International Scholars Journals

African Journal of Soil Science ISSN 2375-088X Vol. 7 (5), pp. 001-004, May, 2019. Available online at www.internationalscholarsjournals.org © International Scholars Journals



*Full Length Research Paper*

# -Amylase production by *Penicillium fellutanum* isolated from mangrove rhizosphere soil

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Accepted 04 March, 2019

**The effects of pH, temperature, incubation time, salinity, sources of carbon and nitrogen were tested in submerged fermentation process in production of -amylase by *Penicillium fellutanum* isolated from coastal mangrove soil. The production medium without addition of seawater and with provision of maltose as carbon source, peptone as nitrogen source, incubated for 96 h, maintained with pH of 6.5 at 30$^{O}$C, was found optimal for production of -amylase by *P. fellutanum.***

**Key words:** -amylase, *Penicillium fellutanum,* mangroves, rhizosphere soil, *Rhizophora.*

## INTRODUCTION

Starch-degrading amylolytic enzymes are of great significance in biotechnological applications ranging from food, fermentation, textile to paper industries (Lin et al., 1997; Pandey et al., 2000). The amylases can be derived from several sources such as plants, animals and microbes. The microbial amylases meet industrial demands; a large number of them are available commercially; and, they have almost completely replaced chemical hydrolysis of starch in starch processing industry (Pandey et al., 2000). The major advantage of using microorganisms for production of amylases is in economical bulk production capacity and microbes are also easy to manipulate to obtain enzymes of desired characteristics (Lonsane and Ramesh, 1990). -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 et al., 20002). Fungal sources are confined to terrestrial isolates, mostly to *Aspergillus* species and to only one species of *Penicillium*, *P. brunneum* (Haska and Ohta, 1994; Pandey et al., 2000).

Strains of *Penicillium* sp. were isolated from the coastal soil of a mangrove habitat. One isolate produced extracellular -amylase which was confirmed by percentage blue value and by identification of the products obtained by starch hydrolysis. Since this natural isolate produced low concentration of amylase, attempts were made to increase the productivity by optimizing the cultural conditions.

## MATERIALS AND METHODS

### Microorganism

The fungus, *Penicillium fellutanum* Biourge., was isolated from rhizosphere soil of a mangrove species, *Rhizophora annamalayana* Kathir., by plating method using Sabouraud Glucose Agar with an antibiotic (Chloromphenicol 0.1 g $l^{-1}$) (Boukhout and Robert, 2003).

### Chemicals

All analytical reagents and media components were purchased from Hi-Media (Mumbai, India) and Sigma chemicals (St. Louis, USA).

### Growth media

For isolation of *Penicillium* sp., Sabouraud Glucose Agar medium containing glucose 20 g, peptone 10 g, agar 20 g, aged seawater 500 ml and distilled water 500 ml was used.

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**Table 1.** Effect of pH on amylase production.

| pH | Amylase activity* (U/ml) |
|---|---|
| 5.0 | 70±2.8 |
| 5.5 | 77±1.7 |
| 6.0 | 88±5.7 |
| 6.5 | 94±2.8 |
| 7.0 | 92±1.1 |
| 7.5 | 80±2.3 |

*One unit of amylase activity was defined as the amount of enzyme which released 1 µmole glucose under the assay conditions. Values are mean±S.E.

**Table 2.** Effect of temperature on amylase production.

| Temperature (°C) | Amylase activity* (U/ml) |
|---|---|
| 20 | 78±1.7 |
| 30 | 98±4.6 |
| 40 | 76±3.4 |

*One unit of amylase activity was defined as the amount of enzyme which released 1 µmole glucose under the assay conditions. Values are mean±S.E.

**Table 3.** Effect of incubation period on amylase production.

| Incubation period | Amylase activity* (U/ml) |
|---|---|
| 24 | 68±2.3 |
| 48 | 80±2.8 |
| 72 | 94±3.4 |
| 96 | 136±1.1 |
| 120 | 120±2.8 |

*One unit of amylase activity was defined as the amount of enzyme which released 1 µmole glucose under the assay conditions. Values are mean±S.E.

