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Enzyme Production by *Aspergillus niger* Isolated from Marine Soil

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The effects of pH, temperature and salinity were tested for enzyme (amylase, protease, cellulase, lipase, laccase and xylanase) production by *Aspergillus niger* isolated from marine sediments. The production medium maintained with pH 7 to 8, at 30°C and salinity 20 ppt found optimal for enzyme production by *A. niger*.

Key words: Marine sediments, *Aspergillus niger*.

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[1-3].

The important development in industrial fermentation lead to the utilization of microbial enzymes in different applications, especially hydrolytic enzymes. The marine biosphere is one of the richest of the earth's innumerable habitats, yet one of the least studied and characterized for microbial flora. Currently, marine microorganisms are considered as untapped sources of metabolites and products, which may possess important properties. They have a diverse range of enzymatic activity and capable of catalyzing various biochemical reactions with novel enzymes. Thus, there is enormous scope for exploring the probabilities of deriving new products of economic importance from the potential marine microorganisms.

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Species of fungi produce a large variety of secondary metabolites which are of considerable interest to the pharmaceutical industry. It is clear that the secondary metabolite production of a species varies significantly with strains isolated from different geographic regions and from different habitats. The influence of genotype and environment on the metabolite production is, however, poorly understood. In the present investigation, the influences of genotypic variability and physiological variability of fungi and environmental parameters of land habitat on the metabolite production were studied.

MATERIAL AND METHODS

Microorganism

The fungi were isolated from 4 stations of Point Calimere by plating method. Totally 62 organisms (Table.1) were isolated from all the four stations. Among them the frequently occurred organisms *Aspergillus niger* was selected for enzyme production.

Screening of fungi for extracellular enzyme production

The method described by Hankin and Anagnostakis[4] and Rohrman and Molitoris[5] were used to detect the production of extracellular enzymes.

Production media employed for the enzymes

The above mentioned fungus was inoculated into enzyme production media such as starch broth (peptone 5 g, beef extract 3 g, soluble starch 3 g) for amylase (alpha amylase) and modified Reese's medium (glucose 0.25 g, casein 0.5 g, yeast extract 0.05 g, K_2HPO_4 1.0 g, $MgSO_4$ – 0.20 g) for protease (alkaline protease).

Modified Czapek's cellulose medium (cellulose – 10.0 g, KNO_3 – 3 g, K_2HPO_4 – 1.0 g, $MgSO_4$ – 0.5 g, $FeSO_4$ – 0.01 g) for cellulase, production medium with coconut oil (tryptone – 1%, yeast extract – 0.5%, NaCl – 0.5% supplemented with $CaCl_2.2H_2O$ – 0.01%, Tween 80-1%) for lipase, Akiba and Horikoshi medium (xylan – 5 g, peptone – 5 g, yeast extract – 1 g, NaCl – 5 g, K_2HPO_4 – 1 g, $MgSO_4$ – 0.2 g, $CaCl_2$ – 0.1 g, Na_2CO_3 – 10 g) for xylanase and liquefied basal medium (1.0 mM ammonium tartarate, 1.0 g of KH_2PO_4, 1.0 g of K_2HPO_4, 1.4 g of $(NH_4)_2SO_4$, 0.5 g of $MgSO_4.7H_2O$, 0.1 g $CaCl_2/2H_2O$, 0.5 ml of vitamin B complex, and 7.0 ml of trace element solution. It also contained 0.2 g of Tween 80) for laccase activity.

Optimization of culture conditions

The factors such as pH, temperature and salinity affecting production of enzyme 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, 6, 7, 8, 9), temperature (10, 20, 30, 40, 50°C) and salinity (0, 10, 20, 30, 40 ppt). The culture filtrate was assayed in triplicate for enzyme activity.

Enzyme assay: Alpha amylase[6]

One ml of starch solution was added with 1 ml of diluted enzyme solution and incubated at 27°C for 15 minutes. 2 ml of dinitrosalicylic (DNS) reagent was added and kept in boiling water bath for 5 minutes. 1 ml of potassium sodium tartrate was added and allowed to cool down in running water. This solution was made upto 10 ml with sterile distilled water and absorbance was read at 560 nm.

