Pak. J. Biochem. Mol. Biol., 2012; 45(2): 99-103

Optimization of cultural conditions for production of amylase from thermophilic *Bacillus* sp.

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Abstract: To exploit the potential of thermotolerant bacteria for the production of starch degrading enzymes, soil samples were obtained from various locations. Samples were screened for extracellular amylase activity on starch containing media. Bacterial cultures showing starch degradation were purified and studied for biochemical and morphological characters. Upon the selection of highest enzyme producing strain, physical and biochemical parameters affecting enzyme production were optimized. It was found that bacterium is an aerophile belonging to class *Bacilli.* It grows best at neutral pH, tolerates temperature up to 62°C with the optimum at 55°C and produces maximum amylase when media is supplemented with 1% starch. The enzyme was found to be active over a wide pH range though optimum activity was found at pH 7.0.

Keywords: Thermotolerant, extracellular, amylase, optimization, cultural conditions.
Received: February 23, 2012 **Accepted:** May 5, 2012
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INTRODUCTION

Starch is among the most abundant polymer on earth and consists of glucose monomer units linked up by α-glucosidic bonds[1]. This biodegradable polymer is hydrolysed by enzymes known as amylases. Amylases are starch-degrading enzymes that catalyze the hydrolysis of glycosidic bonds in low molecular weight products, such as glucose, maltose and maltotriose units[2]. Amylases are among the most important enzymes and are of great significance for biotechnology. They constitute a class of industrial enzymes that share a significant place in international enzyme market[3,4].

Amylases have potential application in a wide range of industrial processes such as bread making, brewing, fermentation, paper, detergent, and pharmaceutical industries[5]. However, with the advances in biotechnology, the amylase application has expanded in many fields such as clinical, medicinal and analytical chemistry, as well as their widespread application in starch saccharification and in the textile, distilling and other industries[2,6,7]. It also plays an important role in biofuel production[8].

Although amylases can be obtained from several sources, such as plants, animals and microorganisms[9,10], enzymes of microbial origin have a broad spectrum of industrial applications as they are more stable than when prepared with other sources[11,12]. The major advantage of using microorganisms for the production of amylases is the economical bulk production capacity and the fact that microbes are easy to manipulate to obtain enzymes of desired characteristics[13].

Although *Bacillus, Aspergillus* and *Rhizopus* species are considered to be the most important sources of industrial amylases[5], it remains a challenging task to obtain a stress tolerant strain in order to be used in industrial processes.

Current study describes the investigation of various soil samples, in order to isolate microbial strains producing amylolytic enzymes, and to characterize the strain productivity, particularly the physical and chemical parameters affecting enzyme production.

MATERIALS AND METHODS

Collection of samples and isolation of amylase producing strains

Soil samples were collected from various locations in sterilized containers. 1% soil suspension was prepared in sterilized saline solution (0.85% NaCl). The suspension was vortexed and 100µl sample was inoculated on sterile Luria agar plates supplemented with 1% starch. Plates were incubated at 37˚C for 18 to 24 hours. The plates were then flooded with iodine solution (Iodine 1.0 gm, KI 2.0 gm, distilled Water 300.0 ml) to detect clear halos of starch degradation.

Biochemical and morphological studies

Bacterial colonies showing starch hydrolysis on starch agar plates were subjected to purification by repeated streaking on same media. Once after purification, cultures were grown on nutrient broth and agar plates for biochemical and morphological analysis according to Bergey's manual[14].

Adaptation of bacterial culture for thermophilic amylase production

A broad range of temperature was used to adapt highest extracellular amylase producing strain for higher temperatures. Culture was inoculated in Luria Broth supplemented with starch and incubated at various temperatures i.e. 40˚C, 50˚C, 60˚C, 70˚C, 80˚C and 90˚C for 24 hours, after 24 hours for estimation of enzyme activity. Strain was repeatedly grown on the selected temperature for stable adaptation.

