Int J Pharm Bio Sci 2013 Jan; 4(1): (B) 575 - 581

Research Article **Microbiology**

International Journal of Pharma and Bio Sciences

ISSN 0975-6299

ISOLATION AND SCREENING OF AMYLOLYTIC *PENICILLIUM* SPECIES FROM SOIL

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ABSTRACT

Amylase is important industrial enzymes having a share of about 25-33% of the total global enzyme market. Amylases are commercially produced from bacteria and filamentous fungi especially from *Bacillus* and *Aspergillus*. Several *Penicillium* species are also known to produce amylases, certain species of which are also recognized as commercial producers. This study was aimed to isolate *Penicillium* species from different soil samples of Assam, India and to screen for the ability to produce amylase. A total of 25 *Penicillium* species were isolated and examined for amylase production ability. Out of the 25 isolates 11 isolates i.e., isolate nos. 1-11 showed good amylase activity in terms of clear zone formation. 7 isolates showed little activity and the rest 7 isolates had no activity. The 11 isolates found to be good in solid starch agar media were also screened in liquid media for amylase producing ability and observed that all the 11 isolates have the ample capacity to produce amylase. This study may generate interest in searching of new indigenous microbial sources for the production of various secondary metabolites like enzymes.

KEY WORDS: Amylase, *Penicillium*, screening, enzyme activity, amylolytic, solid state fermentation.

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INTRODUCTION

During last a few decades, the attention for the exploitation extracellular enzymatic activity among large numbers of microorganisms has been stirred due to great potentiality of microorganisms as the source of industrially important enzymes[2]. The impending use of microorganisms as source of industrially important enzymes is ever increasing and has been accelerating the rate of exploitation of new efficient microorganisms for the production of extracellular enzymes[12]. Amylases are the most important amylolytic enzymes which hydrolyse 1-4 linkages of starch. Sugar, textile, alcohol, detergent, paper and food processing industries such as baking, brewing, preparation of digestive aids, production of cakes, fruit juices, starch syrups etc. are the major industries where extensive use of amylases have been reported [9,16,7,1]. However, due to rapid progress in the field of microbial biotechnology, the applications of amylases have also been extended in many new areas such as clinical, medical and analytical chemistry [6,7]. It is estimated that about 25-33% of the world's enzyme market is occupied by amylases [14,19]. The present projected value of world market is about US$ 2.7 billion and is estimated to increase by 4% annually through 2012. The key industries which utilize about 75% of industrially produced enzymes are Detergents (37%), textiles (12%), starch (11%), baking (8%) and animal feed (6%) [6]. Amylases are found universally in plant, animal and microbial kingdoms. However, microbial sources, especially thermophilic bacteria and mesophilic molds provide industrial need of amylases [7,16]. There are a number of advantages of microbial production of amylases over the other sources specially due to commercial bulk production capacity and easy to manipulate to obtain enzyme of preferred characteristics [7,9]. Although a numbers of species are reported to produce amylase, only a few species of *Bacillus* and *Aspergillus* and their improved strains have dominated application for the production of α-amylases. Moreover, several *Penicillium* species are also studied by various workers for amylase producing ability and it has been found that species like *P. chrysogenum, P. expansum, P. citrinum* ect., have great potentiality as commercial producer of amylases. Due to the widely acknowledged GRAS (generally recognized as safe) status, amylases from fungi are more preferable [9]. Besides, filamentous fungi are found to be more promising producers of various extracellular secondary metabolites including enzymes in solid state fermentation employing agro-industrial wastes which is now regarded as the best fermentation technology for enzyme production [5,16,7]. Therefore, the current assessment dealt with the isolation and screening of local amylolytic *Penicillium* species from different soil habitats for future investigation in industrial processes.

MATERIALS AND METHODS

Isolation of Penicillium species

Soil samples from different habitats such as forest soil, oil degraded land etc. from various places of Assam such as Guwahati, Sivsagar and Lakhimpur were collected randomly. Serial dilution plate method on Czapek dox agar and malt extract agar media was used to isolate *Penicillium* species. The isolated samples were further purified and maintained on agar slants at 4^0C.

Screening for amylase activity of Penicillium isolates

This was done on both solid and liquid culture media.