Alkaline protease[7]

0.2 ml of enzyme sample was taken and made upto 1 ml with distilled H_2O. 3 ml of 0.4 M carbonate – bicarbonate buffer (pH 9) and 1 ml of casein was added. The mixture was incubated for 30 minutes. 2 ml of TCA was added and again incubated at 37°C for 30 minutes. The mixture was filtered through Whatman No.1 filter paper and the clean filtrate was collected. To 0.1 ml of the filtrate 0.5 ml of 0.4 N sodium carbonate and 1 ml of Folin-Phenol were added. The solution was mixed well and incubated at 37°C for 20 minutes. Then, absorbance was read at 660 nm.

Cellulase[8]

0.5 ml of the enzyme extract was added to the test tube containing 0.45 ml of 1% carboxy methyl cellulose and incubated at 55°C for 15 minutes. 0.5 ml of dinitrosalicylic acid reagent (DNS) was added and kept in a water bath for 5 minutes. 0.1 ml of potassium sodium tartrate was added and made up to 5 ml with distilled water. The optical density was measured at 540 nm.

Lipase[9]

250 mg of olive oil was taken into a test tube and added with 2 ml of phosphate buffer (pH 6.3). 1 ml of enzyme sample was added and vortexed for 15 seconds. The mixture was incubated at 37°C in a water bath under static conditions for 30 minutes. 1 ml of concentrated HCl was added and vortexed for 10 minutes. Then, 3 ml of benzene was added and vortexed for 90 seconds. From this 2 ml of benzene layer was taken and added to 1 ml aqueous solution of 5 per cent cupric acetate (pH 6.2). Then it was vortexed for 90 seconds and centrifuged at 5000 rpm for 10 minutes. Clear organic phase of benzene layer was removed and used for the estimation of liberated fatty acids by measuring the optical density at 715 nm.

Xylanase[10]

Enzyme solution (0.5 ml) was added to 2 per cent xylan suspension

(0.5 ml) in 0.1 M acetate buffer, pH 6.0, and the mixtures were incubated at 55°C for 30 minutes. After incubation the mixtures were cooled rapidly in ice water and the insoluble xylan was removed by centrifugation. To the resulting supernatant (0.5 ml), 1 ml of 3, 5-dinitrosalicylate (0.5%) solution was added, and the mixture was kept in boiling water. Colour development was measured using the spectrophotometer at 535 nm. The enzyme activity was expressed as m mol of xylose released per ml/min.