Optimization of media for optimum bacterial growth and enzyme production

To optimize nutrient media for maximum enzyme production**,** several reported media were used (Table 1)[15-17]. Bacterial culture was inoculated in each reported media and subjected to incubation for 24 hours at 55˚C. Enzyme activity was performed by assaying cell free broth at standard assay conditions.

Carbon source

Media were supplemented with 1% carbon sources such as commercially available starch, cornstarch, ground wheat powder, ground rice powder, sago starch; one at a time prior sterilization of media. After selection of carbon source, its suitable concentration to yield maximum enzyme activity was sought by varying concentration from 0-5% in nutrient medium.

Nitrogen Source

Various nitrogen sources (organic and inorganic both) were added in 1% concentration in the selected nutrient medium. Yeast extract, tryptone, peptone, urea, KNO_3, $(NH_4)_2HPO_4$, $(NH_4)_2SO_4$ were added prior media sterilization. Organism was incubated in this media for 24 hours and cell free broth was assayed for enzyme production. Upon selection of a source, its concentration was varied from 0-5% in medium in order to select suitable concentration to support enzyme production.

Optimization of bacterial growth time for maximum enzyme production

Bacterial culture was subjected to varying period of incubation in order to estimate the time required for maximum enzyme production. Inoculated nutrient medium was kept at 55 ˚C for 24-96 hours. Enzyme activity was measured after every 24 hours.

Optimization of pH of nutrient media supporting bacterial growth

Bacterial strain was incubated in nutrient media flasks having a wide pH range. The pH of media was adjusted using 1N NaOH and 1N HCl prior sterilization of media. The flasks were incubated at 55˚C for 24 hours.

Estimation of total protein

Total protein of the sample was estimated by the method of Lowry[18] using Bovine serum albumin as standard.

Estimation of enzyme activity

Units of enzyme activity were measured by Bernfeld method [19]. An amylase unit was defined as the amount of enzyme that produced 1mg of maltose per minute from soluble starch under the assay conditions.

RESULTS AND DISCUSSION

Screening of amylogenic bacterial specie

For the screening of amylase producing microorganisms, soil samples from different locations were tested. Total 92 strains were isolated from soil, and 56 were found to be extracellular amylase producers as they produced clear halos around colonies grown on starch supplemented plates when flooded with iodine solution. Amylogenic cultures were purified and stored in nutrient broth containing 15% glycerol at -20˚C. Highest enzyme producing culture was identified as gram positive, spore forming *bacilli* according to biochemical and morphological characteristics.

Adaptation of bacterial culture for thermophilic amylase production

Culture incubation at various temperatures from 40˚C-90˚C for 24 hours revealed that organism can survive up to 62˚C, however enzyme production was optimal within a range of 50-55˚C. Figure 1 shows the relation of temperature and enzyme production capacity of the producer strain.

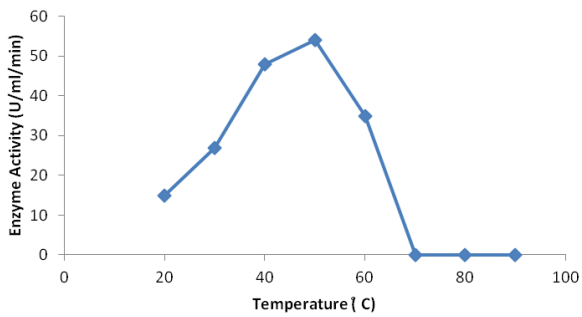

Figure 1: Estimation of enzyme activity after bacterial growth at different temperatures.

Optimization of media for optimum bacterial growth and enzyme production

As elaborated in table 1, various reported media were used to optimize nutrient media for maximum enzyme production. From figure 2 it has been deduced that amylase produced by the bacterium is inductive in nature as very low enzyme activity was obtained in media 1 which lacked starch as carbon source, however, media supplemented with starch (media 2, 3) showed comparatively higher enzyme yield. Cell free broth obtained from media 3 showed highest enzyme yield whereas media 2 showed lower yields of enzyme which could be attributed to solution of complex ions which might have induced inhibitory effect on enzyme production.