I. ***On solid medium :*** For screening of amylase activity, the isolates were grown on the medium containing Peptone 5g, Yeast extract 1.5g, starch 2g, NaCl 5g, Agar 15g per liter. The pH was adjusted at 6.5. The medium was sterilized by autoclaving at 121^0C for 15 minutes. The cultures were inoculated on the

plated medium and incubated for 96 hours at 30^0C. The amylase activity was indicated by a clear zone formation on a dark blue background after exposure to Gram's Iodine solution. The diameter of the clear zones and *Penicillium* colonies were measured.

II. ***On liquid medium :*** Amylase activity of the isolates were screened in liquid medium containing KH_2PO_4 1.5g, $MgSO_4.7H_2O$ 0.5g, $FeSO_4$ 0.01g, Yeast extract 3g, Peptone 5g, Starch 20g per liter. The pH was adjusted at 6.5 and sterilized by autoclaving at 121^0C for 15 minutes. 7 days old culture was used as inoculum. 5ml sterile water was added to the slant and spores were dislodged by an inoculating loop. The spore suspension was vortexed and 1ml of it was transferred to 50ml of the media in a 250ml conical flask. The cultures were incubated for 72 hours at 30^0C in static conditions. The amylase assay was done by starch-iodine method.

III. ***Measurement of amylase activity :*** The culture was filtered through whatmann No. 1 filter paper and the filtrate was used to measure the amylase activity. The procedure was as follows-

i. 1ml of 1% starch solution was taken in a test tube and put in a water bath at 40^0C for 5 minutes.

ii. Then 0.5 ml of the culture filtrate as crude enzyme source was added to the starch solution taken and incubated at 40^0C for 10 minutes.

iii. After 10 minutes of incubation 1 ml 1N HCl was added to stop the reaction.

iv. To this 9.4 ml of distilled water was added.

v. After this 0.1 ml of Gram's Iodine solution was added and shaked.

vi. A control tube was taken without crude enzyme filtrate.

vii. The absorbance was measured by a colorimeter (Elico CL 157) at 610 nm.

viii. The same procedure was also performed at 50^0C.

The amylase activity was calculated by the formula given by Yoo *et al*. (1987) - Activity (unit/ml) = D[(Ro-R)/Ro] x 100 Where, Ro is the absorbance of the substrate-iodine complex in the absence of the enzyme. R is the absorbance of the digest. D is the dilution factor of the enzyme (the enzyme solution can be diluted when necessary so that the ratio Ro-R/Ro was between 0.2 and 0.7). (One unit of enzyme activity was defined as the amount of enzyme required to release 1 µmol of reducing sugar per min from soluble starch under assay conditions)

RESULT AND DISCUSSION

In the study 25 *Penicillium* isolates were isolated from different soil samples collected from various habitats such as forest soil, oil degraded land etc. of Assam which includes Guwahati, Sivsagar and Lakhimpur (Table-1). The isolates were identified by taking references from J.C. Gillman (1975) and Pitt (1979).

Table 1
Screening of Penicillium isolates for amylolytic activity.

Sl. No.	*Penicillium* isolates	Diameter of clearing zone, DCZ (mm)	Diameter of fungal colony, DFC on starch agar (mm)	Hydrolysis activity index = DCZ/DFC
1	*Penicillium* S1	6.8	7.0	0.97
2	*Penicillium* S2	7.5	7.3	1.02
3	*Penicillium* S3	6.9	7.2	0.95
4	*Penicillium* S4	7.1	7.3	0.97
5	*Penicillium* S5	6.9	7.5	0.92
6	*Penicillium* S6	8.0	7.8	1.02
7	*Penicillium* S7	7.0	7.5	0.93
8	*Penicillium* S8	7.5	7.5	1.00
9	*Penicillium* S9	7.3	7.4	0.98
10	*Penicillium* S10	7.0	7.6	0.92
11	*Penicillium* S11	7.2	7.2	1.00
12	*Penicillium* S12	3.5	6.8	0.51
13	*Penicillium* S13	3.2	7.0	0.45
14	*Penicillium* S14	3.3	6.6	0.50
15	*Penicillium* S15	4.0	7.1	0.56
16	*Penicillium* S16	3.7	7.0	0.52
17	*Penicillium* S17	2.9	6.8	0.42
18	*Penicillium* S18	3.3	7.2	0.45
19	*Penicillium* S19	0.0	5.6	0.00
20	*Penicillium* S20	0.0	7.5	0.00
21	*Penicillium* S21	0.0	6.8	0.00
22	*Penicillium* S22	0.0	6.4	0.00
23	*Penicillium* S23	0.0	5.5	0.00
24	*Penicillium* S24	0.0	6.0	0.00
25	*Penicillium* S25	0.0	5.8	0.00