Table 1. Extracellular enzyme activity of soil fungi

S. No.	Fungal species	Enzymatic activity					
		Amylolytic activity	Proteolytic activity	Cellulolytic activity	Lipolytic activity	Xylanase activity	Laccase activity
1.	*Absidia* sp.	+	+	-	+	+	-
2.	Blakeslea sp.	+	-	-	-	-	-
3.	*Rhizopus nigricans*	+	+	-	+	+	-
4.	*Rhizopus* sp.	+	+	-	-	-	-
5.	*Syncephalastrum racemosum*	+	+	-	+	-	-
6.	*Syncephalastrum* sp.	+	-	+	-	+	+
7.	*Chaetomium* sp.	+	-	+	+	-	+
8.	*Emericellopsis* sp.	+	+	+	-	-	+
9.	*Eurotium* sp.	-	+	-	+	-	-
10.	*Thielavia terricola*	+	+	+	+	+	+
11.	*Thielavia* sp.	+	+	-	-	+	-
12.	*Alternaria alternata*	+	+	-	-	-	-
13.	*Alternaria* sp.	+	+	-	-	+	-
14.	*Aspergillus candidus*	-	-	+	-	-	-
15.	*A. carbonareus*	+	+	-	+	-	-
16.	*A. chevalieri*	+	+	-	-	-	-
17.	*A. clavatus*	+	+	+	+	-	+
18.	*A. flaviceps*	-	+	-	-	-	-
19.	*A. flavus*	+	+	+	+	+	+
20.	*A. fumingatus*	+	+	+	+	+	+
21.	*A. granuloses*	-	-	-	-	-	-
22.	*A. humicola*	+	+	-	+	-	+
23.	*A. koningi*	+	-	-	-	+	+
24.	*A. luchensis*	+	+	+	+	-	-
25.	*A. nidulans*	-	-	-	-	+	-
26.	*A. niger*	+	+	+	+	+	+
27.	*A. ochraceous*	+	+	-	+	-	-
28.	*A. oryzae*	+	-	+	-	+	+
29.	*A. sulphureus*	+	-	+	-	-	+
30.	*A. sydowi*	+	+	+	-	-	-
31.	*A. terreus*	+	+	+	+	+	+
32.	*A. terricola*	+	-	+	-	-	-
33.	*A. wentii*	-	-	-	-	-	-
34.	*Bipolaris* sp.	+	+	-	-	-	-
35.	*Cephalosporium* sp.	-	+	-	-	-	-
36.	*Cladosporium diaphanum*	+	+	+	+	-	-
37.	*Cladosporium* sp.	+	+	-	-	-	-
38.	*Curvularia lunata*	+	+	+	+	+	+
39.	*C. indica*	+	+	-	-	+	-
40.	*C. pallescens*	+	+	+	-	-	-
41.	*Curvularia* sp.	-	-	-	-	-	-
42.	*Drechslera indica*	+	+	-	+	-	+
43.	*Drechslera* sp.	+	+	+	-	+	+
44.	*Fusarium moniliforme*	+	-	-	-	-	-
45.	*F. oxysporum*	+	+	+	+	+	+
46.	*F. semitectum*	+	-	+	+	-	-
47.	*Fusarium* sp.	+	+	+	+	+	
48.	*Gliocladium* sp.	-	+	-	+	-	+
49.	*Humicola* sp.	+	+	-	-	+	+
50.	*Nigrospora* sp.	-	-	-	-	-	-
51.	*Penicillium chrysogenum*	+	+	+	+	+	+
52.	*P. citrinum*	+	+	+	+	+	+
53.	*P. funiculosum*	+	+	-	-	-	-
54.	*P. janthinellum*	-	+	+	-	-	-
55.	*Penicillium* sp.	+	+	+	-	-	-
56.	*Trichoderma harzianum*	+	+	+	+	+	+
57.	*T. koningi*	-	+	-	-	-	-
58.	*T. viride*	+	+	+	-	-	-
59.	*Trichoderma* sp.	+	+	-	-	+	-
60.	*Verticillium tenerum*	+	+	+	+	+	+
61.	Brown sterile mycelium	+	-	-	+	-	+
62.	White sterile myceliumss	+	+	-	-	-	-
		50	46	29	27	24	24

+ Presence of enzyme activity, - Absence of enzyme activity

Table 2. Effect of pH on extracellular enzyme activity

pH	Amylase (IU/ml)	Protease (IU/ml)	Cellulase (IU/ml)	Lipase (IU/ml)	Laccase (IU/ml)	Xylanase (IU/ml)
5	1.53	0.48	0.20	0.24	0.024	0.024
6	1.86	0.62	0.34	0.31	0.041	0.031
7	2.50	1.91	0.43	0.49	0.043	0.049
8	4.51	2.46	0.26	0.28	0.032	0.046
9	3.21	1.88	0.18	0.19	0.019	0.024

Table 3. Effect of temperature (°C) on extracellular enzyme activity

Temperature (°C)	Amylase (IU/ml)	Protease (IU/ml)	Cellulase (IU/ml)	Lipase (IU/ml)	Laccase (IU/ml)	Xylanase (IU/ml)
10	1.39	1.92	0.98	0.08	0.007	0.007
20	2.08	2.63	1.25	0.41	0.017	0.033
30	3.45	3.12	1.65	0.64	0.037	0.041
40	3.01	2.01	1.15	0.17	0.031	0.031
50	2.62	1.26	0.87	0.75	0.009	0.021

Table 4. Effect of salinity on extracellular enzyme activity

Salinity (ppt)	Amylase (IU/ml)	Protease (IU/ml)	Cellulase (IU/ml)	Lipase (IU/ml)	Laccase (IU/ml)	Xylanase (IU/ml)
0	1.621	1.92	0.38	0.18	0.018	0.021
10	2.052	2.62	0.464	0.23	0.022	0.024
20	3.961	3.67	0.91	0.35	0.029	0.028
30	2.641	2.80	0.63	0.20	0.014	0.019
40	1.821	1.42	0.31	0.12	0.009	0.006

Laccase[11]

Laccase activity was assessed in 1.0 ml of reaction mixture containing catechol as substrate in 50 mM sodium phosphate buffer (pH 5.0). 0.2 ml of enzyme extract was added to it. The progress of the reaction was monitored at 440 nm for 10 minutes.

