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SCREENING AND OPTIMIZATION OF EXTRACELLULAR AMYLASE PRODUCTION FROM PLANT GROWTH PROMOTING RHIZOBACTERIA

Arun Karnwal
Bhojia Institute of Life Sciences, Budh, Teh. Baddi, Solan, H.P. India, 173205
E-mail: arunkarnwal@gmail.com

Abstract
Total 50 Fluorescent Pseudomonas was screened for their ability to produce extracellular alpha-amylase and fifteen were selected for further studies due to amylolytic activity on starch agar plates. Of these 50 isolates, Pseudomonas fluorescence APK10 was selected for the further studies due to its higher amylase activity levels. P. fluorescence APK10 showed amylase activity at a wide range of pH (5.0 to 9.0) and temperature (20ºC to 40ºC). The optimum incubation time for amylase production was recorded as 24hrs (1050U/ml/min), while no activity detected after 96hrs of incubation. Higher alpha-amylase production was recorded at pH 7.0 (1500U/ml/min) and 30ºC (2000U/ml/min). P. fluorescence APK10 showed varied levels of enzyme activity with different starch concentrations. 2.0% starch concentration was the optimum concentration for maximum enzyme production (2500U/ml/min), while higher concentration above 2.5% reduced the enzyme activity. Alpha-amylase production varied greatly with different carbon source. Amongst the sugars, fructose, maltose, glycerol and trehalose encouraged maximum amylase production in comparison to starch, lactose, sucrose, glucose, mannitol, arabinose and xylose. While ribitol, raffinose, and sorbitol based medium was showed no or negligible enzyme activity. Results of this study revealed that isolated amylase was considered to be thermostable and active in a wide range of pH.

Keywords: Amylolytic activity. Food Industry. Pseudomonas. Reducing sugars. Starch hydrolysis.

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1. INTRODUCTION

In recent years the potential of using microorganisms as biotechnological sources of industrially relevant enzymes has stimulated interest in the exploration of extracellular enzymatic activity in several microorganisms (Abu E.A. *et al.,* 2005; Akpan, I. *et al.*,1999; Lin L.L. *et al.*,1998; Pandey P. *et al.,* 2000). Amylases are a group of enzymes that have been found naturally in plants, animals and several microorganisms like bacteria (Busch J.E., 1997; Haseltine C. *et al.*, 1996; Young M.H. *et al.,* 2001) and fungi (Fadel M., 2000; Wang B.D. *et al*., 2001).

Two major classes of amylases, α-amylase and β-amylase, have been identified in microorganisms. In microorganisms, α-amylase is extracellularally produced and secreted in the surrounded medium that randomly cleaves the 1, 4-α-D-glucosidic linkages between adjacent glucose units in the linear amylose chain and ultimately generate glucose, maltose and maltotriose units (Kandra L., 2003; Mitchell D.A., Lonsane B.K., 1994; Pandey P. *et al.*, 2000).

Amylase has been derived from several fungi, yeasts, bacteria and actinomycetes. However, enzymes from fungal and bacterial sources have dominated applications in industrial sectors (Pandey P. *et al.*, 2000).

The hydrolyzed products are widely applied in the food, paper, baking, brewing, detergent and textile industries (Nigam P., Singh D., 1995). With the advent of new frontiers in biotechnology, the spectrum of α-amylase application has expanded into many other fields, such as clinical, medical and analytical chemistry (Pandey P. *et al.*, 2000).

This research is aimed to isolate α-amylase producing *Pseudomonas fluorescence* strains from different plant rhizospheres and analyze their α-amylase producing ability with different parameters.

2. MATERIAL AND METHODS

Isolation & Screening of Microorganism

Pseudomonas fluorescence was isolated from different plant rhizospheres. Serial dilution agar plate method was followed for the isolation of *Pseudomonas* species on succinate agar medium. Each isolate showed generation time between 3-3.5h (which indicated the fast growing nature of isolates), pigmentation and biochemical reactions as described in Bergey's Manual of Determinative Bacteriology (Holt J.G., 1994).

Starch agar plate method was used to check the α-amylase producing ability of isolated strains. Isolated strains were grown on nutrient agar plates (containing 1%(w/v) starch) for 24 hrs at 35±2°C. After the proper incubation, bacterial plates were stained with Gram's iodine solution (2% iodine and 0.2% potassium iodide) and largest halo-forming zone was considered as the most promising strain and was chosen for further investigation.

The growth medium or production media used for α-amylase production was composed of 20.0g Soluble Starch, 5.0g Beef Extract, 10.0g Tryptose, 1.0g $MgSO_4.7H_2O$, 10.5g $(NH_4)_2SO_4$, 0.3g $MgSO_4$, 0.5g $CaCl_2$, 0.004g $MnSO_4$, 0.004g $ZnSO_4$, and 2.0g NaCl per liter. The pH was maintained at 7.0 with 1N NaOH and was autoclaved at 121°C for 15 minutes.

