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Optimization of α - Amylase Production of *Bacillus Stearothermophilus* KDP from Sago Industry Waste

J. Sasi Premila[1] and K. Dhandayuthapani[2*]

[1]Research and Development Centre, Bharathiar University, Coimbatore, Tamil Nadu India – 641 046

[2]PG Department of Plant Biology and Biotechnology, Arignar Anna Govt. Arts College, Cheyyar, Tamil Nadu, India – 604 407.
*Corresponding author: kdpani_bio@yahoo.co.in; +91 9976133537

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Abstract

Thermophilic and amylolytic aerobic bacteria was isolated from soil sample collected from sago industry waste through a selective enrichment procedure at 55 °C with starch as the carbon source and identified as *Bacillus stearothermophilus* KDP by biochemical test with Bergey's manual. We aimed to study the Influence of certain nutritional and environmental factors on amylases production by this newly isolate. Optimum pH and temperature for the maximum amylase production by *B. stearothermophilus* was found at 7.0(6.2 U/m1/min) and 55^0C (8.2 U/ml/min) respectively. Carbon source arrow root present media showed the maximum enzyme production (8.8 U/mL). Among different nitrogen sources the yeast extract presented media increased the enzyme yield (9.2 U/mL). Stimulatory and Inhibitory effects of metal ions such as Se, Ni, Mg, Zn and Cu were tested. Stimulatory effect was observed in Se and Ni present media.

Keywords: Amylase, *Bacillus stearothermophilus,* Nitrogen sources, Carbon sources, pH, Temperature, Metal ions

Introduction

Amylases [α-amylase, β-amylase and glucoamylase (GA)] are among the most important enzymes in present day biotechnology. α - Amylases (EC 3.2.1.1, 1, 4-α-D-glucan glucanohydrolase) were classified in family 13 of glycosyl hydrolases and hydrolyzes starch, glycogen and related polysaccharides by randomly cleaving internal α-1, 4-glucosidic linkages to produce different sizes of oligosaccharides. They have diverse applications in a wide variety of industries such as food, fermentation, textile, paper, detergent, pharmaceutical and sugar industries (Gupta *et al*., 2003).

To meet the current largely increased demand, studies on the cost effective production of industrially important enzymes have become the need of today. Microorganisms are the most important sources for enzyme production; they made significant contribution to the production of foods and beverages in the last four decades. Selection of the right organism plays a key role in high yield of desirable enzymes (Pandey, 1990; Syed *et al*., 2009).

The level of enzyme activity produced by an organism from a natural environment is often low and need to be elevated for industrial production. Increase in enzyme levels is often achieved by mutation of organism and media optimization. The microorganisms used for enzyme production are grown in fermenters using as optimized growth medium. Both solid state and submerged fermentation are applied commercially. The enzymes produced by the microorganism may be intracellular or secrete into the extracellular medium.

Each application of α-amylase requires unique properties with respect to specificity, stability, temperature and pH dependence (Hmidet, *et al*., 2010). The amylase exhibited activity at a wide range of pH and temperature, desirable characteristics which can lead to its application in detergents as additive and in textile desizing. Among bacteria, *Bacillus sp* is widely used for thermostable α-amylase production to meet industrial needs. They are known to be good producers of thermostable α-amylase, and these have been widely used for commercial production of the enzyme for various applications.

Therefore, any improvement in the enzyme production, extracellular activity and thermo stability or activity will have a direct impact on the process performance, economics and

feasibility. Since the natural isolate produced very low concentration of amylase, in the present study an attempt was made to increase the productivity by optimizing the nature and relative concentration of carbon and nitrogen source with supplemented metal ions.

Materials and Methods

Microorganism

Thermophilic microorganism *Bacillus stearothermophilus* KDP used in this study which was isolated from sample collected from sago industry waste, Rasipuram, Tamil Nadu, India and identified by Goodfellow, (1989) methodology. This strain was routinely maintained on Luria-Bertani agar (LB) plates and stored at 4°C.

