

Original Research Article

Optimization of Enzyme Production in *Trichoderma viride* using Carbon and Nitrogen source

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ABSTRACT

Keywords

Aspergillus sp;
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chitinase and
xylanase.

In the present study, the soil samples were collected from mangrove environment of Maravakadu, Thanjavur District. The fungal species were isolated by plating method, in 50% sea water containing potato dextrose Agar medium. Totally 10 fungal species were isolated and identified from the soil sample. Out of 10 isolates, nine species belonged to *Aspergillus* sp. and one was *Trichoderma* sp. The production of asparaginase, chitinase and xylanase from *Trichoderma viride* by using solid state fermentation. The production of enzymes by *Trichoderma viride* was optimized by using fermentation medium containing different substrates. The maximum asparaginase production was observed on wheat bran, coffee husk and urea containing medium. The chitinase production was maximum in coffee husk containing media. Wheat bran produced the maximum level of xylanase. This study revealed that mangrove environment provides impressive diversity of fungi in the East Coast of India and are unexplored for microbial resources.

Introduction

The variety and galaxy of fungi and their natural beauty occupy prime place in the biological world and India has been the cradle for such fungi. Only a fraction of total fungal wealth has been subjected to scientific scrutiny and mycologists have to unravel the unexplored and hidden wealth one third of fungal diversity of the globe exist in India.. Fungi are not only beautiful but play a significant role in the daily life of human beings besides their utilization in industry, agriculture, medicine, food industry, textiles,

bioremediation, natural cycling, as biofertilizers and many other ways. Fungal biotechnology has become an integral part of the human welfare.

Mangroves constitute the second most important ecosystem. Mangrove forests of India are dispersed in tropical as well as subtropical conditions. Mangrove fungi are the second largest group among the marine fungi. One fourth of the world coastline is dominated by mangroves, which are distributed in 112 countries and

territories comprising about 181,000 sq km (Spalding *et al.*, 1997).

Chitinase producing fungi especially *Trichoderma* species can be effective as biocontrol agents against some plant pathogenic fungi (Hjelford and Tronsmo, 1998). An important part of the mechanism involved in the antagonism of *Trichoderma* sp. and fungal pathogens appears to be production of fungal cell wall lytic enzymes including chitinases. (Chet *et al.*, 1998).

The technique of solid state fermentation (SSF) involves the growth and metabolism of microorganisms on moist solids in the absence of any free-flowing water. SSF offers distinct advantages including economy of the space simplicity of the media. No complex machinery, equipments and control systems; greater compactness of the fermentation vessel owing to a lower water volume greater product yields; reduced energy demand; lower capital and recurring expenditures in industry; easier scale up of processes; lesser volume of solvent needed for product recovery; superior yields; absence of flown build-up; and easier control of contamination due to the low moisture level in the system (Babu *et al.*, 1995, Arima *et al.*, 1964; Lonsane *et al.*, 1985).

Mangroves provides unique opportunities for mycologists to explore fungal diversity and exploit their ecological medicinal and Industrial potential, Fungi are well recognized to produce a wide variety of chemical, several of which are most valuable pharmaceuticals, agrochemicals and industrial products. The world of fungi provides a fascinating and almost endless source of biological diversity which is a rich source for exploitation.

Hence in the present investigation attention is focused on the diversity of fungi in soil sample and their enzyme activities.

Materials and Methods

Sample Collection

The marine sediment soil sample was collected from mangrove environment of Maravakadu, Thanjavur District (Plate: I).

Isolation

One gram of marine sediment soil sample was diluted serially in distilled water. Potato Dextrose Agar medium (PDA) was prepared and sterilized in an autoclave at 121°C for 15 minutes. The medium was incorporated with streptomycin sulphate solution (1%) and poured in to the petri plates. After solidification 0.1 ml of serially diluted soil samples were inoculated in to the medium. The inoculum was spread uniformly and kept undistributed in dust free chamber at room temperature for a period of 3-5 days. The fungal colonies were counted. Pure cultures were maintained in potato dextrose agar medium.

Identification and Photomicrography

Morphological features of fungi were photographed using Nikon Microscope. All the fungi were identified with referring the standard manual (Gilman, 1957).

Enzyme studies

Among the fungal isolates *Trichoderma viride* was selected for enzyme studies of asparaginase, chitinase and xylanase using different substrates.

Production of enzymes in *Trichoderma viride* by using carbon and Nitrogen Source

The available carbon and nitrogen substrates such as Wheat bran, Rice bran, Coffee husk, Glycerol and Urea. Were used in the present study.

Asparaginase activity

Asparaginase liquid medium was prepared and autoclaved at 121°C for 15 minutes the test cultures were inoculated into cooled asparaginase liquid medium. Crude enzyme was used for enzyme assay. The assay mixture containing 0.25 ml of crude enzyme extraction; 1.25 ml of 0.2M borate buffer was added and the mixture was incubated at 35°C for 30 minutes. The reaction was stopped by the addition of 0.5ml of 15% TCA and the assay mixture was subjected to centrifugation. The supernatant (1ml) was mixed with 4 ml of sterilized distilled water free from ammonia. To this 0.5 ml of Nessler's reagent was added and the colour intensity read in a spectrophotometer at 450 nm. One enzyme unit was defined as the amount of enzyme, which liberates 1µmol of ammonia per minute under the optimal conditions. Soluble protein in culture filtrate was estimated by Lowry *et al.* (1951) measured at 650nm.

