer in ice bath. After the ammonium sulfate being completely solubilized, the solution was kept for precipitation at 4°C for 24 h. Thereafter, the solution was centrifuged for 10 minutes by using cold centrifuge machine (10,000 rpm, 0 ˚C) in order to collect the precipitates. After centrifugation the precipitates were dissolved in 2 mL of 50 mM phosphate buffer (pH 7.0) and its amylolytic activity and total protein content was determined. Furthermore, ammonium sulfate was added in the left behind supernatant to obtain 50 % saturation and the same process of precipitate collection was repeated. The precipitates showing highest specific activity was taken in the pretreated dialysis tube against 50 mM phosphate buffer (pH 7.0) and left at 4°C for 24 h with three buffer changes. After 24 h, the desalted sample was collected and analyzed for its specific activity.

Enzyme assay and Total Protein Determination

The amylase activity was determined using starch as a substrate by DNS method (Miller *et al.,* 1959). The reaction mixture contained 1 mL of 1 g% starch prepared in 50 mM phosphate buffer of pH 7.0 to which 0.1 mL of partially purified enzyme was added. The reaction was allowed to proceed for 20 minutes at 50 ˚C. Thereafter, 1 mL DNS was added, and the tubes were subsequently boiled for 5 minutes.

The total protein of different samples during partial purification was analyzed by Lowry's method (Lowry *et al.,* 1951). Bovine serum albumin was used as standard.

Enzyme unit

One Unit of amylase is referred to as "the amount of enzyme required to release 1µmol of reducing sugar (glucose) from the substrate (starch) in one minute at 50˚C"**.**

Characterization of amylase

Time course for optimal reaction

To determine the enzyme-substrate reaction time, enzyme was allowed to react with the substrate for different time intervals (5, 10, 15, 20, 25, 30 minutes) and amylase activity was measured as described.

Optimum substrate concentration for maximum amylase activity

Amylase activity was measured by incubating enzyme with different concentrations of starch (0.5, 1, 1.5, 2, 2.5, 3 %) for 20 minutes and activity was measured under standard conditions.

Optimum temperature for maximum amylase activity

The optimum temperature of amylase activity was determined by performing standard enzyme assay at different temperatures starting from 37˚C to 60˚C with an increment of 5 ˚C.

Optimum pH for maximum amylase activity

In order to evaluate the effect of reaction pH on amylase activity, enzyme was incubated with substrate prepared in phosphate buffer (50 mM) of different pH including pH 5, 6, 7, 8, 9. Enzyme activity was then measured under standard assay conditions.

Effect of metal ions on amylase activity

The inhibitory or stimulatory effect of $CaCl_2$, $MgCl_2$, $BaCl_2$, KCl, $CdCl_2$, NaCl, $HgCl_2$ and $BaCl_2$ was determined by mixing and incubating equal volumes of partially purified enzyme and 1 mM metal ion solutions at room temperature for 30 minutes. Thereafter, 1 mL of enzyme-metal solution was used as Test for amylase activity. Activity of untreated enzyme was taken as Control.

RESULTS AND DISCUSSION

Partial Purification of Amylase

In the present study, amylase from *Bacillus* ARF was subjected to partial purification using ammonium sulfate fractionation technique. It was observed that 40 g % $(NH_4)_2SO_4$ showed maximum precipitation of enzyme with specific activity of 174.8 U/mg and 2.6-fold purification (Table 1). The dialysis step increased the specific activity and fold purification up to 392 U/mg and 5.9, respectively. Bano *et al.* (2011) has also reported increase in fold purification by using 40% ammonium sulfate. Whereas 5.6-folds purification was reported by Ozdemir *et al.* (2011) by using 65% ammonium sulfate. Several investigators reported purification by 80% ammonium sulfate as well (Schokker *et al.,* 2012; Roy *et al.,* 2014).

Time course for optimal reaction

Activity of amylase from *Bacillus* ARF was increased with the increase in incubation time of enzyme with 1.5 g % substrate (starch) and showed maximum activity when incubated for 20 minutes at 50 ˚C beyond which the enzyme activity was found to be declined (Fig. 1). Kharkrang and Ambasht (2012) also reported 20 minutes for hydrolysis of starch by α-amylase. Incubation time of 60 minutes between amylase and its substrate has also been reported by Sethi *et al.* (2016).

