Purification of Amylase Produced by Thermophilic *Bacillus* sp. TS9

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Received: 5th March, 2020 / Revised: 29th April, 2020 / Accepted: 4th May, 2020 / Published: 24th June, 2020

Abstract

Amylases are the starch degrading enzymes obtained from diverse sources such as plants, animals and microorganisms including bacteria and fungi. But the microbial amylases are preferred over other sources. The thermophilic alpha amylase obtained from bacterial species has applications in many industries. Six thermophilic bacteria Microbacterium sp. TS1, Pseudomonas sp. TS2, Alishewanella sp.TS3, Rheinheimera sp. TS5, Bacillus sp. TS9 and halophilic Bacillus sp. HS7 were isolated from Shahdrah and Kheiwera regions of Pakistan and their 16s RNA identified through molecular techniques. All the isolates were screened for amylase quantitatively and qualitatively. The Thermophilic Bacillus sp. TS9, based on its higher production of amylase, was selected for purification and characterization. The bacteria produced clearance zone of 2.7cm in diameter on 1% starch supplemented agar plate and the maximum specific activity of 2.45U/mg was obtained after 72 hours incubation at 45° C and pH 8. The extra-cellular amylase was then purified by ammonium sulphate precipitation and gel permeation chromatography. The specific activity of amylase obtained from Bacillus sp. TS9 increased to 4.47U/mg (1.82 fold) through partial purification, and 15.92U/mg (6.48 fold) through column chromatography. The current Study points out a valuable approach to purifiy the amylase and consequently improves the activity of amylase which have numerous applications in many industries.

Keywords: Amylase, Column Chromatography, Ammonium sulphate precipitation, Thermophiles, Enzymes.

Introduction

Extremophiles are the organisms that have adapted to a variety of extreme environments depending on which they can be divided into various classes like thermophiles, psychrophiles, halophiles, alkalophiles and acidophiles. Some extremophiles can also be found in fields of sulphur, deep-sea hydrothermal vents, low

nutrients and oxygen environments, high and low intensity of light and pressure and presence of heavy metals **(Ladenstein and Antranikian, 1998; Niehaus *et al.*, 1999; Ventosa, 2006)**. The adaptation of extremophilic microorganisms is due to their physiology and genetic makeup that allow them to tolerate extreme environment **(Aguilar, 1996)**. The thermophilic microorganisms because of their ability to grow at higher temperatures possess unique properties due to which they produce high end products that are physically and chemically stable even with low growth **(Kikani *et al.*, 2010)**. A number of products obtained from extremophilic microorganisms are of importance in biotechnology some of which are already being used commercially **(Tango and Islam, 2002)**. For example, the biocatalysts and enzymes obtained from thermophiles are advantageous are used for the degradation of polymeric compounds like starch, chitin, cellulose, xylan and pectin under high temperature **(Burg, 2003)**.

Microorganisms, for the catalytic activities of their enzymes, have been used since ancient times in the food industry to make food products like vinegar, cheese, wine, beer, sourdough, etc. and in other industries such as the preparation of linen, leather and indigo and more recently in pharmaceuticals, beverages, and even in waste water treatments **(Wiseman, 1985)**. Besides the whole microorganisms, enzymes isolated from animal tissues such as calves, rumen, from plant tissues such as papaya fruit or by microorganisms are increasingly used. However, the enzymes used in these industries are not well-purified and well characterized **(Kirk *et al.*, 2002)**. Recently, microorganisms (bacteria and fungi) are used to produce variety of enzymes such as amylases, proteases, cellulases, xylanases, lipases, pectinases etc for industrial uses **(Mukesh *et al.*, 2010)**. Microbial enzymes are preferred over enzymes produced by plants and animals because of several advantages including their ease of production and isolation on a large scale, genetic manipulation, low production costs, and advanced techniques of growing them in controlled environment to maximize the yield of their end products **(Hasan *et al.*, 2006)**.