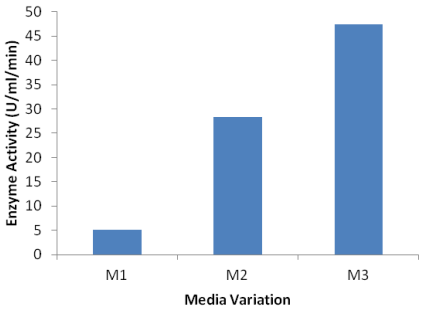

Figure 2: Estimation of enzyme activity in different reported media.

Table 1: Composition of different nutrient media.

Media Components	Media 1 (g/l)	Media 2 (g/l)	Media 3 (g/l)
Starch	-	10	15
Sucrose	20	-	-
Peptone	-	10	
Tryptone	10	-	-
Yeast extract	5.0	-	5
NaCl	5	-	-
KCl	-	0.5	-
$FeSO_4$	-	0.01	-
$MgSO_4$	-	0.5	5
$NaNO_3$	-	3	-
KH_2PO_4	-	1	-
K_2HPO_4	-	-	5
$CaCl_2$	-	0.1	-
pH	7.0	7.0	6.0

Carbon source

From various sources of starch used for enzyme production, commercially available soluble starch showed maximum enzyme activity in cell free broth (Figure 3).

After the selection of starch as carbon source, its concentration was varied from 0-5% in nutrient media to determine the concentration suitable for higher enzyme yields (figure 4). The media supplemented with 1% starch supported maximum enzyme yield.

Nitrogen source

Producer strain was grown in media provided with various nitrogen sources (organic and inorganic both) keeping rest of all media components constant. It was found that among all sources, yeast extract and tryptone gave almost same enzyme yield (Figure 5).

Inorganic nitrogen sources were found not much supportive for amylase production. Therefore tryptone with a concentration of 1% was selected as nitrogen source in media for higher enzyme production (Figure 6).

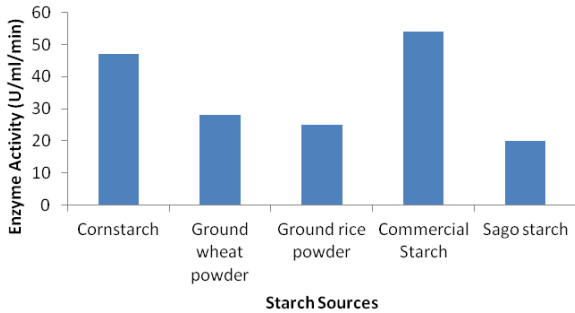

Figure 3: Estimation of enzyme activity in medial supplemented with different starch source.

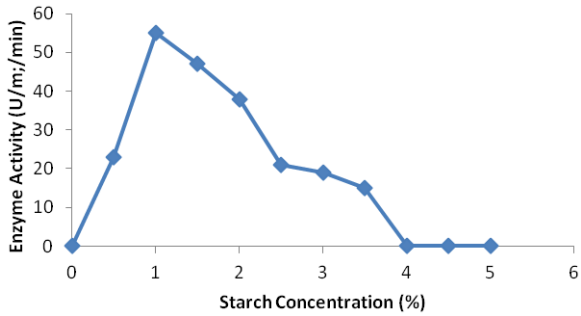

Figure 4: Estimation of enzyme activity at varying starch concentrations.

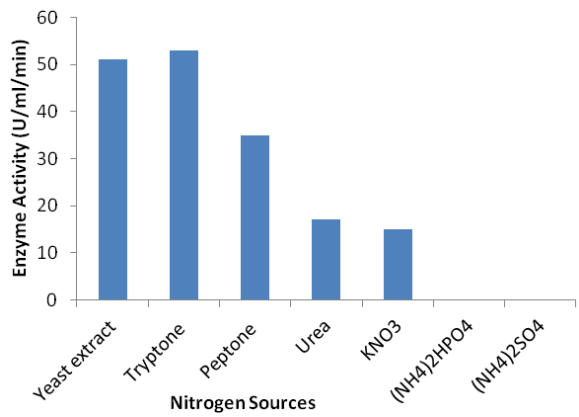

Figure 5: Estimation of enzyme activity in medial supplemented with different nitrogen sources.