Table 1. Partial Purification of Amylase by Ammonium Sulphate.

	Enzyme Activity (U/ml/min)	**Total Protein (mg/ml)**	**Specific Activity (U/mg) (S.A=E.A/T.P.)**	**Fold Purification (S.A of PPT/S.A of CFF)**
CFF	255±12.75	3.39±0.169	66±3.33	1±0.001
40% Precipitates	376±18.80	2.15±0.107	174.8±8.74	2.6±0.132
40% Desalted Precipitates	601±21.53	1.53±0.076	392±19.60	5.9±0.241
50% Desalted Precipitates	240±11.07	1.07±0.053	224±11.20	3.39±0.169

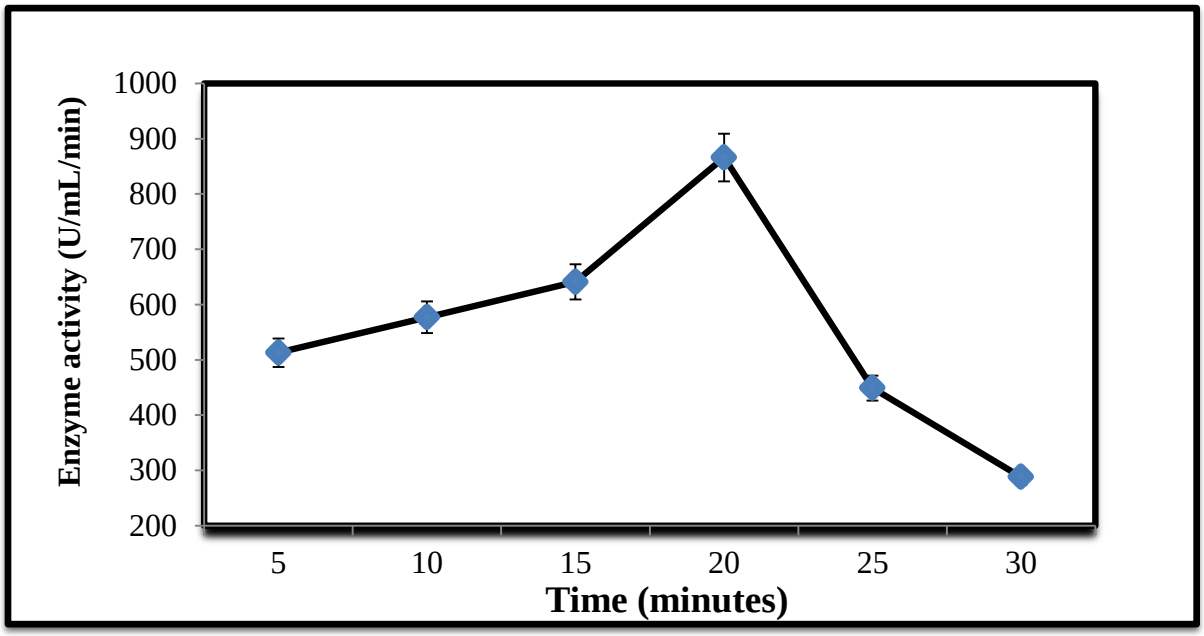

Fig. 1. Effect of Time Course for Enzyme Substrate Reaction on Amylase.

Effect of substrate concentration on amylase activity:

The kinetic parameters of amylase from *Bacillus* ARF have been investigated using starch as a substrate. Maximum amylase activity was observed at 1.5 g % starch; beyond this concentration no increase in activity was observed (Fig. 2).

Lineweaver–Burk plot was plotted (Fig. 3) using Graph pad prism in order to determine the catalytic efficiency of enzyme. The Vmax and Michaelis–Menten constant, Km of amylase was found to be 2437 U/mL/min and 5.73 mg/mL, respectively. Km value actually determines the affinity and strength of substrate binding with the active sites of enzymes. The Km value of amylase from *Bacillus licheniformis* has been reported as 8.3 mg/ml using 1 %

soluble starch as a substrate (Haq *et al.*, 2010). On the other hand, Tabassum *et al.* (2014) reported 3.4 mg/mL as the Km value of amylase *Bacillus licheniformis* RT7PE1.