Fermentation Media

Media for enzyme production was starch with the following composition: (g/L):- $NaNO_3$, 3.0; $MgSO_4$. $7H_2O$, 0.5; KCl, 0.5; KH_2PO_4, 1.0; $FeSO_4$ $7H_2O$, 0.01; $CaCl_2$ 0.1; soluble starch 10.0; 1L distilled water and pH 7.0.To optimize the following physical and chemical parameters the experiments were carried out in 250ml Erlenmeyer flasks contained 100ml media and the cultures were incubated with shaking 250rpm for 48 h. Cells were harvested from the culture broth by centrifugation at 10000 x g for 25 min and the supernatants was used as sources of extracellular amylases.

Effect of pH and temperature on α-amylase production

The physical parameters such as pH and temperature were optimized by varying its range from 4 to 8 and 30 to 60^0C respectively. Cultures were incubated with shaking 250rpm for 48 h.

Effect of different carbon sources on α-amylase production

The effect of different carbon sources such Arrow root, Fructose, Lactose, Mannitol, Starch, Sucrose and Xylose on α-amylase production by *B. stearothermophilus* KDP was investigated. Each carbon source effect was studied separately by added 1 % (w/v) initial concentration to the above said fermentation media (without starch). The cultures were incubated with shaking 250rpm at the optimized temperature 55^0C and pH 7.0 for 48 h.

Effect of different nitrogen sources on α-amylase production

The Nitrogen source taken for the present investigation is Ammonium Hydrogen phosphate, Sodium nitrate, Potassium nitrate, Peptone, Yeast extract, meat extract, Urea respectively to study the effect on amylase production by *B. stearothermophilus* KDP. Each nitrogen source effect was studied separately by added 1 % (w/v) initial concentration to the above said fermentation media (without $NaNO_3$). The cultures were incubated with shaking 250 rpm at the optimized temperature 55^0C and pH 7.0 for 48h.

Effect of metal ions on α-amylase production

Effects of different metal ions such as Se^{2+}, Ni^{2+}, Zn^{2+}, Mn^{2+} and Cu^{2+} on *α*-amylase production was tested by amended with 1 mM/L final concentration of each metal ions separately in the standard culture media. The cultures were incubated at the optimized temperature 55^0C and pH 7.0 for 48 h.

α-Amylase assay

The activity of α-amylase was assayed by incubating 0.5 ml enzyme with 0.5 ml soluble starch (1%, w/v) prepared in 0.1 M sodium phosphate buffer (pH 7.0). After incubation at 37^0C for 60 min the reaction was stopped by the addition of 2 ml of 3-5-dinitrosalicylic acid reagent (Bernfeld, 1955) and absorbance was measured in a UV-Visible spectrophotometer (Elico). One unit (U) is defined as the amount of enzyme which releases 1mol of reducing end groups per minute in 0.1 M sodium phosphate buffer (pH 7.0) with 0.5% (w/v) soluble starch as substrate at 37^0C.

Statistical Analysis

Correlations analysis (Karl Pearson) and ANVOVA (one way) was performed to find the significations of parameters by Software - MINITAM Release 12.2.

Results and Discussion

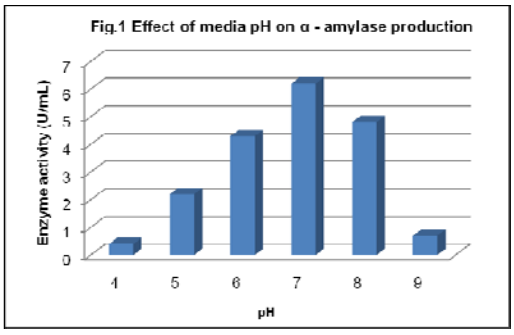
Characteristics and identification of α-amylase producing strain

The cells were swollen, motile rods occurring in chains and utilization of carbohydrates such as Glucose, Maltose, Lactose, and Mannitol positive. The organism was oxidase-positive, catalase-positive, Voges Proskaeur (add creatine as catalyst with reagents) reaction- negative, starch hydrolysis - positive, attacked glucose oxidative with acid and gas production, showed growth at

55^{0}C, but not grew in 6.5% NaC1. The organism was identified as *Bacillus stearothermophilus* and designated with suffix as KDP. Almost all microorganisms of the *Bacillus* genus synthesized alpha amylase, thus this genus has the potential to dominate the enzyme industry (Shah Ali UI Qader *et al*., 2006).