Amylases are starch metabolizing enzymes that break down starch molecules into small glucose units **(Paula and Magalhaes, 2010)**. Amylases can be further classified into two categories. i) Endoamylases and ii) Exoamylases. Endoamylases catalyse hydrolysis of the interior of starch molecules in a random manner, thus yielding linear and branched chains oligosaccharides (glucose, dextrin and maltose) of varying lengths. Exoamylases catalyse non-reducing ends of the starch molecules yielding short end products **(Gupta *et al.*, 2003)**. Amylases are the second largest class of commercially produced enzymes after proteases, constituting 25-30% of world enzymes market and hold a lot of significance in biotechnology **(Rajagopalan and Krishnan, 2008; Reddy *et al.*, 2003)**. Extracellular α-amylase is mostly obtained from *Bacillus* species, such as *Bacillus subtilis, Bacillus stearothermophilus, Bacillus amyloliquefaciens* and *Bacillus licheniformis* **(Haq *et al.*, 2010)**. The *Bacillus* strain is the most preferred source to produce thermophilic α-amylase because it has short generation time, and its environmental and genetic manipulation is easy. Moreover, the thermophilic α-amylase is preferred over mesophilic amylase because thermophilic amylases are more stable and can maintain their stability and activity at elevated temperature thus having longer life in

industrial reactors which is very economical **(Daniel *et al.*, 1981)**. Thermostable α-amylase maintains its activity at high temperatures such as in starch processing industries, these amylases are used in liquefaction process performed at 80-90°C and gelatinization process carried out at 100-110°C **(Rasooli *et al.*, 2008)**. Also, the allowance of the processes to be performed at elevated temperatures reduces the risk of contamination, imparts better solubility of substrate in media and efficient mixing of nutrients **(Mamo *et al.*, 1999).**

The significance of the present study was to purify and enhance the activity of thermophilic α-amylase isolated from thermophilic Bacillus sp. from a hot spring at Shahdrah, Islamabad.

Material and Methods

Bacterial Strains

Five thermophilic strains *Microbacterium* sp. TS1, *Pseudomonas* sp. TS2, *Alishewanella* sp. TS3, *Rheinheimera* sp. TS5, *Bacillus* sp. TS9 and one halophilic strain *Bacillus* sp. HS7, previously isolated in the Microbiology Research Laboratory, Department of Microbiology, Quaid-i-Azam University, Islamabad, were used in the current study.

Qualitative Test for Amylase

For qualitative analysis of amylase, the six bacterial strains were streaked on starch supplemented agar plates **(Beffa *et al.*, 1996)** and incubated for 24-48 hours at 45° C. The plates were then flooded with iodine crystals and were observed for the formation of clear zones of hydrolysis around the amylase producing colony.

Quantitative Test for Amylase

For quantitative analysis of amylase, inoculum of *Alishewanella* sp.TS3, *Rheinheimera* sp.TS5, *Bacillus* sp. HS7 and *Bacillus* sp. TS9 was prepared. *Microbacterium* sp. TS1, *Pseudomonas* sp. TS2 were not further processed due to production of small quantity of enzyme qualitatively.

Preparation of Inoculum: For the preparation of inoculum, nutrient broth was prepared in 100 ml Erlenmeyer flasks and then autoclaved at 15 psi pressure and 121° C for 15 to 20 minutes. Then a loop full of respective bacterial culture from starch supplemented agar plates was transferred to the nutrient broth and incubated at 45° C for 24 to 48 hours, 150 rpm.

Production Medium for Amylase: The composition of amylase production media is given in Table 1.

Table 1: Composition of Amylase Production

Chemicals	Quantity (g/l)
Starch	10
Yeast extract	2
$MgSO_4 .7H_2 O$	0.5
$K_2 HPO_4$	1
NaCl	5

pH was adjusted to 8 by the addition of 1N NaOH and 1N HCl using pH meter (Sartorius). Then the production medium was autoclaved.

Inoculation of Production Media: About 10% of inocula of *Alishewanella* sp. TS3, *Rheinheimera* sp. TS5, *Bacillus* sp. HS7 and *Bacillus* sp. TS9 was transferred to 6 separate 250 ml flasks containing 100 ml production medium for amylase. Incubated in shaker incubator at 45° C, 150 rpm and samples were taken at 0-minute time point and then after 24, 48, 72, 96, 120 and 144 hours of incubation.

