ISSN 2277-8330 *(Electronic)*

*J.Bio.Innov*1(3),pp:48-57,2012

www.jbino.com

COMPARATIVE STUDY OF KINETIC PARAMETERS OF BACTERIAL AND FUNGAL AMYLASES

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(Received on Date: 2nd March 2012 Date of Acceptance: 7th April 2012)

ABSTRACT

Bacterial amylase from *Bacillus subtilis* and fungal amylase from *Aspergillus niger* were potential source for industrial exploitation in the field of amylase production. Hence, this study on bacterial amylase and fungal amylase was done to compare the kinetic parameter of both the types of amylases. 30-60% ammonium sulfate precipitation has done which showed 90 % of purification of both the partially purified bacterial and fungal amylases. Dialysis was done which showed 98% of purification of the types of amylases (bacterial and fungal amylase). pH optima were 8.2 for bacterial amylase and 6.6 for fungal amylase. Optimum temperature was 50ºC for bacterial amylase and 30ºC for fungal amylase. Optimum time of incubation for maximal activity was 30 minutes for bacterial amylase and 50 minutes for fungal amylase. 10% of NaCl was found to be good activator which enhanced both the bacterial and fungal amylases. It was showed that bacterial amylase is more stable at alkaline pH and thermostable as compared to fungal amylase.

Key words:

Bacterial amylase, Fungal Amylase, *Bacillus subtilis*, *Aspergillus niger*.

Number of Figures : 6 **Number of References :** 22

INTRODUCTION

Amylases are enzymes, which hydrolyze starch molecules to give diverse products including dextrins, and progressively smaller polymers composed of glucose units (Windish *et al*., 1965). Amylases are in the class of industrial enzymes having approximately 25% of the enzyme market (Sindhu *et al*., 1997; Rao *et al*., 1998). α-Amylases is thermostable enzyme (80-90°C) to economize processes (Sindhu *et al*., 1997) and hence the most widely used thermostable enzymes are the amylases in the starch industry. Thermostable enzymes isolated from thermophilic organisms have found a number of commercial applications because of their overall inherent stability (Poonam and Dalel, 1995; Crab and Mitchinson, 1997; Sarikaya *et al*., 2000). α-Amylases are universally distributed throughout the animal, plant and microbial kingdoms. However, enzymes from fungal and bacterial sources have dominated applications in industrial sectors (Pandey *et al*., 2000). Several *Bacillus* sp. and thermostable *Actinomycetes* including *Thermomonospora* and *Thermoactinomyces* are versatile producers of the α-amylases (Ben *et al*., 1999). The genus *Bacillus* produces a large variety of extracellular enzymes of which amylases and proteases are of significant industrial importance. An extremely thermostable α-amylase is available from the mesophile, *B. licheniformis* (Morgan *et al*., 1981). Alkaliphilic *Bacillus* strains often produce enzymes active at alkaline pH, including alkaline α-amylase, protease and carboxymethylcellulase (Horikoshi, 1996).

In the present study, an attempt is made to compare the kinetic parameters of the α-amylase produced by bacteria, *Bacillus subtilis* and fungi, *Aspergillus niger*. The α-glycosidic bond is very stable having a spontaneous rate of hydrolysis at room temperature (Wolfenden *et al*., 1998). The catalytic mechanism of the α-amylase family is that of α- retaining double displacement (Van der Maarel *et al*., 2002)**.** α-Retaining mechanism is the characteristic feature of the enzymes from the α-amylase family. They vary widely in their reaction specificities. The attachment of different domains to the catalytic site or to extra sugar binding sub-sites around the catalytic site is the prime reason for these differences (Van der Maarel *et al*., 2002). The catalytic domain-A is the most conserved domain in the α-amylase family. It consists of an amino terminal (β/α) 8- barrel structure. The structure of taka-amylase was reported by Matsuura *et al*., 1984 and structure of acid-amylase was reported by Boel *et al*., 1990.