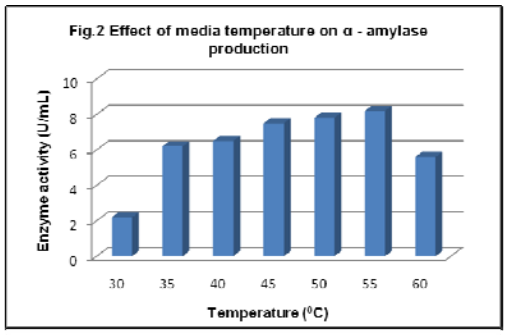
Effect of media pH on α - amylase production

Among the physical parameters, the pH of the growth medium plays an important role by inducing morphological change in the organism and in enzyme secretion. The pH change observed during the growth of the organism also affects product stability in the medium. Most of the Bacillus strains used commercially for the production of α-amylases by SmF have an optimum pH between 6.0 and 9.0 for growth and enzyme production (Burhan *et al*., 2003; Asgher et al., 2007). In our study *B. stearothermophilus* was cultivated at different initial pH (range from 4 to 9) with the standard media. The maximum α - amylase 6.2 U/mL was found at pH 7 grown cultures (Fig.1). Further increase or decrease in the pH resulted decrease in the activity of amylase. Since the correlation analysis showed high degree of positive correlation optimum pH of α - amylase production media was 7.0. This result is in good agreement with *B. cereus* and *B. subtilis* (Prabakaran and Pugalvendhan, 2009) and the newly isolated *Bacillus* from soil in Iran (Iraj Rasooli *et al.,*2008) in which the maximum α - amylase enzyme activity was found at pH 7.0. Majority of the bacteria produce their extracellular amylase at neutral pH only.



Effect of media temperature on α - amylase production

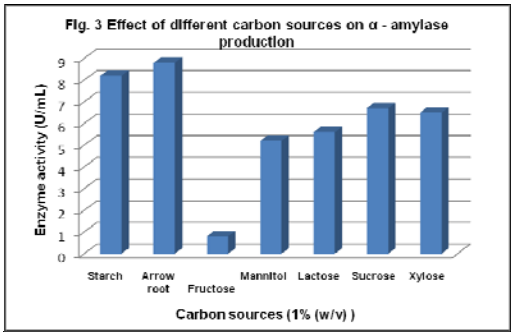
Among bacteria, *B. subtilis, B. stearothermophilus, B. licheniformis*, and *B. amyloliquefaciens* are known to be good producers of thermostable α-amylase, and these have been widely used for commercial production of the enzyme for various applications (Om Prakash and Jaiswal, 2010). In the present investigation the effect of different initial temperature on α - amylase production was studied by grown the strain *B. stearothermophilus* at temperature range from 30 to 60^{0}C. The bacterium grown satisfactorily and produced the enzyme at temperature range from 45 to 55^{0}C and statistical analysis also showed the high degree of correlation (γ = +0.997) for these temperature but the maximal α-amylase activity was achieved at 55^{0}C (8.2 U/mL) (Fig.2). Since the maximum α - amylase production was obtained at 55^{0}C this new isolate is thermophilic and possessed the ability to produce thermostable α-amylase. A reduction in enzyme activity was observed at temperatures above 55^{0}C. The statistical analysis revealed that the influence of temperature on amylase production is related to the growth of the organism. A wide range of temperature (35–80^{0}C) has been reported for optimum growth and α-amylase production in bacteria (Burhan *et al*., 2003; Konsula & Liakopoulou-Kyriakides, 2004; Asgher *et al*., 2007).



Effect of different carbon sources on α - amylase production

Carbon source in the form of either monosaccharide or polysaccharides may influence the production of amylase enzyme. Many researchers have shown that different carbon sources have varied influence on the production of extracellular enzymes especially among amylase producing strains (Vijayabaskar *et al.,* 2012). In our present investigation different carbon sources were used to study their influence on amylase production (Fig.3). The influence of arrow root (8.8 U/mL) and starch (7.6 U/mL) was more than the other carbon sources tested. ANOVA indicated that the carbon source arrow root and starch was more significant than other carbon