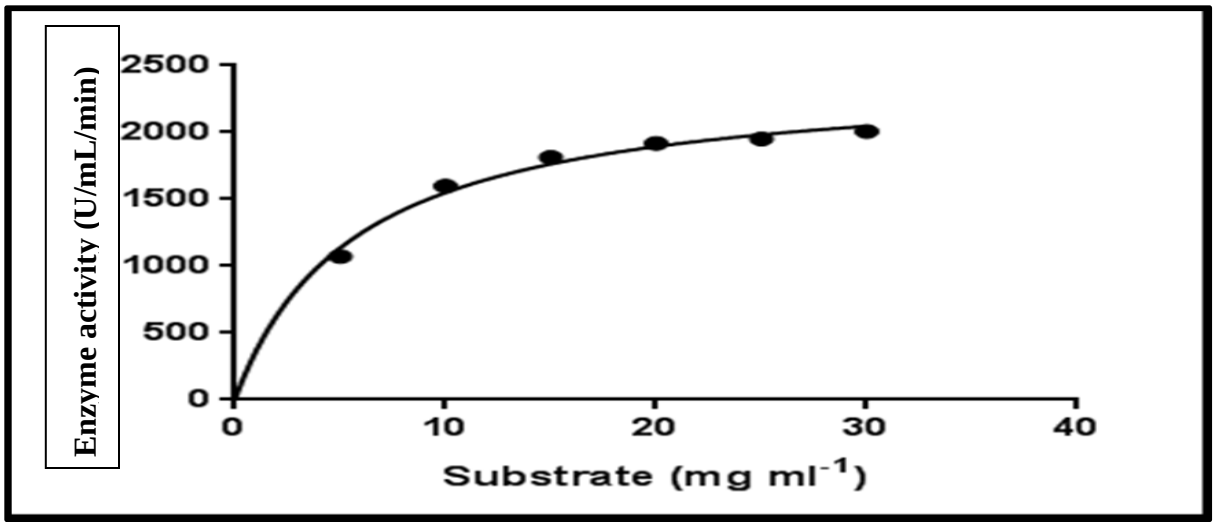

Fig. 2. Michaelis-Menten Plot of Amylase Produced from *Bacillus* ARF (Graph Pad Prism).

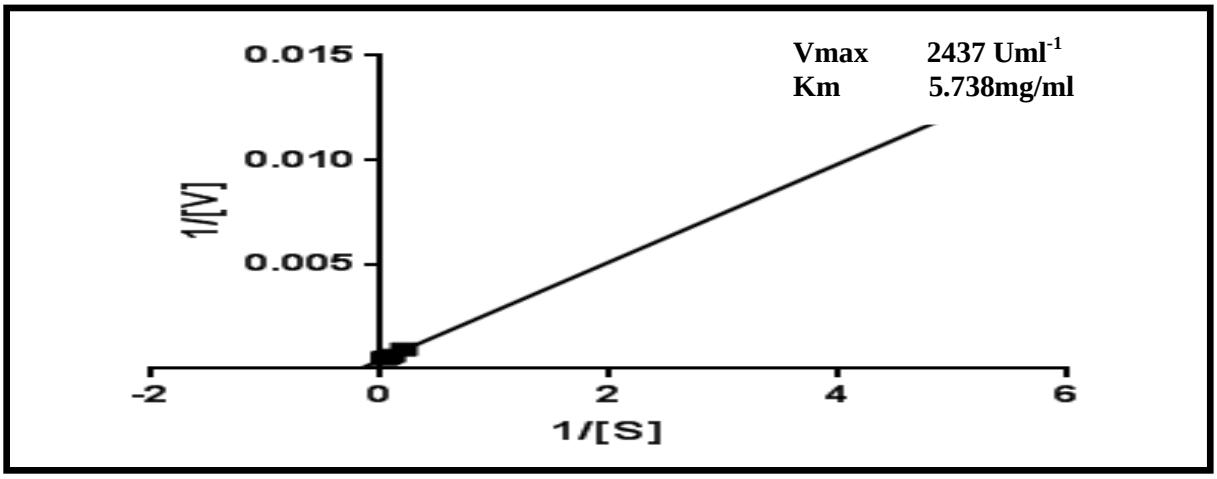

Fig. 3. Lineweaver–Burk Plot of Amylase Produced from *Bacillus* ARF (Graph Pad Prism).

