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Production of Extra Cellular α-amylase using *Bacillus megaterium isolated* from White Mangrove (*Avicennia marina)*

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ABSTRACT

Bacillus megaterium isolated from leaves of *Avicennia marina* (Forssk.) was screened for their ability to produce α-amylase, studied in submerged fermentation by using an Adlof-Kuhner orbital shaker. The levels of amylase production detected in culture supernatants varied greatly with the type of carbon source used such as starch, lactose and glucose. Effect of different nitrogen sources revealed that peptone increase the enzyme yield. The enzyme activity increased between 1.5 and 3 g L^{-1} of yeast extract concentration and optimal concentration of peptone for the production of amylase was detected as 6 g L^{-1}. The maximum enzyme activity was obtained under optimum conditions of an incubation period of 72 h, an incubation temperature of 35°C and pH of 6.5.

Key words: α-amylase, *Avicennia marina*, *Bacillus megaterium*, submerged fermentation, starch

INTRODUCTION

Enzymes are among the most important products acquired for human needs in the areas of industrial, environmental and food biotechnology through microbial sources. Alpha amylase is a hydrolytic enzyme and in recent years, interest in its microbial production has increased dramatically due to its wide spread use in food, textile, baking and detergent industries (Asgher *et al*., 2007). α-amylase has been derived from several fungi, yeast, bacteria and actinomycetes; however enzymes from fungi and bacteria sources have dominated applications in industrial sectors (Pandey *et al*., 2000). The major advantage of using microorganisms for production of amylases is an economical bulk production capacity and microbes are also easy to obtain enzymes of desired characteristics. The production of amylases by fermentation has been thoroughly investigated and it's affected by a variety of physiochemical factors. Spectrum of applications of α-amylase has widened in many sectors such as clinical, medicinal and analytical chemistry. Besides their use in starch saccharification, they also find applications in baking, brewing, detergent, textile, paper and distilling industry (Ramachandran *et al*., 1978). *Bacillus megaterium* has been ideal for studies of cell structure, protein localization, sporulation, and membranes (McCool and Cannon, 2001).

Mangroves are known to stabilise coastal sediments through their aboveground aerial root complex. Mangroves inhabit intertidal zones with high salinity (Liang *et al*., 2008) and can tolerate a wide range of salinities under natural conditions. The mangrove's adaptations to landward depression and seaward depression localized topographic differences were important in view of changes in intertidal hydrology, the latter being linked to changes in topography. The grey

mangrove *Avicennia marina* has the ability to adapt its pneumatophores to micro-topographical irregularities in the otherwise regularly sloping intertidal zone (Dahdouh-Guebas *et al*., 2007). *Avicennia marina* (Forssk.) Vierh. is the most common species planted for mangrove restoration and is highly salt tolerant (Khan and Aziz, 2001).

The present investigation reported the optimization of physio-chemical factors such as pH, temperature, carbon and nitrogen sources for the production of amylase using *Bacillus megaterium* first time isolated from leaves of white mangrove Avicennia marina under submerged fermentation.

MATERIALS AND METHODS

Isolation and screening of micro organism: The rod shaped, mainly aerobic spore-forming Gram positive bacteria Bacillus megaterium was isolated from the aerial part of leaves of white mangrove Avicennia marina in the Vellar estuary of Southeast coast of Tamil Nadu (11°29' N Lat, 79°46' E Long) India during the month of August 2010. The primary screening of the strain was done by starch agar plate method (Shaw *et al*., 1999). The selection of thermophilic bacteria was done by growing them on a medium containing 2% Bactotryptone, 1% Bacto yeast extract, 1% NaCl and 2% agar at pH 7.0. The screening of bacteria capable of producing starch digesting enzymes was done by growing them on medium containing 1% soluble starch, 0.2% yeast extract, 0.5% peptone, 0.05% $MgSO_4$, 0.05% NaCl, 0.015% $CaCl_2$ and 2% agar at pH 7.0. The plates were stained with Gram's iodine solution (2% 2I and 0.2% potassium iodide) and largest halo-forming zone was considered as the most promising strain. Later microbiology of the isolated strain was determine according to the methods describe in Barges manual of systematic bacteriology (Seneath *et al*., 1986) and strain maintained on nutrient agar slant at 4°C for further studies. All the analytical chemicals and media components were purchased from Hi-media (Mumbai) and Sigma chemicals (USA).