Upon optimization of all media components, media 3 was modified by supplementation of starch 10g/l and tryptone 10g/l; rest of all components were remained same.

Optimization of bacterial growth time for maximum enzyme production

Incubation time was varied from 24-96 hours to optimize time interval required for maximum enzyme production. Upon estimation of enzyme activity it was found that 72 hours incubation showed higher enzyme yield than any other time interval (Figure 7).

Optimization of pH of nutrient media supporting bacterial growth

Media pH was varied to a wide range in order to determine optimum pH at which enzyme production would be maximum. Figure 8 shows that the strain yields higher amount of enzyme at neutral pH and increasing or decreasing the pH above or below this adversely affected on enzyme production.

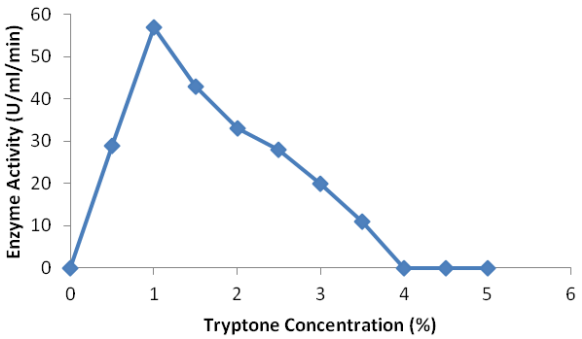

Figure 6: Estimation of enzyme activity at varying tryptone concentrations.

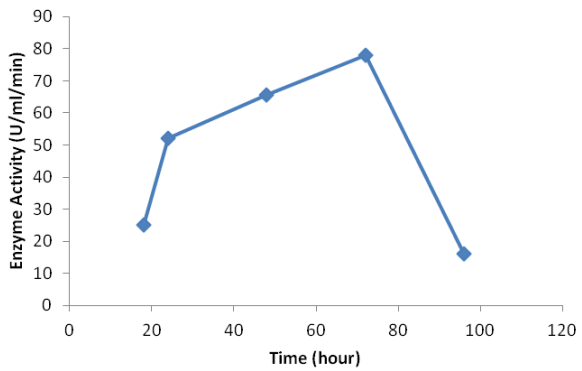

Figure 7: Estimation of enzyme activity at varying incubation time intervals.

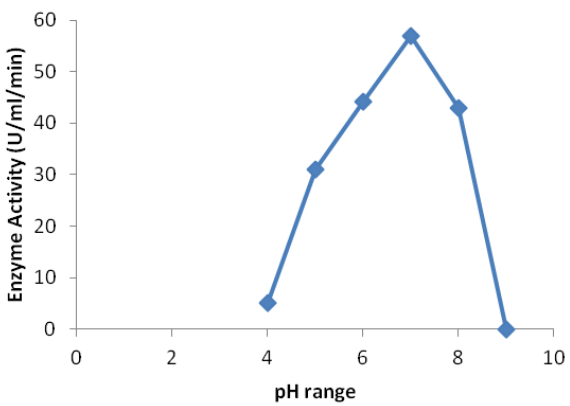

Figure 8: Estimation of enzyme activity at varying pH range.

CONCLUSION

In view of the importance of amylases in industrial biotechnology and the distinct characteristics of enzymes isolated from various strains of *Bacillus* specie, current study focused at isolation of amylase producing thermotolerant *Bacillus* specie and to optimize culture conditions for optimal production of the enzyme. Producer strain was subjected to different nutrient media, varying substrate concentrations, pH values and incubation time periods. It was found that enzyme production was maximum when provided with starch and tryptone as major carbon and nitrogen source respectively. Yeast extract, $MgSO_4$ and K_2HPO_4 were also added to supplement bacterial growth and enzyme production. Bacterium was able to produce maximum enzyme yield at 55˚C with media pH at 7.0.