These *Penicillium* isolates were screened for their ability to produce amylase. Screening was carried out on both solid and liquid media. At first the screening was done by incubating the cultures on starch agar plates. Amylase activity was determined after flooding the plates with Gram's Iodine solution. The ability starch degrading activities of *Penicillium* isolates were assessed in terms of diameter of clear zone (DCZ) *I* diameter of fungi colony (DFC) ratios [12]. Table-1 shows the DCZ*/*DFC ratios of all the *Penicillium* isolates. Out of the 25 isolates 11 isolates i.e., isolate nos. 1-11 showed good amylase activity in terms of clear zone formation and hydrolysis activity index. 7 isolates (isolate nos. 12-18) showed little activity and rest 7 isolates (isolate nos. 18-25) had no activity. The 11 isolates i.e., isolate nos.

1-11 showed DCZ/DFC ratio between 0.9 - 1.2 (Table-1). However, clear zone formation due to amylase action cannot be correlated quantitatively with the amount of enzyme produced. Therefore, the selection of amylase producers using starch plates can only be made partially. More efficient amylolytic strains can be selected on biochemical basis [12].

Table 2
Amylase activity of eleven *Penicillium* isolates after screening on starch agar plates.

Sl. No.	*Penicillium* Isolates	Amylase activity (units/ml)	
		40°C	50°C
1	*Penicillium* S1	24.21	32.08
2	*Penicillium* S2	21.24	26.33
3	*Penicillium* S3	22.38	30.20
4	*Penicillium* S4	20.76	23.70
5	*Penicillium* S5	25.56	31.25
6	*Penicillium* S6	26.32	35.42
7	*Penicillium* S7	18.78	20.70
8	*Penicillium* S8	16.21	25.18
9	*Penicillium* S9	18.70	25.10
10	*Penicillium* S10	17.81	23.57
11	*Penicillium* S11	21.80	29.10

The 11 isolates of *Penicillium* showing better amylase activity in terms of clear zone formation were further evaluated for the ability to produce amylase enzyme in liquid media. Table-2 depicts the enzyme activity of the 11 isolates of *Penicillium* species. The observations showed that all the 11 isolates were able to produce amylase in liquid cultures containing starch. *Penicillium* S6 exhibited highest enzyme activity of 26.32 and 35.42 units/ml both at 40^0C and 50^0C respectively. On the other hand, the lowest enzyme activity was shown by *Penicillium* S8 at 40^0C and *Penicillium* S7 at 50^0C respectively. It was also seen that the enzyme activity of all the isolates was higher at 50^0C than 40^0C.This is a very good sign since industrial applications require high temperature activity of amylases. It is enumerated from the spectrum of *Penicillium* isolates employed for amylase production in liquid culture media that all the 11 isolates found better in solid medium in plating method possessed high potential for amylase production in liquid medium. These preliminary outcomes are supported by the findings of various workers like Sindhu *et al*., 2009 ; Balkan and Ertan, 2006 ; Kathiresan and Manivannan, 2006 ; Ikram-ul-Haq *et al*., 2002 ; Sohail *et al*., 2005 ; Bakri *et al*., 2009 ; Tripathy *et al*., 2011 etc**.** Further studies and optimization of various cultural conditions for amylase production will be required to find out the most potent amylolytic isolate of *Penicillium* examined. Moreover, characterizations of the enzyme produced will determine the applicability of such amylases. Though several bacterial and fungal species of *Bacillus* and *Aspergillus* are serving our commercial need of amylases, still newer sources of amylase producing microorganisms are in search to get enzyme with novel properties to compensate the growing demand for this enzyme.

CONCLUSION

Amylases are one of the most important enzymes having highest range of applications in various industries which alone share more than 30% of the enzyme market with an increasing demand day by day. Thus the

search for new and novel amylase producing microorganism is very much relevant in the present context. The *Penicillium* isolates showing capability to produce amylase in preliminary study may turn out to be hyper amylase producing commercial strain during further studies. Employment of Solid State Fermentation technique for amylase production also holds high potentiality to use various agro-industrial as well as organic wastes which can contribute to the management of solid wastes in addition. There is immense probability to find out hyper amylase producing microorganism from North Eastern region of India, since no such extensive investigations are done from this region. This probability lies in the fact that this region is included in one of the three biodiversity hot spots in India.

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