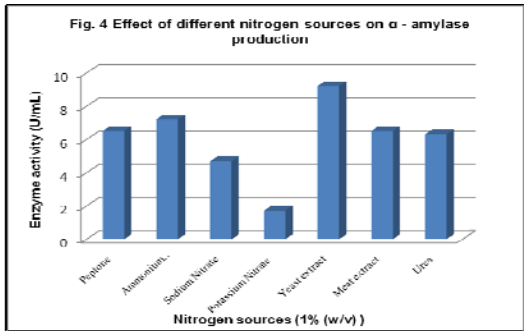
sources because the calculated value (0.22) was lesser than the table value (1.84). Similarly Syed *et al.,* (2009) studied the effect of different carbon sources such as sucrose, starch, lactose, xylose, fructose, glucose, on α -amylase activity by *Streptomyces gulbargensis* DAS 131. Among the carbon source starch at a concentration 1% (w/v) only showed maximum yield of 2,216.6 U/mL. Santos and Martins (2003) reported that increasing starch concentration in the medium beyond 1% did not increase enzyme activity.



Very lowest enzyme production was observed (0.8U/mL) in fructose present media. Synthesis of amylases in most species of the genus *Bacillus* is repressed by readily metabolizable substrates such as glucose (Lin *et al*., 1998). Haseltine *et al.,* (1996) reported a repression of a-amylase synthesis by glucose addition in *Sulfolobus solfataricus* and described that glucose prevents α-amylase gene expression and not merely secretion of preformed enzyme.

Effect of different nitrogen sources on α -amylase production

Nitrogen sources are the most important secondary energy compounds for microorganism's growth and their production. The nature of these compounds and the concentration used may stimulate or down regulate the production of enzymes. The amylase synthesis by several microorganisms has been correlated to the presence or absence of different nitrogen sources and various amino acids in the growth medium. Organic sources like yeast extract, peptone usually have stimulating effects (Hamilton *et al.*, 1999; Hewitt and Solomons, 1996). In the present study evaluated the effect of different nitrogen sources on *B. stearothermophilus* α – amylase production. Among the nitrogen sources tested yeast extract amended media showed the maximum production of α -amylase was 9.2 U/mL (Fig.4). ANOVA of this experiment showed not significant for other nitrogen sources except yeast extract since their calculated value (0.18) was lesser than table value (2.01). Asgher et al., (2007) reported that the growth and synthesis of α-amylase by *B. subtilis* JS-2004 was favored by 1.0 % of yeast extract. Yeast extract was the best nitrogen source for amylase production, probably due to its high content in minerals, vitamins, coenzymes and nitrogen components (Vijayabaskar *et al.,* 2012).



Effect of different metal ions on α –amylase production

The influence of 5 kinds of metal ions on the α-amylase activity was studied (Fig.4). The maximum activity (98%) was recovered 1 mM/L of Ni^{2+} present media. This was similar to the results from *Streptomyces gulbargensis* (Syed *et al*., 2009). The next maximum was founded to be at 1mM/L of Se^{2+}. These metal ions are more significant than others. The remaining metal ions showed the inhibitory effect. Many metal ions often influence the activity of α-amylase (De Azeredo et al., 2004). Ni^{2+}, Cd^{2+}, Zn^{2+}, and Hg^{2+} ions strongly inhibited the enzymatic activity by 82, 91, 100, and 100%, respectively. On the other hand, in *Bacillus sp*. TS-23, Ni^{2+} and Cd^{2+} slightly inhibited amylase activity (Asgher *et al*., 2007).

Conclusion

The enzymes of amylase family have great significance due to its wide area of potential application. In the present investigation, B. *stearothermophilus* was isolated from sago industry waste. This also produced thermostable α-amylase. The optimum pH and temperature was found to be 7.0 and 55^0C respectively. Among different carbon and nitrogen sources arrow root and yeast extract supported to the maximum yield. The metal ions Ni^{2+}, and Se^{2+} enhanced the production at 1mM/L concentration. By this

investigation the above said conditions are the optimum for the maximum α- amylase production and which can be used to design new production media.

References

Asgher,M., Javaid Asad, M., Rahman S.U. and Legge, R.L. 2007. A thermostable α-amylase from a moderately thermophilic *Bacillus subtilis* strain for starch processing. *J. Food Engineering*,79: 950–955.

Bernfeld, P. 1955. Amylases alpha and beta. In: Colowick SP, Kaplan ON (eds) Methods in enzymology. Academic Press, New York, 140–146.