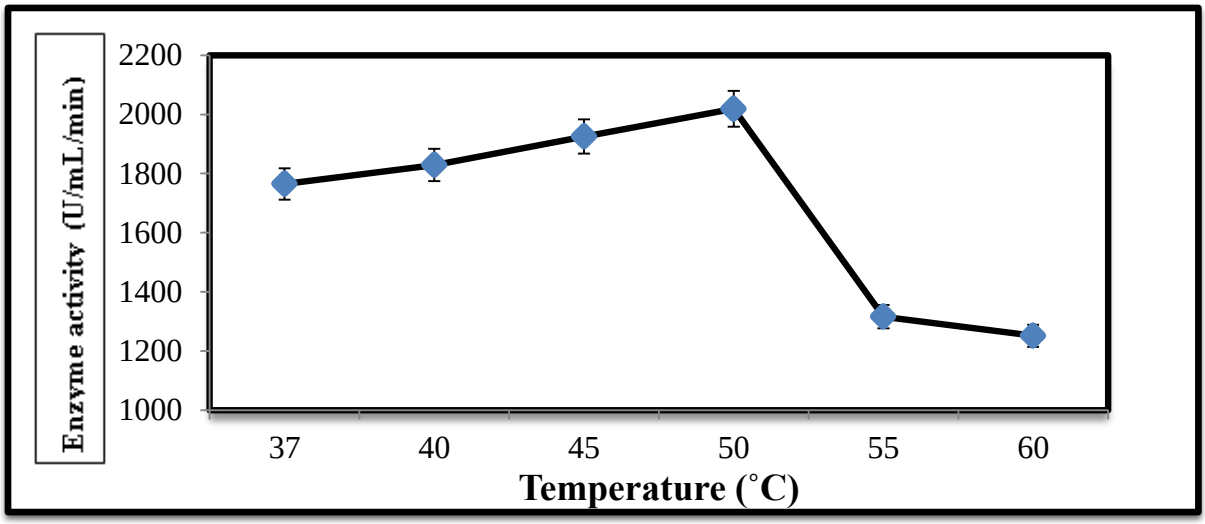

Fig. 4. Effect of Temperature on Amylase Activity.