**Table 4.** Effect of various carbon sources at 1% on amylase production.

| Carbon sources | Amylase activity* (U/ml) |
|---|---|
| Glucose | 139±5.19 |
| Galactose | 138±1.73 |
| Lactose | 138±2.3 |
| Maltose | 146±2.6 |
| Sucrose | 136±3.4 |
| Xylose | 140±4.61 |

*One unit of amylase activity was defined as the amount of enzyme which released 1 µmole glucose under the assay conditions. Values are mean±S.E.

**Confirmation of amylase production**

Amylase production by *Penicillium* spp. was confirmed on starch agar plates containing peptone 5 g, yeast extract 1.5 g, soluble starch 2.0 g, NaCl 5.0 g, agar 15 g, aged seawater 500 ml and distilled water 500 ml (Capuccino and Sherman, 2001). Culture was maintained for 72 h at 30°C and pH 6.5. Amylase production was detected after flooding the plates with iodine solution (Hols et al., 1994).

**Production medium**

The composition of production medium used was soluble starch 50 g, yeast extract 0.5 g, $KH_2PO_4$ 10 g, $(NH_4)_2SO_4$ 10.5 g, $MgSO_4$ 0.3 g, $CaCl_2$ 0.5 g, $FeSO_4$ 0.013 g, $MnSO_4$ 0.004 g, $ZnSO_4$ 0.004 g, CoCl 0.0067 g, aged seawater, 500 ml, and distilled water 500 ml. The pH was adjusted to 6.5 and the media were sterilized in an autoclave for 15 min at 121°C. The media were inoculated with a loop-full of spore suspension *of P. fellutanum* and then incubated at 30°C in an orbital shaker set at 100 rpm for 72 h. The media were centrifuged at 5,000 g for 15 min to obtain crude enzyme solution.

**Enzyme assay**

Amylase assay was made by using a reaction mixture (4 ml) consisted of 1 ml of enzyme solution and 2 ml of soluble starch in phosphate buffer, pH 6.5 (Wood and Bhat, 1988). The mixture was incubated for 10 min at 30°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 µ mole glucose).

**Optimization of culture conditions**

The factors such as temperature, pH, salinity, sources of carbon and nitrogen affecting production of amylase were optimized by varying parameters one at a time. The experiments were conducted in 200 ml Erlenmeyer flask containing production medium. After sterilization by autoclaving, the flasks were cooled and inoculated with culture and maintained under various operational conditions separately such as pH (5.0, 5.5, 6.0, 6.5, 7.0, 7.5), temperature (20, 30, 40°C), incubation period (24, 48, 72, 96, 120 h), carbon source ( glucose, galactose, maltose, lactose, sucrose and xylose each at 1%), nitrogen source (peptone, beef extract, yeast extract, meat extract and casein each at 0.5%) and salinity (0, 40, 50, 60, 70, 80, 90, 100% sea water). After 72 h (expect for incubation period effect), the culture filtrate was assayed in triplicate for amylase activity.

## RESULTS

The 72 h culture of *P. fellutanum* exhibited amylase activity of 94 U/ml, at pH 6.5 and 30°C. The activity was about 31% higher at pH 6.5 than pH 5.0; and 30% higher at 30°C than 40°C (Tables 1 and 2). When the culture was incubated at 96 h, the maximum activity detected was 136 U/ ml. There was a 2 fold increase in activity at 96 h incubation as compared to 24 h (Table 3).

Among carbon sources, maltose was the best to enhance the enzyme activity of 146 U/ml which was 7% higher than sucrose (Table 4) . Among nitrogen sources, peptone was ideal to increase the enzyme activity of 150 U /ml, which was about 9% higher than yeast, meat and casein (Table 5) .The activity, was about 10 fold high in absence of seawater, as compared to 100% seawater (Table 6).