MATERIALS AND METHODS

Preparation of potato dextrose agar (PDA) and potato dextrose broth (PDB): Potatoes (200 gm) were peeled, sliced and 500 ml of distilled water was added into it. This mixture was boiled till tender, crushed and filtered through four layers of cheese cloth. PDA was prepared by adding 2% dextrose and 2% agar to the potato extract and made up the final volume up to 1 liter with double distilled water. The solution was autoclaved for 30 minutes. After autoclaving, PDA slants were prepared. PDB was prepared by 2% dextrose to the

extract and autoclaved for 30 minutes. These agar plates were incubated at room temperature for about 12 -14 days. For bacteria, one loop of *Baciilus subtilis* strain was spread on Nutrient Agar plates containing 1 % w/v soluble starch and incubated at 30ºC for 24 hours. For bacteria, streaking of subcultured bacteria was done on a plate of Nutrient Agar containing 1 % starch. For fungi, subcultured fungus was transferred to Potato Dextrose Agar slants. Allowed bacteria to grow for 24 hours and fungi to grow for 72 to 120 hours, then stored in the refrigerator until needed.

Fungal amylase production: Scraped with a wire loop to loosen the spores and inoculated the medium with 0.5 ml spore suspension of fungi.

Medium composition (g/L) is as follows -

Media Composition	Quantity
KH2PO4	1.4
NH4NO3	10
KCL	0.5
MgSO4 ·7H20	0.1
FeSO4 ·7H2O	0.01
Soluble starch	20

Adjusted pH to 6.5. Autoclaved it and allowed to cool down to room temperature. Incubated at 37ºC for 72 hours in incubator shaker (Toya Ekunsaumi, 2009 & Abe J. *et al.*, 1988).

Bacterial Amylase production:

Grow bacteria in Nutrient Agar and added a loop of bacterial culture into the amylase production medium.

Medium for bacterial amylase production (g/L):

Media Composition	Quantity
Bacteriological Peptone	6
MgSO4·7H2O	0.5
KCL	0.5
Starch	1

Mixed well and kept into 100 ml Erlenmeyer flasks Sterilize to autoclave for 30 minutes (Toya Ekunsaumi, 2009).

Extraction of Enzyme from bacteria: Poured the bacterial culture into centrifuge tubes and centrifuged it for 20 minutes at 5000 rpm. Decanted the supernatant and discard the pellet. Supernatant was contained the crude enzyme.

Extraction of Enzyme from Fungi: Poured the whole content of the flask containing the growing fungus through a funnel fitted with Whattman number 1 filter paper. The filtrate was contained the crude amylase.

Standard Curve of Maltose:

Prepared 10 dilutions ranging from 0.3 to 5 µg of maltose concentration. Added 1 ml of dinitrosalicylic acid color reagent into each dilution. Incubated in boiling water bath for 5 minutes and added 1 ml of sodium potassium tartarate (40%). Then, cooled at

room temperature and 10 ml distilled water was added into each test tube and mixed well. Read at A_{575} and extrapolated A_{575} vs µg of maltose concentration.

Test of Amylase Activity

Added 0.25ml of 1M acetate buffer and 0.25 distilled water in 1.25ml of 1% starch. Then 0.25 ml of crude enzyme extract of bacterial and fungal amylase was added into it. Incubate the reaction mixture was incubated for 10 minutes at 30°C. Then, 1 ml of dinitrosalicylic acid color reagent was added and incubated in boiling water bath for 5 minutes and added 1 ml of sodium potassium tartarate (40%). Then, cooled at room temperature and 10 ml of distilled water was added and mixed well. Read at A_{575}.

Ammonium sulphate precipitation

Ammonium sulfate fractionation of crude extract of was performed at three saturation levels viz; 0-30%, 30-60% and 60-90%. Ammonium sulfate was added to the enzyme extract according to the required saturation levels by continuous stirring (0-4^0C) in ice bath till the saturation was not reached. After the complete saturation, the mixture was centrifuged at 14000 rpm for 20 minutes and supernatant was discarded. The pellet was pipette out and dissolved in sodium acetate buffer (pH 4.5).