RESULTS AND DISCUSSION

Mangrove environment is organically very rich due to the presence of high amount of dissolved and particulate organic matter which offer diversity of niches and microorganisms. Microorganisms from these areas play an important role in the biodegradation of the enormous amount of dead plant material through their extracellular enzyme producing ability[12]. It is well known that the microorganisms possess remarkable adaptive capacity and may develop resistance and or degradative ability to any given organic compounds. Similarly the mangrove sediments are known to harbour heterogeneous groups of microflora which potentially excrete degradative enzymes, such as cellulases, amylases, pectinases[13] and phenol oxidase[14] etc.

Rai and Chowdhery[15] found that the fungal species isolated from the mangroves had a higher rate of cellulolytic activity when compared to their normal soil counterparts, and suggested that the unique prolonged impact of mangrove environment might have resulted in the higher amount of cellulolytic enzyme production. There is direct correlation between the cellulase activity and the degrading capability of fungi[16-18]. In the present investigation, among the 62 species of fungi screened for their enzyme producing ability 50, 46, 29, 27, 24 and 24 and 22 species showed amylolytic; proteolytic, cellulolytic, lipolytic, xylanolytic and laccase activity respectively.

Extracellular amylases, lipases, proteases, cellulases, xylanases, laccase, polygalacturanase, pectate lyase, lignin peroxidase, manganese peroxidase, urease and chitinase were assayed by different *Phaeoacremonium* spp. and *Phaemoniella chlamydospora* isolates[19]. In the present study, the dominant species such as *Aspergillus niger* isolated from Point Calimere showed significantly high enzymatic activity.

The enzymatic activity of the fungi varied in relation to pH, salinity, temperature and nutrients was studied in the laboratory in relation to pH, salinity, temperature and nutrients by several workers[15,20-24].

Effect of pH

Among the physical parameters, pH of the medium plays an important role on production of microbial enzymes. Garg[25], Chaery *et al.*[26] and Alva *et al.,*[27] studied the influence of enzyme such as cellulase and gluoamylase. pH on the production of extracellular, the maximum production of enzyme was achieved at pH 7. The present study revealed that the pH 7-8 was more favourable than other pH levels for the production of amylase protease, cellulase, lipase, xylanase and laccase.

The enzyme producing ability of the test organisms varied in relation to pH of the medium. Among the 5 different pH tested, the maximum enzyme activity was observed at pH 8 in amylase (4.51 IU/ml) and protease (2.46 IU/ml), while pH 7 favoured the enzyme production for cellulase (0.43 U/ml), lipase (0.49 U/ml), xylanase (0.043 IU/ml) and laccase (0.049 U/ml) (Table 2).

Effect of temperature

The influence of temperature on amylase production is related to the growth of microbes. Temperature optimum for amylase was in the range between 25 and 37°C for the mesophilic fungi[28-29]. The present study recorded 30°C as optimal for enzyme production which agrees with earlier findings.

Maximum enzyme production was observed at 30°C for amylase (3.45 U/ml), protease (3.12 U/ml), cellulase (1.65 U/ml), lipase (0.64 U/ml), xylanase (0.037 U/ml) and laccase (0.041 U/ml) (Table 3).

Effect of salinity

Variation in the salinity of the medium altered the growth rate of the test organism and enzyme production. There was decrease in the growth and enzyme producing ability observed in relation to the increased salinity of the medium.

Among the 5 different salinity levels used, 20 ppt was suitable for the growth of the organism and enzyme production. Maximum enzyme production was 3.96, 3.67, 0.91, 0.35, 0.029, 0.28 IU/ml for amylase, protease, cellulase, lipase, xylanase and laccase respectively (Table 4).

It has been reported that the cellulolytic activity of some fungi isolated from Sunderban mangroves was influenced by salinity[25]. All the fungal isolates were capable of producing considerable amount of cellulase. Highest cellulolytic activity was recorded in *Chaetomium globosum* at zero per cent salt level, whereas *Aspergillus terreus* showed highest activity at 60% salt level. The present study also showed that salinity influenced the production of extracellular enzyme such as amylase, protease, cellulase, lipase, xylanase and laccase. It was found that the activity of the medium, salinity at 20 ppt were favoured the enzyme production. It lends support to previous findings[15,20-21].

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