Burhan,A., Nisa, U., Gokhan, C., Omer, C., Ashabil, A. and Osman, G. 2003. Enzymatic properties of a novel thermophilic, alkaline and chelator resistant amylase from an alkalophilic *Bacillus* sp. isolate ANT-6. *Process Biochemistry,* 38: 1397–1403.

De Azeredo L.A.I., Freire, D.M.G., Soares, R.M.A., Leite, S.G.F. and Coelho, R.R.R. 2004. Production and partial characterization of thermophilic proteases from *Streptomyces* sp isolated from Brazilian cerrado soil. *Enzyme Microb Technol.,* 34:354–358.

Goodfellow, M. 1989. Supergenic classification of actinomycetes. In: Williams, S.T, Sharpe, M.E., Holt, J.G. (eds) Bergey's manual of Systematic Bacteriology, Vol 4. Williams and Wilkins, Baltimore, 2333–2339.

Gupta, R., Gigras, P., Mohapatra, H., Goswami, V.K. and Chauhan, B. 2003. Microbial α-amylases: a Biotechnological prospective. *Process Biochemistry*, 38:1599–1616.

Hamilton, L. M., Kelly, C. T. and Fogarty, W. M. 1999. Production and properties of the raw-starch-digesting α-amylase of *Bacillus* sp. IMD- 435. *Process Biochemistry*, 35: 27–31.

Heseltine, C., Rolfsmeier, M. and Blum, P. 1996. The glucose effect and regulation of α–amylase synthesis in the hyperthermophilic archeon *Sulfolobus solfataricus*. *J. Bacteriol*., 178:945-950.

Hewitt, C. J. and Solomons, G.L. 1996. The production of α -amylase (E.C.3.2.1.1.) by *Bacillus amyloliquefaciens*, in a complex and a totally defined synthetic culture medium. *Journal of Industrial Microbiology*, 17: 96–99.

Hmidet, N., Maalej, H., Haddar, A. and Nasri, M. 2010. A Novel α-Amylase from *Bacillus mojavensis* A21 Purification and Biochemical Characterization. *Appl. J. Biochem. Biotechnol*., 162:1018–1030.

Iraj Rasooli, Shakiba Darvish, Alipoor Astaneh, Hojjat Borna and Kamal Azizi Barchini, 2008. A thermostable α-amylase producing natural variant of *Bacillus* Spp. Isolated foam soil in Iran. *American Journal of Agricultural and Biological Sciences,* 3:591-596.

Konsula, Z and Liakopoulou-Kyriakides, M. 2004. Hydrolysis of starches by the action of an α-amylase from *Bacillus subtilis*. *Process Biochemistry*, 39: 1745–1749.

Lin, L.L., Chyau, C.C., and Hsu, W.H. 1998. Production and properties of a raw-starch-degrading amylase from thermophilic and alkaliphilic *Bacillus* sp. TS-23. *Biotechnology and Applied Biochemistry*, 28: 61–68.

Om Prakash and Nivedita Jaiswal 2010. α-Amylase an ideal representative of thermostable enzymes., *Appl. Biochem. Biotechnol*., 160:2401–2414.

Pandey, A., 1990. Improvement in solid-state fermentation for glucoamylase production. *Biol. Wastes*, 34:11–19.

Prabakaran, G. and Pugalvendhan, R. 2009. Production and immobilization of alpha amylase by using *Bacillus subtilis*. *Recent Research in science and Technology*, 1(4):189-194.

Santos, E.O. and Martins, M.L.2003. Effect of the medium composition on formation of amylase by *Bacillus* sp. *Broz. Arch. Boil. Technol*., 46:1516-1520.

Shah Ali UI Qader, Saeeda Bano, Afsheen Aman, Noman Syed, Abid Azhar, 2006. Enhanced Production and extracellular activity of commercially important amylolytic enzyme by a newly isolated strain of *Bacillus* sp. AS-1. *Turk. J. Biochem*., 31 (3): 135–140.

Syed, D.G., Dayanand Agasar and Ashok Pandey, 2009. Production and partial purification of α amylase from a novel isolate *Streptomyces gulbargensis*, *J. Ind. Microbiol. Biotechnol*., 36:189–194.

Vijayabaskar, P., Jayalakshmi D. and Shankar, T. 2012. Amylase production by moderately halophilic *Bacillus cereus* in solid state fermentation. *African Journal of Microbiology Research*., 6(23):4918-4926.

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