Dialysis

The partially purified extracts of bacterial and fungal amylase were poured into the dialysis tubing and kept overnight at 0-4^0C in 10mM Tris-HCL buffer (pH 7.5). Volume of buffer should be 50 times more than volume of enzyme extract. The total volume of the sample was noted after dialysis and its activity is measured at $A_{575\ nm}$

% Purity of Amylase: The percent purity of Amylase enzyme can be given by the following formula:

$$\frac{(\text{Specific Activity of Purified Amylase} - \text{Specific Activity of Crude Extract})}{\text{Specific Activity of Crude Extract}} \times 100$$

Kinetic parameters of purified bacterial and fungal amylases

The various kinetic parameters were studied. The parameters were effect of incubation time, effect of pH, effect of temperature and effect of NaCl concentration.

Effect of time of incubation on bacterial and fungal amylases activity

The effect of time on the activity of the enzyme was studied by performing the enzyme assay at different Time (10 min- 80 min), the optimum incubation time was determined by incubating the enzyme with the buffer and the time described above at 30ºC and then carrying out the enzyme activity by DNS test at $A_{575\ nm}$.

Effect of pH on bacterial and fungal amylases activity

The effect of pH on the activity of the enzyme was studied by performing the enzyme assay at different pH using acetate buffer and phosphate buffer (pH 3.0-9.0),

the pH stability of the enzyme was determined by incubating the enzyme with the buffer described above for 15 minutes at 30ºC and then carried out the enzyme activity by DNS test at $A_{575\ nm.}$

Effect of temperature on bacterial and fungal amylases activity

Optimum temperature needed for enzyme activity was estimated by incubating the reaction mixture for 15 minutes at different temperatures (10-80° C). The temperature stability of the enzyme was determined by incubating the enzyme solution at temperatures in the range of 10-80° C for 15 minutes and estimating the residual activity of the treated enzyme by DNS test at $A_{575\ nm}$.

Effect of NaCl concentration on bacterial and fungal amylases activity

The effect of NaCl on the enzyme activity was studied by performing the enzyme assay at different concentration of NaCl (2% - 16%), the NaCl activity of the enzyme was determined by incubating the enzyme with NaCl solution for 15 minutes at 30ºC and then carrying out the enzyme activity by DNS test at $A_{575\ nm}$.

RESULTS & DISCUSSION

Standardization

Figure 1. was showed the standard curve of maltose.

Ammonium sulfate Precipitation

The crude extract was fractionated into three fractions viz. 0-30%, 30-60% and 60-90% ammonium sulfate saturation levels. These three fractions were assayed for bacterial and fungal amylases activity. Figure 2 was showed that 30-60% fraction was found to have maximum activity for both the types of amylases (bacterial and fungal amylases).

Dialysis

The crude extract was dialyzed and the enzyme activity was observed. It was showed the enzyme activity after dialysis with 98% of purity in both types of amylase (Bacterial and fungal amylase).

Kinetic parameters of bacterial and fungal amylases

Time of incubation: The reaction mixture of amylase was incubated for varied time intervals from 10 to 80 minutes. Figure 3 was showed that 30 minutes of time of incubation was found the optimum time of incubation for bacterial amylase and 50 minutes for fungal amylase. This result was comparable with the reports of Fogarty *et al*., 1979 and Hamilton *et al*., 1999.

pH optima: The pH of the reaction mixture was varied from 3 to 9 as shown in Figure 4. A distinct peak corresponding pH 6.5 in bacterial amylase and fungal amylase was obtained. This result was comparable to previous reports such as α-Amylases are generally stable over a wide range of pH from 4 to 11 (Fogarty *et al*., 1979; Vihinen *et al*., 1989; Hamilton *et al*., 1999; Saito *et*

al., 1973;Khoo *et al*., 1994) as well as stability of α-amylases in a narrow range have also been reported (Coronado *et al*., 2000; Robyt *et al*., 1971).