Assessment of Enzyme Production and Growth of the Bacteria

Amylase is an extracellular enzyme, therefore, the cells were centrifugation at 14,000 rpm for 15 minutes and consequently crude enzyme extract (supernatant) was obtained. The α-amylase was qualitatively assayed by Bernfeld method with some modifications **(Bernfeld, 1955)**. For determination of enzyme units, 1 ml of cell free supernatant (crude enzyme) was mixed with 1 ml of 0.02 M potassium phosphate buffer containing 1% starch and incubated at 40° C for 1 hour. Then 2 ml of DNS reagent was added and placed in boiling water (99.9°C) for 10 minutes. Optical density was measured at 540 nm using UV-Visible spectrophotometer (Agilent) using maltose as a standard. One unit of enzyme activity can be defined as the amount of enzyme required to release 1mg of maltose in one hour at 40°C, pH 8, under the standard assay conditions. The protein concentration of the sample was measured by Lowry's method using BSA as a standard **(Lowry *et al.*, 1951)**. Growth of TS3, TS5, HS7 and TS9 was measured spectrophotometrically at 600 nm after every 24 hours for one week.

Purification of α-Amylase

Based on qualitative and quantitative tests, thermophilic *Bacillus* sp. TS9 was selected for further purification of α-amylase. The broth was centrifuged to remove cells from the supernatant followed by ammonium sulphate precipitation and gel permeation chromatography.

Preparation of Inoculum of *Bacillus* sp. TS9: About 50 ml of nutrient broth was prepared in 250 ml Erlenmeyer flask and then autoclaved at 121° C and 15 psi pressure for 15-20 minutes. Then a loop full of *Bacillus* sp. TS9 was transferred from starch supplemented agar plate to the nutrient broth and then incubated at 40°C for 24-48 hours in shaker incubator.

Preparation of Production Medium: Then 500 ml production medium for α-amylase was prepared in 1000 ml Erlenmeyer flask and then autoclaved.

Inoculation of Production Medium with *Bacillus* sp. TS9: Then 50 ml of *Bacillus* sp. TS9 inoculum was transferred to 500 ml of α-amylase production medium and then incubated for 72 hours at 45°C, at 150 rpm. After 72 hours of incubation, the broth was centrifuged at 10,000 rpm for 30 minutes in order to remove the cells from the broth. CFS (cell free supernatant) was obtained containing α-amylase and the supernatant was further processed for the purification.

Ammonium Sulphate Precipitation

Ammonium sulphate salt was added to the supernatant containing α-amylase produced by *Bacillus* sp. TS9 to bring 40% saturation at 4°C followed by stirring for 15 minutes and then centrifugation at 10,000 rpm for 20 minutes. The supernatant and pellets were dissolved in potassium phosphate buffer at pH 6.9 and assayed for amylase activity. The pellets showing higher enzyme activity and protein contents were collected and processed for further studies.

Gel Permeation Chromatography

For the separation of different molecules sephadex G-75 was used. The gel was soaked in 0.02M potassium phosphate and buffer containing 0.02% sodium azide for 3 days. After swelling of the gel, it was de-aerated and then the required column (0.9x60 cm) was packed with it. The column was washed by passing 20-30 ml of buffer through it. The volume of the column was

determined by 0.5% dextran blue solution. After washing the column with buffer, 2 ml of sample (precipitates dissolved in 0.02 M potassium phosphate buffer) was applied to column and 20 fractions (3 ml each) were collected through automatic fraction collector. Total protein content in the fractions was then determined by taking its OD at 280 nm. Protein estimation and enzyme assay of the fractions was also done. Fractions having high activity was pooled and processed for further study.