Production of α-Amylase

About 10 ml of production media (starch broth) was inoculated with a loop full of growing culture of *P. fluorescence* individually and incubated at 35±2°C in BOD incubator shaker set at 140 rpm for 24 hrs. After 24 hrs incubation, 10 ml culture was transferred into 40 ml of sterile production media and incubated at 35±2°C in BOD incubator shaker set at 140 rpm for 24 hrs. For obtaining the crude enzyme, incubated culture media was centrifugation at 10,000 rpm for 10 min at 4°C and this cell free filtrate was stored in a deep-freezer (BlueStar In Co.) at -20°C.

Enzyme Assay

α-Amylase assay was made by using a reaction mixture (5 ml) consisted 1 ml of enzyme (crude extract/fermented broth supernatant) and 2 ml (1.0%) solution of soluble starch in 50 mM phosphate buffer (pH 7.5) was incubated at 30°C for 10 minutes. 1 ml of 3, 5 dinitrosalicylic acid was used to stop the enzymatic reaction, followed by boiling for 10 min and to develop brown color. The final volume was made to 5 ml with distilled water and the absorbency measured at 540 nm with a spectrophotometer (Systronics-UV-vis double beam 2202). A calibration curve of absorbency and concentration of glucose was established with known amount of glucose, which work as standard curve. One unit (U) of amylase was defined as the amount of enzyme that liberates one micromole of reducing sugars, measured as glucose per min under the conditions of assay.

Protein Determination

The protein concentration of the fermented broth supernatant was determined by the Lowry method (Lowry O.H., 1951) with bovine serum albumin as standard.

3. RESULTS AND DISCUSSION

Total 50 *Pseudomonas* isolates were isolated from different plant rhizospheres. These isolates were gram negative, citrate positive, oxidase positive, catalase positive, produce fluorescence under UV light, able to hydrolyze casein, indole positive, able to use glucose, mannitol, fructose, arabinose, trehalose, glycerol, xylose and starch as carbon source. Of these 50 isolates fifteen were showed amylolytic activity on starch agar plates. Of these 15 isolates *Pseudomonas fluorescence* APK10 was formed the largest halo-forming

zone (Figure 1) and was considered as the most promising strain and was chosen for further investigation.

Effect of Incubation Period on α-Amylase Production

Production of alpha amylase and cell mass at different incubation time are plotted and shown in Figure2. Maximum α-amylase production was obtained at 24 hrs of incubation. (Sonjoy *et al.* 1995) reported that higher enzyme production was observed at short incubation period. Same results also noticed by Kathiresan and Manivannan (Kathiresan K., Manivannan S., 1996) with *Penicillium fellutanum* isolated from mangrove rhizosphere soil. It was found that cell mass was increased with the increment of incubation time but at the same time enzyme production was declined, and after 96 hrs no activity was observed (Figure 2).

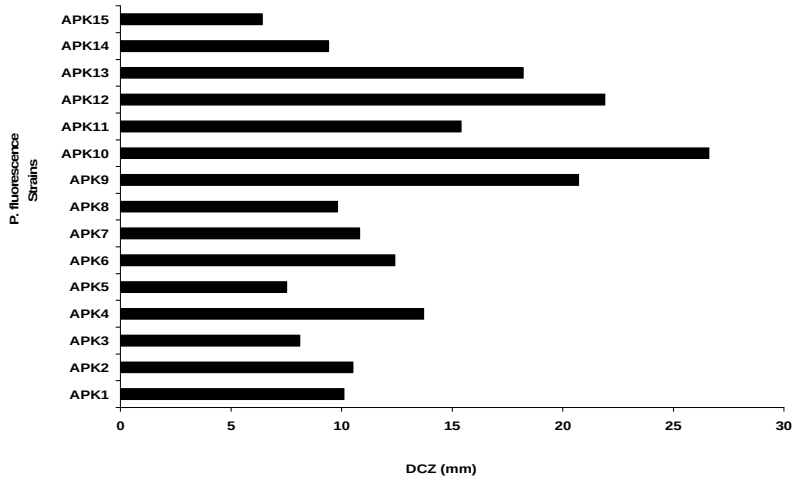

Figure 1: Diameter of clear zone (DCZ) of amylolytic *P. fluorescence* strains on Starch agar plates isolated from different plant rhizospheres