REFERENCES

1. Machovic M and Janecek S. In: *Industrial Enzymes, Structure, Function and Applications*, Editors: Polaina J and MacCabe AP, Springer, The Netherlands. 2007; pp 3-4.
2. Gupta R, Gigras P, Mohapatra H, Goswami VK and Chuahan B. Microbiol α-amylases: a biotechnological perspective. *Process. Biochem.* 2003; 38: 1599-1616.
3. Reddy NS, Nimmagadda A and Sambasiva Rao KRS. An overview of the microbial α-amylase family. *Afr. J. Biotechnol.* 2003; 2: 645-648.
4. Rajagopalan G and Krishnan C. Alhpa-amylase production from catabolite depressed *Bacillus subtilis* KCC103 utilizing sugarcane bagasse hydrolysate. *Bioresour Technol.* 2008; 99: 3044-3050.
5. Pandey A, Nigam P, Soccol CR, Soccol VT, Singh D and Mohan R. Advances in microbial enzymes. *Biotechnol. Appl. Biochem.* 2000; 31: 135-152.
6. Rao DM, Swamy AVN and SivaRamaKrishna G. Bioprocess Technology Strategies, Production and Purification of Amylases: An overview. *Internet J. Genomics Proteomics.* 2007; 2: 1540-2630
7. Chi MC, Chen YH, Wu TJ, Lin LL. Engineering of a truncated α-amylase of *Bacillus sp*. Strain TS-23 for the simultaneous improvement of thermal and oxidative stabilities. *J Biosci Bioeng*. 2010; 109:531-538
8. Qin Y, Fang Z, Pan F,Zhao Y, Li H, Wu H and Meng X. Significance of Tyr302, His235 and Asp194 in the α-amylase from *Bacillus licheniformis. Biotechnol Lett*. 2012; 34:895-899
9. Vihinen M and Mantsala P. Microbial Amylolytic Enzymes. *Crit. Rev.in Biochem. and Mol. Biol*. 1989; 24(4): 329-418.
10. Fogarty WM and Kelly CT. In: *Microbial Enzymes and Biotechnology*, Second Edition, Elsevier Science Publishers, London. 1990; pp 71-98.
11. Tanyildizi MS, Ozer D and Elibol M. Optimization of alpha amylase production by *Bacillus sp*. Using response surface methodology. *Process Biochem.* 2005; 40: 2291-2296.
12. Negi S and Banerjee R. Amylase and protease production from *A. awamori*. *Food Technol. Biotechnol*. 2006; 44(2): 257-261.

13. Souza PM and Oliveira e Magalhaes P. Application of microbial alpha amylase in industry – A review. *Braz. J. Microbiol.* 2010; 41: 850-861.
14. Holt JG, Krieg NR, Sneath PHA, Staley JT and William ST. In: *Bergey's manual of determinative bacteriology*, Nineth Edition, Editor: Wikins W, Wavely Company, Baltimore. 1994; pp.787.
15. Rupinder KG and Jagdeep K. A thermostable glucoamylase from a thermophilic Bacillus sp.: characterization and thermostability. *J. Ind. Microbiol. Biotechnol*. 2004; 31: 540-543.
16. Llori MO, Amund OO and Omidiji O. Short communication: purification and properties of a glucose forming amylase of Lactobacillus brevis. *World J. Microbial. Biotechnol.* 1995; 11:595-596.
17. Kunamneni A, Permaul K and Singh S. Amylase production in solid state fermentation by the thermophilic fungus Thermomyces langinosus. *J. Biosci. Bioeng*. 2005; 100(2): 168-171.
18. Lowry OH, Rosenbrough NJ, Farr AL and Randall RJ. Protein Estimation with the Folin-Phenol Reagent. *J. Biol. Chem.* 1951; 193: 265-275.
19. Bernfeld P. In: *Amylases, α and β: Methods in Enzymology*, vol.1,Editors: Colwick SP and Kaplan NO, Academic Press, New York. 1955; pp 149-150.