Results

Qualitative Test for Amylase

Microbacterium sp. TS1 and *Pseudomonas* sp. TS2 did not produce significant amount of amylase qualitatively, therefore, they were not processed further while the remaining four strains produced clear zone of hydrolysis around the colonies indicating the production of amylase. The largest zone of hydrolysis was 27 mm produced by thermophilic bacterial strain *Bacillus* sp. TS9, followed by *Rheinheimera* sp. TS5 producing 24mm diametric zone of hydrolysis and *Bacillus* sp. HS7 produced zone having diameter of 22 mm and *Alishewanella* sp. TS3 having diameter of 20 mm as given in figure 1.

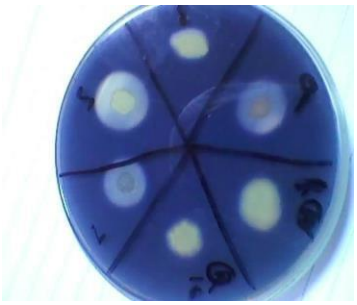

Figure 1. Qualitative Test of Amylolytic Bacterial Strains

Quantitative Test for Amylase

After qualitative test, quantitative test for the amylase production was done in order to select the best amylase producing strain for further studies. The growth OD and enzyme activities of the four bacterial strains studied are shown in figure 2. For *Bacillus* sp. TS9, *Rheinheimera* sp. TS5 and *Bacillus* sp. HS7 the log phase occurred during 24-72 hours incubation and maximum growth OD of 4.69 and 3.34 mg/ml respectively obtained after 72 hours incubation. Moreover, the enzyme production in both species increased with increase in growth and maximum amylase was produced in the late log phase after 72 hours incubation showing maximum specific activity of 2.45 U/mg, 1.555 U/mg and 1.59 U/mg, respectively. At further incubation the bacteria entered the stationary phase and then decline phase. The enzyme production also followed the curve (figure 2-3). *Alishewanella* sp TS3 entered the log phase at 24 hours and its maximum growth was obtained after 48 hours of incubation, at which point the enzyme's specific activity was 1.27U/mg. The enzyme production followed the growth curve showing a growth dependent pattern (figure 4).

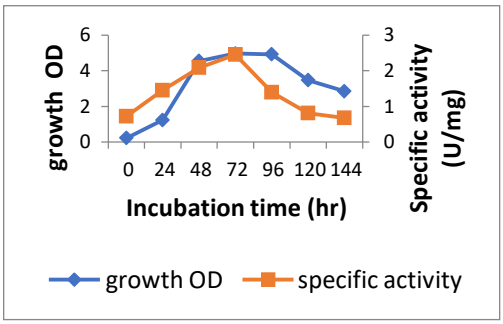

Figure 2. Growth OD and Amylolytic Activity of *Bacillus* sp. TS9

The growth curve of *Rheinheimera* sp. TS5 shows that its log phase also occurred for 24-72 hours of incubation and maximum growth obtained after 72 hours incubation. The amylase production by *Rheinheimera* sp. TS5 also increases with increase in growth as its production is growth dependent and maximum enzyme production showing peak specific activity of 1.555 U/mg obtained after 72 hours of incubation. By further incubation the Rheinheimera sp. TS5 entered in to the stationary phase and then decline phase, where enzyme production also lowered depicted in figure 3.

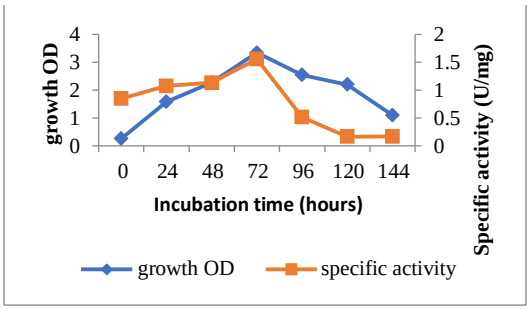

Figure 3. Growth OD and Amylolytic Activity of *Rheinheimera* sp. TS5

The growth curve of *Alishewanella* sp TS3 shows that its log phase started after 24 hours of incubation and remained for 48hors as maximum growth obtained after 48 hours of incubation. As the amylase production by *Alishewanella* sp TS3 was also growth dependent, therefore, maximum enzyme was produced after 48 hours of incubation with peak specific activity of 1.27U/mg. Its growth and enzyme production reduced when incubated for 72-144 hours as shown in figure 4.