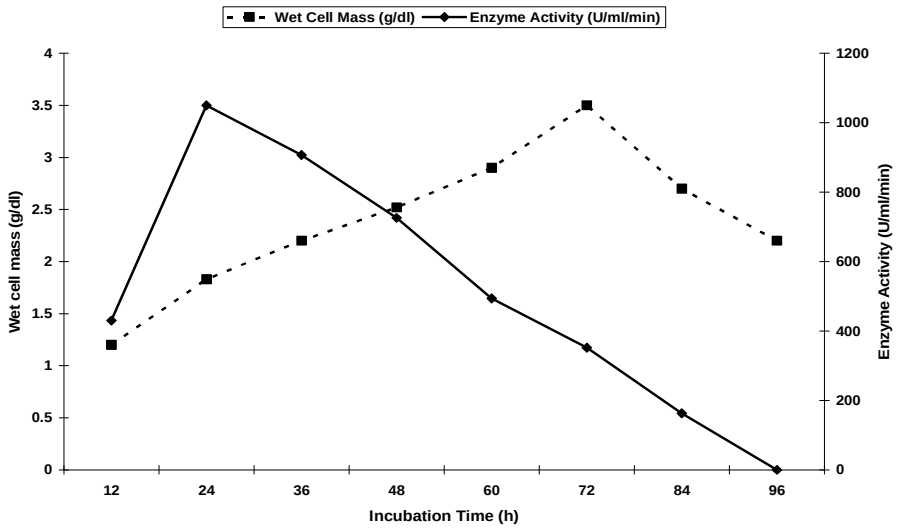

Figure 2: Effect of Incubation period on α-Amylase production

Effect of Starch Substrate Concentration on α-Amylase Production

Bajpai and Bajpai (Bajpai P., Bajpai P., 1989) reported that starch concentration greatly influence α-amylase production. To investigate the effect of various concentrations of starch, *Pseudomonas fluorescence* APK10 was incubated in the medium containing 0.5%, 1.0%, 1.5%, 2.0%, 2.5%, 3.0%, 3.5% starch concentration (Figure 3). Our strain showed higher enzyme production from 1.5% to 2.5% starch concentration while at 3.5% starch enzyme production was decreased. Santos and Martins (Santos E.O., Martins M.L.L., 2003) observed that higher starch concentration in fermentation medium did not increase the enzyme production.