Temperature Optimum: Temperature optimum of amylase was determined by varying the temperature from 10 to 80^{0}C. The enzyme was found to show maximum activity at 30^{0}C as shown in figure 5. This result was found to be comparable to earlier reports, the temperature optimum for the activity of α-amylase is related to the growth of the microorganism and α-amylase was thermostable enzyme and thermostabilities are affected by many factors like presence of calcium, substrate and other stabilizers (Vihinen *et al*., 1989).

NaCl Concentration

The effect of salt on the enzyme activity was seen by addition of NaCl at various concentration ranging from 2-16%. Figure 6 was showed the maximum activity was obtained at 10% of NaCl. And NaCl act as activators to enhance the amylase activity in the reaction medium. No reports are available on to the study of effect of salt concentration on α-amylase activity. The enzyme produced by the organism also has several beneficial properties needed for an industrial enzyme. It shows activity and stability over a wide range of pH and temperature values. The enzyme is highly compatible with commercial detergents and retains almost complete activity in their presence. Hence, this enzyme may also be used along with the new formulations of detergents to make it more effective and also to make it more eco-friendly. However, scale up studies is needed for the development of an economic bioprocess for commercial exploitation of this organism for amylase production.

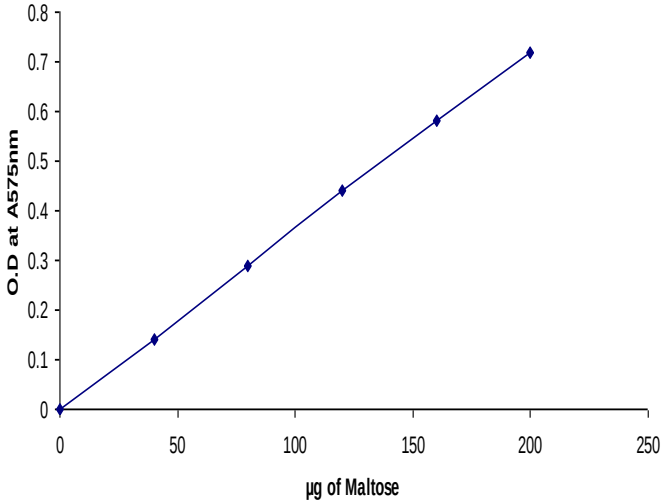

Figure 1. Standard curve of Maltose

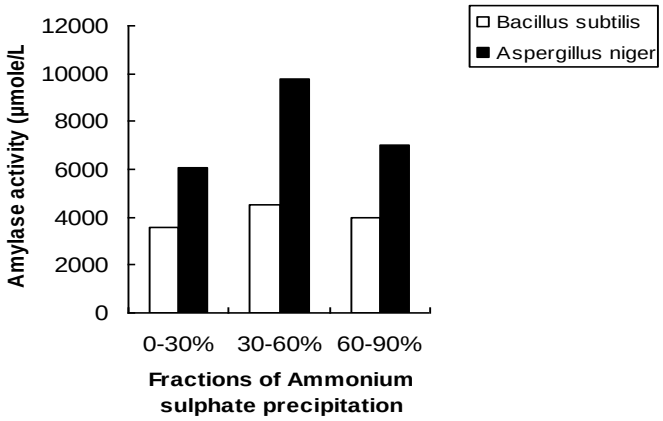

Figure 2. Ammonium sulphate precipitation of bacterial amylase (Bacillus subtilus) and fungal amylase (Aspergillus niger)

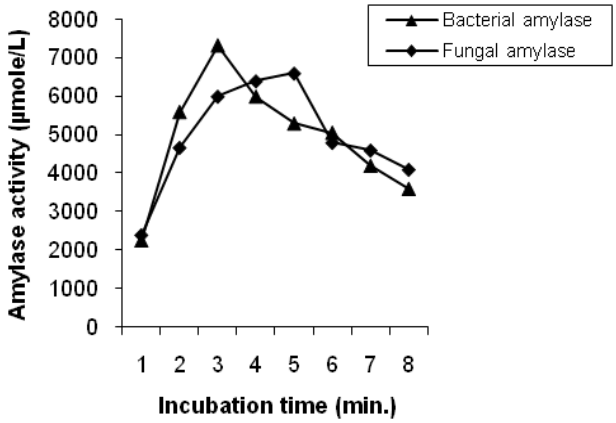

Figure 3. Effect of incubation time on activity of bacterial amylase (*Bacillus subtilus*) and fungal amylase (A*spergillus niger*)