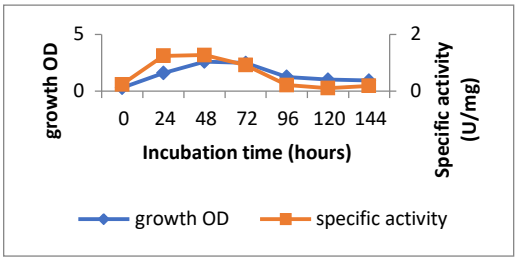

Figure 4. Growth OD and Amylolytic Activity of *Alishewanella* sp TS3

The growth curve of *Bacillus* sp. HS7 shows that its log phase started after 24 hours of incubation and remained for 72 hours. Maximum growth of *Bacillus* sp. HS7 occurred after 72 hours and also amylase production by *Bacillus* sp. HS7 increases with increase in growth. The maximum amylase production with maximum specific activity of 1.59 U/mg obtained after 72 hours of incubation. Then further declines in growth and enzyme production by *Bacillus* sp. HS7 occurred when incubated for 96-144 hours as given in figure 5.

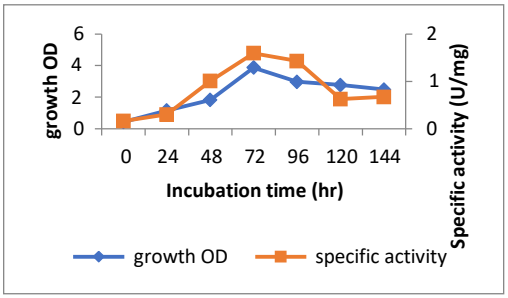

Figure 5. Growth OD and Amylolytic Activity of *Bacillus* sp. HS7

Purification of Amylase from Thermophilic Bacillus sp. TS9

Among the four amylase producing strains, thermophilic *Bacillus* sp. TS9 was

selected for the further investigations on the basis of qualitative and quantitative tests. The precipitates obtained at 10% ammonium sulphate showed significantly higher amylolytic activity and higher protein content. The precipitates showed maximum specific activity 4.47U/mg as depicted in figure 6. The partially purified enzyme obtained through ammonium sulphate were further purified through column chromatography. One peak was obtained having specific activity of 15.9 U/mg as shown in Figure 7. The fractions 1, 2, 3 and 4 showing the highest activities were pooled together and were further characterized. The purification fold and recovery are explained in Table 2.

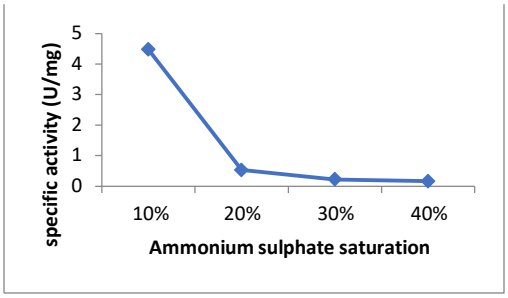

Figure 6. Amylolytic Activities of Precipitates Obtained at Different Concentration of Ammonium Sulphate

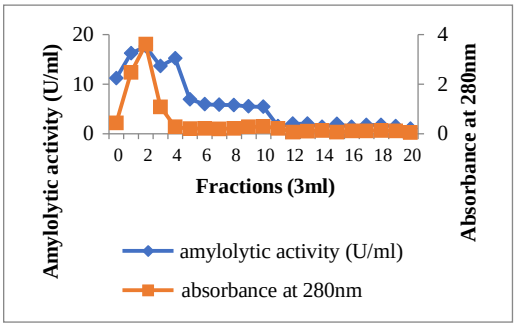

Figure 7. Amylolytic Activity, Protein Estimation and Total Protein Content of the Fractions Obtained Through Column Chromatography

Discussion

Five thermophilic and one halophilic bacterial strains obtained from soil and water samples from different areas of Pakistan (Kheiwera and Shadrah spring) were compared for their amylase production. These strains were previously characterized and identified through gram staining, biochemical tests and molecular analysis of 16s rRNA. Through qualitative test on starch supplemented agar plates, it was found that all these strains were producers of amylase.