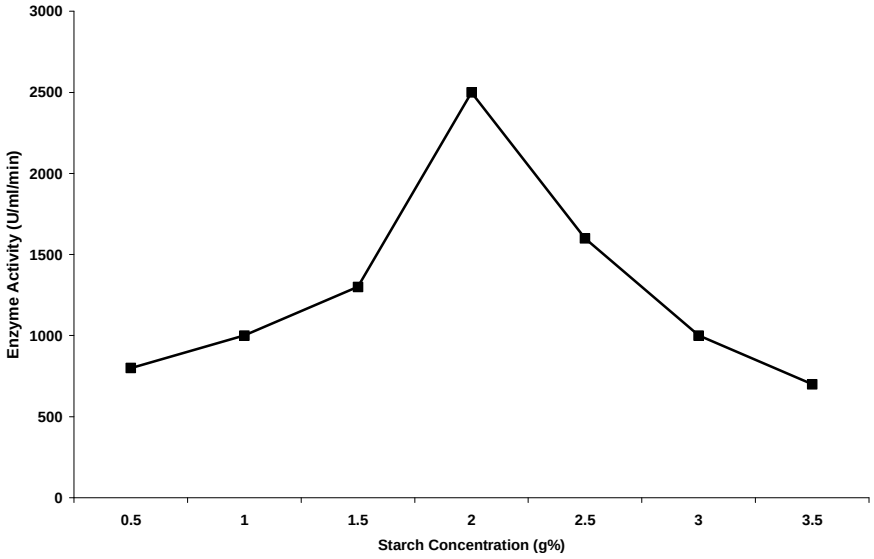

Figure 3: Effect of Starch Substrate Concentration on α-Amylase Production

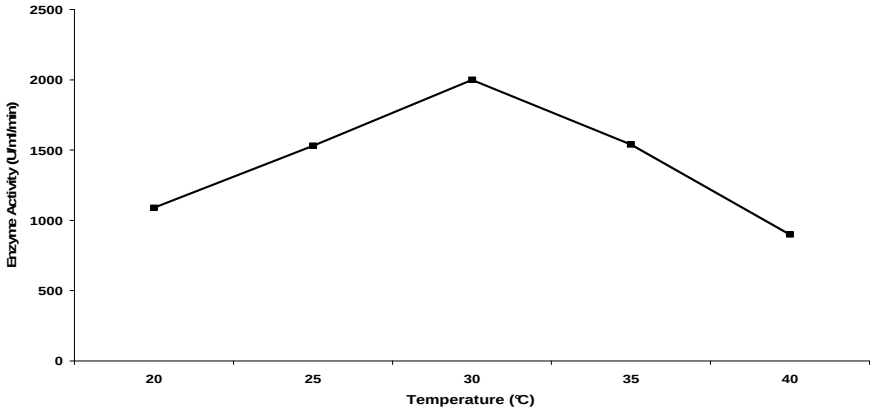

Figure 4: Effect of Temperature on α-Amylase Production

Effect of Temperature on α-Amylase Production

Temperature is the most important factors, which markedly influence enzyme activity. Production of enzyme was determined at different temperature ranges from 20-40°C (Figure4). Optimum temperature for maximum α-amylase activity was recorded at 30ºC. Further increase in temperature resulted in decrease in the activity of amylase. Many workers (Bajpai P., Bajpai P., 1989; Gupta R., 2003; Kundu A.K. *et al.*, 1973; Lin L.L. et al., 1998; Shah A. *et al.*, 2006 ; Ueno S. *et al.*, 1987) were recorded that 25-37°C temperature range showed optimum temperature for amylase production in mesophilic microorganism. Present study recorded 30°C as optimal temperature for α-amylase production with *Pseudomonas fluorescence* APK10, which agrees with earlier findings (Figure4).

Effect of pH on α-Amylase Production

Effect of pH on α-amylase production was recorded from pH range 5.0-9.0. It was noticed that *Pseudomonas fluorescence* APK10 showed good amount of enzyme activity from 6.0-8.0, while at pH 7.0 higher enzyme activity was recorded (Figure5).

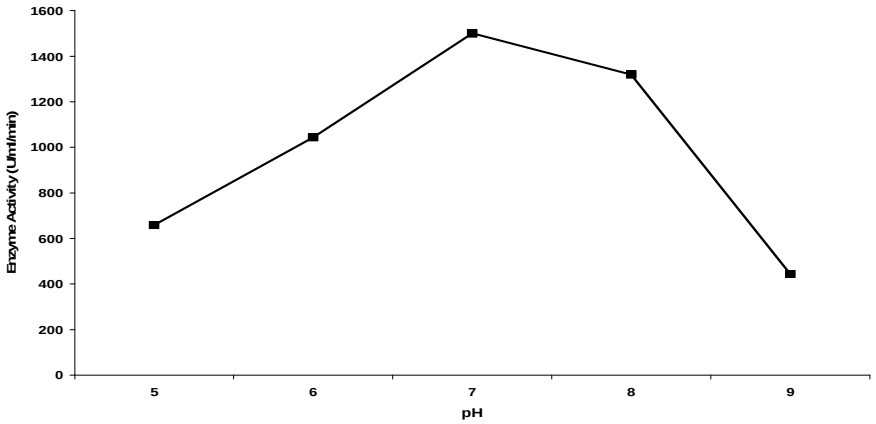

Figure 5: Effect of pH on α-Amylase Production

This suggests that the enzyme would be useful in processes that require wide range of pH change from slightly acidic to alkaline range and vice-versa. Bajpai and Bajpai (Bajpai P., Bajpai P. , 1989) was observed that at pH 3 to 11 *Bacillus licheniformis* TCRDC-B13 showed good growth, although bacterial growth start decreasing as the pH increases. The pH change observed during the bacterial growth affects product stability in the medium (Anto H. *et al*., 2006; Gupta R. *et al*., 2003). It has been observed (Anto H. *et al*., 2006; Castro P.M.L. *et al*., 1992; Gupta R. *et al*., 2003; Kathiresan K., Manivannan S., 2006; Kundu A.K. *et al*., 1973) that optimum pH for enzyme production and bacterial growth range between 6.0 and 7.0.

Effect of Pure Soluble Sugars on α-Amylase Production

The nature and amount of carbon source in culture media is an important factor for the growth and production of extracellular amylase in bacteria. To investigate the effects of various carbon sources on α-amylase production, *Pseudomonas fluorescence* APK10 strain was grown in different media containing starch, lactose, fructose, sucrose, glucose, maltose, mannitol, ribitol, raffinose, sorbitol, arabinose, glycerol, trehalose, and xylose as pure carbon sources. It was recorded that enzyme activity is higher in fructose, maltose and glycerol based medium

(Table 1) while in ribitol, raffinose, and sorbitol based medium enzyme activity was not recorded.

Table 1. Effect of Pure Soluble Sugars on α-Amylase Production

Pure Carbon Source	Enzyme Activity (U/ml/min)
Starch	90
Lactose	140
Fructose	390
Sucrose	190
Glucose	200
Maltose	380
Mannitol	120
Ribitol	10
Raffinose	0
Sorbitol	10
Arabinose	40
Glycerol	240
Trehalose	215
Xylose	200

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