**Table 5.** Effect of various nitrogen sources at 0.5% on amylase production.

| Nitrogen sources (0.5%) | Amylase activity* (U/ml) |
|---|---|
| Yeast extract | 138±2.3 |
| Meat extract | 140±4.61 |
| Beef extract | 138±2.4 |
| Peptone | 150±2.8 |
| Casein | 138±2.3 |

*One unit of amylase activity was defined as the amount of enzyme which released 1 µmole glucose under the assay conditions. Values are mean±S.E.

**Table 6.** Effect of salinity on amylase production.

| Salinity (% of sea water) | Amylase activity*(U/ml) |
|---|---|
| 0 | 184±2.3 |
| 40 | 157±4.04 |
| 50 | 150±1.1 |
| 60 | 130±2.8 |
| 70 | 104±2.3 |
| 80 | 85±2.3 |
| 90 | 45±4.1 |
| 100 | 18±4.01 |

*One unit of amylase activity was defined as the amount of enzyme which released 1 µmole glucose under the assay conditions. Values are mean±S.E.

## DISCUSSION

The media optimization is an important aspect to be considered in the development of fermentation technology. However, there are only a few reports concerning the optimization of media composition especially for fungal strains in amylase production (Quang et al., 2000).

Among physical parameters, pH of the growth medium plays an important role by inducing morphological changes in microbes and in enzyme secretion. The pH change observed during the growth of microbes also affects product stability in the medium (Rani Gupta et al., 2003). Most of the earlier studies revealed the optimum pH range between 6.0 and 7.0 for the growth of bacterial strains and enzyme production (Rani Gupta et al., 2003; Kundu et al., 1973; Castro et al., 1992). This is also true of strain of *P. fellutanum* used in the present study. However, *Aspergillus oryzae* released amylase only in alkaline pH above 7.2 (Yabuki et al., 1977).

Temperature optimum for amylase was found to be in a range between 25 and 37°C for the mesophilic fungi (Kundu et al., 1973; Ueno et al., 1987; Rani Gupta et al., 2003) and the present study recorded 30°C as optimal, which agrees with earlier findings. The influence of temperature on amylase production is related to the growth of microbes.

The incubation period varies with enzyme productions (Smitt et al., 1996). Short incubation period offers potential for inexpensive production of enzymes (Sonjoy et al., 1995). In the present study the amylase activity increased steadily and reached maximum at 96 h of incubation (Table 3), as against a short duration of 24 h in the case of bacteria (Dharani Aiyer, 2004).

-Amylase is an inducible enzyme and is generally induced in the presence of carbon sources such as starch, its hydrolytic product, or maltose (Yabuki et al., 1977; Tonomura et al., 1961; Lachmund et al., 1993; Morkeberg et al., 1995). Glucose has been known to induce only a minimal level of amylase by *A. oryzae* (Ars and Baile, l977). Still the role of glucose in production of amylase is controversial. Xylose has been reported to strongly repress amylase production, although the carbon source supports good growth in *A. nidulans* (Arst and Baile, 1977). Contrary to this, xylose induced amylase production (140 U/ml) by *P. fellutanum* (Table 4). Most reports available on the induction of -amylase in different strains of fungal species suggest that the general inducer molecule is maltose which increases many fold enzyme activity (Eratt et al., 1984). However, there is no statistically significant variation among the carbon sources used in our study (Table 4).

Organic nitrogen sources are preferred for the production of -amylase. A maximum -amylase production was supported by yeast extract, peptone or beef extract (Hamilton et al., 1999; Emanuilova and Toda, 1984; Krishnan and Chandra, 1982; Hayashida et al., 1988). Although, peptone appears to be ideal source, there is no statistically significant variation among the nitrogen sources used in our study (Table 5).

Even though the fungal strain was isolated from coastal soil, it produced low concentration of amylase when production medium prepared with 100% sea water (Table 6). Hence, it can be a terrestrial species; facultatively halophilic in nature. The nature of culture conditions and composition of media for optimal production of -amylase by *P. fellutanum* has been developed in this study.

## ACKNOWLEDGEMENTS

The authors are thankful to the authorities of Annamalai University and the Ministry of Environment and Forests, Govt. of India, New Delhi for financial support.

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