Table 2. Purification fold and recovery of partially purified and purified amylase obtained from *Bacillus* sp. TS9

Amylase obtained from TS9	Total proteins (mg)	Enzyme activity (units)	Total activity (U/ml)	Specific activity (mg/ml)	Purification Fold	Recovery
Crude	0.72	6.7	16.9	2.45	-------	-------
Partially purified	0.6	10.23	25.61	4.47	1.82	83.3%
Purified	0.3	17.6	43.69	15.92	6.48	41.6%

Through qualitative and quantitative analysis, the *Bacillus* sp. TS9 was comparatively best producer of amylase, so it was selected for further study. The phylogenetic analysis of the isolated strain revealed that 99% of nucleotide sequence was similar to thermophilic Bacillus specie, by blasting in NCBI.

The production of extracellular amylase by thermophilic *Bacillus* sp. isolated from soil samples **(Kim *et al.*, 2012; Fattah *et al.*, 2012; Rasooli *et al.*,** 2008) and hot springs (**Oczan *et al.*,** 2010; **Fooladi and Sajjadian**., 2009) has been reported in several studies from different parts of the world.

In the current study the quantitative analysis done for 6 days (144 hours) revealed that the maximum production of amylase was obtained on 3rd day (72 hours), and the unit/ ml of the extracellular amylase after 72 hours was 16.95U/ml with specific activity 2.45U/mg determined through maltose standard curve and beyond that limit its stationary phase started, followed by its decline phase. It might be because of deprivation of nutrients in the media. Previously different groups studying thermophilic *Bacillus* species have arrived at different results regarding maximum growth and enzyme activities at 24, 48 and 72 hours (**Akcan *et al.*,** 2011; **Verma *et al.*,** 2011; **Gaur *et al.*,** in 2012; **Cordeiro *et al*.,** 2002).

In current study, the amylase produced by *Bacillus* sp. TS9 was partially purified and precipitated out from the supernatant at 10% saturation of ammonium sulphate with the increase in the specific activity of about 1.8 folds. Similarly**, Kim *et al.*,** in 2012 also reported the partial purification of amylase produced by thermophilic *Bacillus* specie at 10% saturation of ammonium sulphate from culture supernatant, which supports our study. **Thippeswamy *et al.*,** in 2006 reported that the partial purification of amylase produced by *Bacillus* specie from culture supernatant was partially purified at 40% of ammonium sulphate saturation and **Kumar *et al.*,** in 2012 reported that the amylase was precipitated out from the culture supernatant at 30% of ammonium sulphate saturation. The extracellular enzyme can be salted out from the culture supernatant at different saturations of ammonium sulphate depending on the number and position of polar groups, pH of the solution and molecular weight of the solution.

In the current study, after partial purification, the amylase produced by thermophilic *Bacillus* sp. TS9 was further purified through gel filtration method using chromatographic technique and it was employed by using sephadex G75 column. A single band was obtained showing increase in specific activities of about 6.5 folds and 41.6% recovery. The fractions demonstrated showed protein content and enzyme assay were pooled together and further the purified amylase was characterized. **Lim *et al.*,** in 2020 reported 13.2 folds purification with 60% recovery of α-amylase isolated from *E. coli* and *Bacillus* sp. purified through affinity column chromatography. Moreover, **Baltas *et al.*,** in 2016 also reported 28.9 folds purification with 74.6% recovery of α-amylase isolated from *Anoxybacillus thermarum* A4 purified through sephadex G100 gel filtration chromatography.

Conclusion

The study can be concluded as among five bacterial strains thermophilic *Bacillus* sp. TS9 was comparatively good producer of amylase extremozyme. Continuous fermentation process can be used for the production of α-amylase thermophilic

Bacillus sp. TS9 for extended log phase because enzyme was growth dependent. Furthermore, the amylase produce by thermophilic bacterial strain can withstand in harsh conditions during industrial bioprocess and can be used in food and feed industries.

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