Enzyme production medium: The composition of production medium used was soluble starch 50 g, yeast extract 0.5 g, KH_2PO_4 10 g, $(NH_2)_2SO_4$ 10.5 g, $MgSO_4$ 0.3 g, $CaCl_2$ 0.5 g, $FeSO_4$ 0.013 g, $MnSO_4$ $0.7H_2O$,0.004 g, $ZnSO_4$ $.2H_2O$ 0.004 g, $CaCl_2$ 0.0067 g and 1000 mL of distilled water. The pH was adjusted to 6.8 and the media were sterilized in an autoclave for 15 min at 121°C. The media were inoculated with a loop full of growing culture of *Bacillus megaterrium* and then incubated at 32°C in an orbital shaker set at 100 rpm for 24 h. The media were centrifuged at 8000 g, for 10 min at 4°C to obtain cell free filtrate.

Enzyme assay: Amylase assay was made by using a reaction mixture 4 mL consisted of 1 of enzyme solution and 2 mL of soluble starch in phosphate buffer, pH 6.8 (Wood and Bhat, 1988). The mixture was incubated for 10 min at 32°C. Level of reducing sugars was determined by Dinitrosalicylate method (Miller, 1959) and is expressed in units (one unit is the amount of enzyme which releases 1 μmole glucose.

Optimization of culture conditions: The factors such as temperature, pH, source of carbon and nitrogen affecting production of amylase were optimized by varying parameters one at a time. The experiments were conducted in 200 mL of Erlenmeyer flask containing production medium. The optimum pH was determined by adjusting pH of the media at a range from 4.5-7.0. The media was incubated at 26-36°C for 24-120 h to check preferred temperature and incubation time for enzyme production. Similarly, the effect of various carbon sources viz. (glucose, galactose, maltose, lactose

and sucrose 1%) and nitrogen sources namely peptone, beef extract, yeast extract and casein each at 0.5% were tested. Each experiment was carried out in triplicate and their values were averaged.

RESULTS AND DISCUSSION

Isolation and screening of amylase producing microorganism: The production of extra cellular α-amylases by submerged fermentation has been thoroughly investigated and it is affected by a variety of physiochemical factors. Many bacteria produce extra cellular amylase during the fermentation of starch. The organism was isolated from the leaves of a white mangrove species by serial dilution and screened by zone hydrolysis method and later identified as *Bacillus megaterium* (Table 1) according to Bergey's manual. The attached bacterial variations may be related to atmospheric and leaf temperature and probable inhibiting substance released by the plant leaves. The isolation of heterotrophic marine bacteria from the leaves of *Avicennia marina* had also been made by Mahasneh (2002). *B. megateium* was also capable of Polyhydroxybutarate accumulation up to 65% of the cell dry weight when grown in a synthetic medium based on sucrose and ammonium feed (Sabra and Abou Zaid, 2008). After 72 h of incubation, every 1 h interval, the culture filtrate was analyzed for amylase activity. In addition *Bacillus megaterium* was isolated and identified from the cassava dumpsites and used for amylase production (Oyeleke and Oduwole, 2009).

Effect of pH and temperature: The pH of the growth medium plays an important role in terms of inducing enzyme production. Similarly our study reported the amylase production varied at different range of pH. The best pH for growth was 6.5 where as the optimum temperature was 32°C and strain was capable to produce amylase by hydrolyzing starch. The activity of amylase was about 35% higher at pH 6.5 than pH 5.0 (Fig. 1) and 30% higher at 35°C than 40°C (Fig. 2). Some of the researchers previously reported the optimum pH of amylase production from *Bacillus* sp. at 7 (Vidyalakshmi *et al*., 2009; Alamri, 2010) and 7.5-8.5 (Amoozegar *et al*., 2003). Hence that, pH for the amylase production from *Bacillus* sp., ranges between 6.5-8.5. Although the optimum temperature for α-amylase production by *Bacillus megaterium* and *Bacillus* sp. exhibited at 60°C (Takasaki, 1989) and 70°C (Asgher *et al*., 2007). Moreover, the highest α-amylase activity produced by *Bacillus megaterium* was achieved in our investigation at the temperature of 35°C and pH of 6.5.

Table 1: Biochemical characterization of isolated bacteria isolated from auxiliary buds of white mangrove

Characteristics	*B. megaterium*
Gram staining	+
TSI	Slant - Butt +
Mannitol	Acid
Motility	Motile
Indole test	-
Methyl red test	-
V.P. test	-
Citrate test	-
Urease test	-
Oxidase test	+
Catalase test	+
H_2S	-