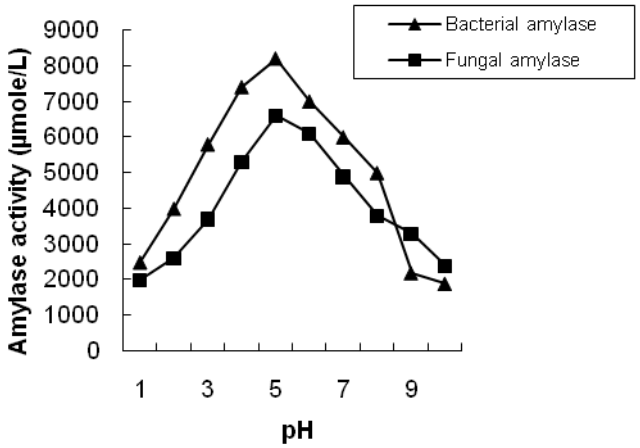

Figure 4. Effect of pH on activity of bacterial amylase (*Bacillus subtilus*) and fungal amylase (A*spergillus niger*)

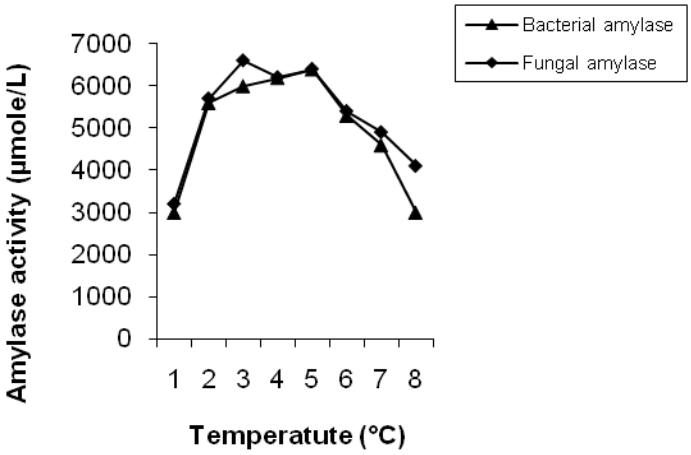

Figure 5. Effect of temperature on activity of bacterial amylase (*Bacillus subtilus*) and fungal amylase (A*spergillus niger*)

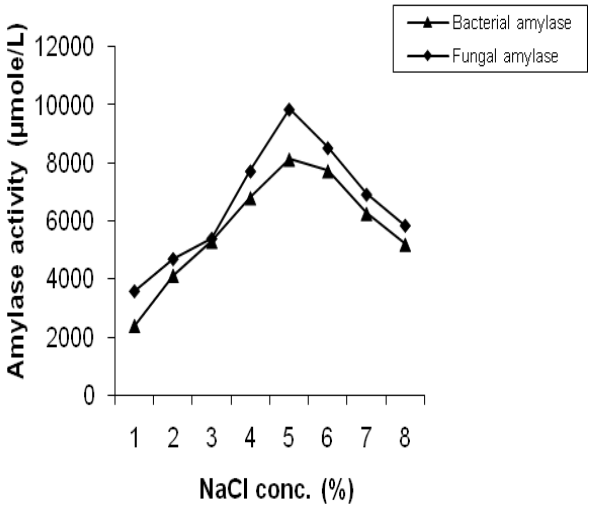

Figure 6. Effect of NaCl concentration activity of bacterial amylase (*Bacillus subtilus*) and fungal amylase (A*spergillus niger*)

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