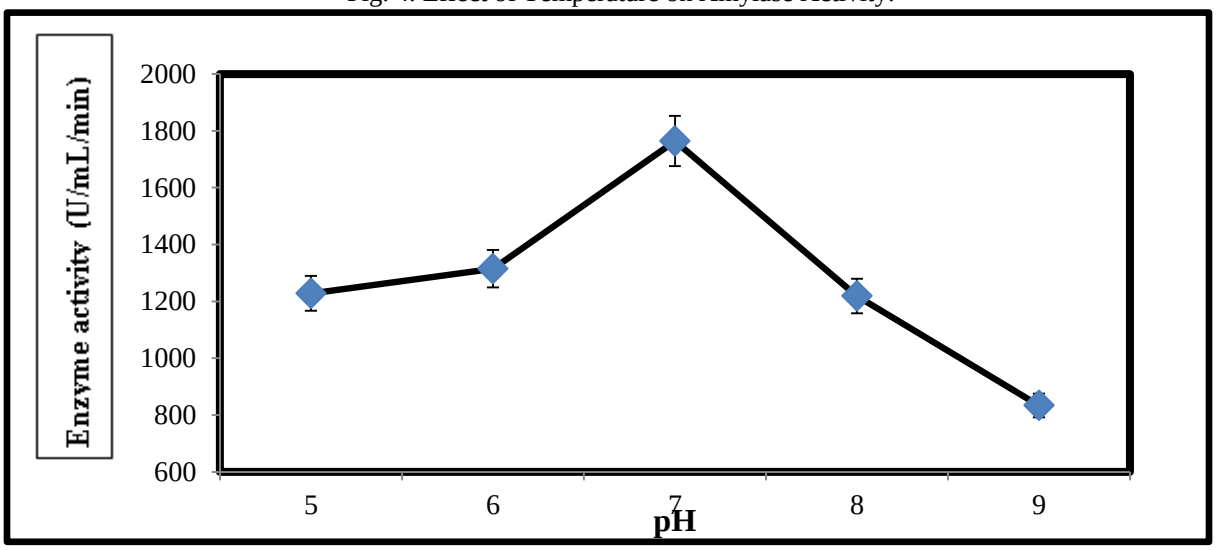

Fig. 5. Effect of pH on Amylase Activity.

Table 2. Effect of Metal Ions on Amylase Activity.

	Enzyme Activity (U/mL/min)	**Residual Activity (%)**
CONTROL (Untreated enzyme)	1347 ± 57.35	100 ± 4.32
TEST {Metal ion (1mM)Treated}		
$MgCl_2$	32± 1.6	2 %± 0.001
$CdCl_2$	417± 20.85	30 %± 0.015
NaCl	1219± 50.95	90 %± 0.045
KCl	1712± 55.6	127 %± 0.064
$BaCl_2$	642± 22.1	48 %± 0.024
$CaCl_2$	1362± 38.1	101 %± 0.050
$HgCl_2$	0.0	-

Effect of temperature on amylase activity

In the present work, initially as the temperature was increased from 37 to 50 °C, enzyme activity was also found to be increased and maximum activity was achieved at 50˚C (Fig. 4). It has been reported that temperature has a significant impact on the active or inactive confirmation of enzyme (Gomaa, 2013).With the increase in temperature, enzyme substrate collision rate is increased due the increased level of kinetic energy resulting in the formation of enzyme-substrate complex (Mohammadi *et al.*, 2010). Beyond 50 °C, a sharp decline in enzyme activity was observed that might be due to the heat induced cleavage of the peptide bonds in enzyme protein (Sethi *et al.*, 2016).

Our results agree with Wanderley *et al.* (2004) and Liu *et al.* (2015) who reported 50 °C as an optimum temperature for amylase activity. An optimum temperature of 70 and 80 °C has also been reported by several investigators (Hernandez-Heredia and Moral, 2016; Kiran *et al.*, 2018).

Effect of pH on amylase activity

pH of the reaction mixture is a factor that affects the ionic state of active sites in enzymes. The activity of the partially purified enzyme was measured by using substrate prepared in phosphate buffer of different pH ranging from 5–9. Amylase showed optimum activity at pH 7.0 but remained active at both acidic and basic pH values as it retained about 74 % activity at pH 6.0 and 69 % activity at pH 7.0 as shown in Fig. 5. This makes the enzyme a perfect candidate to be utilized in food, detergent and textile industries. Similar finding was reported by Saini *et al.* (2017).

Effect of metal ions on amylase activity

Metal ions affect the activity of amylase as some of them act as activators and some behave as inhibitors of enzyme. In the present study, KCl was found to be the only strong activator of amylase whereas Hg^{+2}, Mg^{+2}, $Cd^{+2}$and Ba^{+2} acted as strong inhibitors. Only 10 % activity was decreased in the presence of Na^{+2}. Whereas, amylase showed no activity at all in the presence of Hg^{+2} (Table 2).The inhibition by metal cations could be due to interruption in binding affinities of those cations that are normally involved in maintaining the active confirmation of enzyme (Roy *et al.,* 2014). Amylase is known to be dependent on calcium ions for its optimal activity and stability (Gupta *et al.,* 2003) but in the present study, calcium ions did not significantly enhance the activity of the enzyme. Amylase was found to retain about 99 % of its activity in the absence of Ca^{+2} that categorized it as calcium independent amylase. In contrast, Ca^{+2}has been reported as an activator of carbohydrases by various investigators (Xian *et al.,* 2015; Sachdev *et al.,* 2016).

Conclusion

The results concluded that *Bacillus ARF* is a good source of thermostable, pH tolerant amylase and it can be utilized in many industries which process starch at high temperature such as paper industry, textile industry, food industry and detergent industry etc.

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