+: Positive; -: Negative

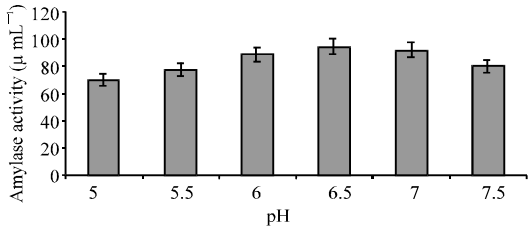

Fig. 1: Effect of pH of anylase production

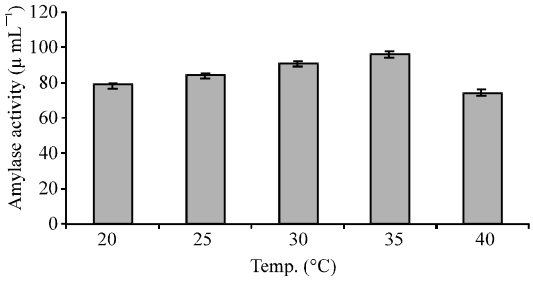

Fig. 2: Effect of various range of temperature on anylase production

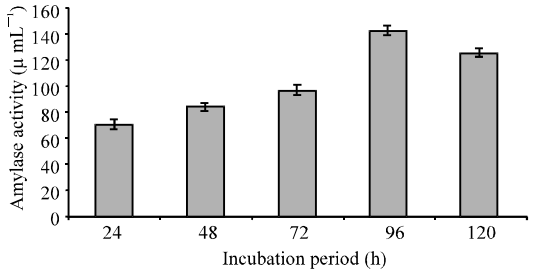

Fig. 3: Anylase production on various incubation period

Effect of time course on amylase production: When the culture was incubated at 96 h, the maximum activity detected was 156 U mL^{-1}. There was a two fold increase in activity at 96 h incubation as compared to 24 h (Fig. 3). Extension beyond the optimum time course was generally accompanied by a decrease in the growth rate and enzyme productivity, which gradually declined after 96 h of incubation (Alamri, 2010). The short incubation period for *Bacillus* sp. compared with other bacteria and fungi offers the potential for inexpensive enzyme production (Bernfeld, 1955).

Effect of carbon sources: A number of carbon sources (1% w/v) were tested in order to determine their effect on growth and α-amylase production. Among the effect of various carbon sources maltose was the best to enhance the amylase activity of 156 U mL^{-1} which was 7% higher than other carbon sources are shown in Fig. 4. The similar observation had been reported by Thomas *et al.* (1980). Growth and enzyme production did not alter when potato and corn starch were used as carbon sources. The same findings were reported by Oliveira *et al.* (2007). They mentioned that the induction of α-amylase requires substrates having α-1, 4 glucoside bond, including starch and maltose. The biosynthesis of α-amylase in most species of the genus *Bacillus*

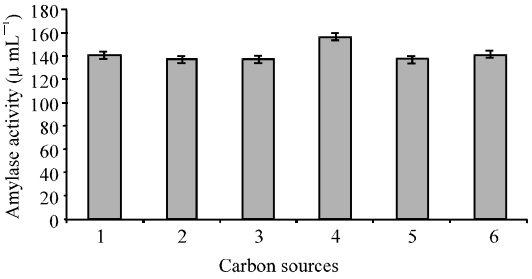

Fig. 4: Anylase production on different carbon sources. 1: Glucose, 2: Galactose, 3: Lactose, 4: Maltose, 5: Sucrose, 6: Xylose

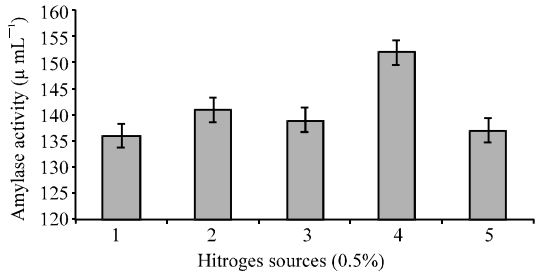

Fig. 5: Anylase production on different nitrogen sources. 1: Yeast extract, 2: Neat extract, 3: Beef extract, 4: Peptone, 5: Casein

was represed by readily metabolizable substrates, especially glucose, by a mechanism of catabolite repression (Lin *et al*., 1998). In addition *Bacillus licheniforms*, starch used as carbon source for amylase production (Adeyanju *et al*., 2007). Besides, Sarikaya and Gurgun (2000) reported that the highest α-amylase yield obtained by the addition of Na-citrate and sucrose for the strains of *Bacillus subtilis* and *Bacillus amyloliquefaciens*. Among different carbon sources disaccharides are more suitable for amylase production compared to polysaccharides.

Effect of nitrogen sources: Among the nitrogen sources, peptone was ideal to increase the enzyme activity of 140 U mL^{-1} which was about 10% higher than casein, meat and yeast (Fig. 5). The enzyme was susceptible to reagents that react with thiol groups and had an exo-action on starch yielding maltose with a p-anomeric configuration. It is concluded that the principal starch-hydrolysing enzyme from *B. megaterium* is a 1, 4-α-glucan maltohydrolase similar in its properties to other *Bacillus*, plant, and amylases.

CONCLUSION

The present study revealed white mangrove (*Avicennia marina*) leaves also a one of the habitation for microbial population. The production of amylase from *Bacillus megaterium* showed significant application for industrial enzyme production.

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