Chitinolytic activity

Conical flasks (250ml) containing 150ml of chitinase medium were sterilized by autoclaving at 120° for 30 minutes. Seven agar plugs (5mm diameter) were taken from the edges of 5 to 14 days – old colonies were used to inoculate the flasks and these were incubated at 24°C in dark up to 37 days. Culture medium was filtered through a whatmann paper No.1

filter followed by filtration through 0.2µm Millipore filter. Filtered culture supernatant was then lyophilized and kept at - 20°C until use. Chitinolytic activity was assayed by using Dackman *et al.*, (1989) method.

Xylanase Activity (Takashi nanmoriet *al.*, 1990)

Xylanase positive cultures were inoculated into the xylan containing broth. The broth was incubated for 3 -7 days for production of xylanase. After incubation xylanase activity was determined, 0.5 ml of broth was taken and it was mixed with 0.5 ml of 2% xylan suspension in 100 mM tris-HCl buffer (pH 7.0). The broth was incubated at 55°C for 30 min. and kept in ice water. The insoluble xylan was removed by centrifugation. After centrifugation 0.5 ml supernatant was taken and 1 ml of 3,5, di-nitrosalicylate (0.5 ml) solution was added. The mixture was kept in boiling water bath. The colour intensity was measured at 535 nm. One unit of activity was defined as amount of enzyme required to liberate 1µmol of xylose per minute under the assay condition. Xylose acts as a standard solution.

Estimation of Protein

Protein concentration were determined according to (Lowry *et al.* 1951)

Results and Discussion

Microbial source such as fungi are well recognized to produce a wide variety of chemical sources, several of which are most valuable pharmaceuticals, agrochemicals and industrial products. The world of fungi provides a fascinating and almost endless source of biological diversity which is a rich source for

exploitation. Mangrove forests of India are dispersed in tropical as well as subtropical conditions. Marine fungi play an important role in nutrient regeneration cycle as decomposers of dead and decaying organic matter in the estuaries. Although mangroves are dominant feature of Indian coastline and provide niches and habitats for many marine organisms very little is known about the fungi associated with them till recently.

In the present study totally 10 fungal species were isolated from soil sample of Maravakadu mangroves. All are belonged to Deuteromycetes and *Aspergillus* was the dominant genera including 9 species and one *Trichoderma viride*. A few researchers have studied the mycoflora in mangal soil. Stolk (1955) reported two new species from African mangrove soil. Swart (1963) examined the culturable mycoflora of mangrove soils of Eastern Africa and reported *Cladosporium*, *Alternaria*, *Aspergillus*, *Penicillium*, *Phoma*, *Septonema*, *Robillardo* and *Periconia* from mangrove soils

Among the 10 fungal isolates *Trichoderma viride* was selected for enzymatic activities include asparaginase, chitinase and xylanase. *Trichoderma* sp. possesses innate resistance to most agricultural chemical inducing fungicides, although individual strains differ in their resistance. Some lines have been selected or modified to be resistant to specific agricultural chemicals most manufacturers of *Trichoderma* strains for biological control have extensive lists of susceptibilities or resistance to a range of pesticides.

Trichoderma sp. is highly efficient producer of many extracellular enzymes. They are used commercially for the production of cellulose and other enzymes that degrade complex polysaccharides. They are frequently used in the food and

textile industries. Cellulases from these fungi are used in “biostoning” of denim fabrics to give rise to the soft whitened fabric – stone – washed denim. The enzymes are also used in poultry feed to increase the digestibility of hemicelluloses from barley or other crops. As noted, these fungi are used with or without legal registration for the control of plant diseases. There are several reputable companies that manufacture government registered products.

Considering these significances of *Trichoderma* sp. the present work was carried out on *T. viride* for enzymatic activities by using various substrates such as wheat bran, rice bran, coffee husk, glycerol and urea in fermentation medium.

Fungi Isolated from soil

A total number of 10 fungal species belonging to two genera such as *Aspergillus*, and *Trichoderma* were isolated by plating method. Among them *Aspergillus* was the dominant genera represented by 9 species and one species was belonged to *Trichoderma* (Table 1) (Plate: II,III and IV).

In the present study maximum production of asparaginase was produced by *Trichoderma viride* in wheat bran, coffee husk and urea containing asparaginase medium. Maximum asparaginase production level (0.053 IU/ml) was observed at 62 hrs in wheat bran, coffee husk and urea containing media, followed by (0.052 IU/ml) and (0.050 IU/ml) observed in rice bran and glycerol containing media respectively (Table: 2) (Fig:1). L-Asparaginase is produced by a large number of microorganisms that include *E. coli* (Dersert *et al.*, 1994; Mercado and Arenas, 1999) filamentous

Table.1 Isolation of fungi from Mangrove Soil

S.No.	Fungal isolates
1.	<i>Aspergillusflavus</i>
2.	<i>A.fumigatus</i>
3.	<i>A.luchuensis</i>
4.	<i>A.nidulans</i>
5.	<i>A. niger</i>
6.	<i>A.sulphureus</i>
7.	<i>A. wentii</i>
8.	<i>A.terreus</i>
9.	<i>A.variecolor</i>
10.	<i>Trichodermaviride</i>

Table.2 Asparaginase production in *Trichoderma viride* using Carbon and Nitrogen Sources

S.No.	Carbon and Nitrogen Sources	Enzyme activity (IU/ml)
1.	Wheat bran	0.053
2.	Rice bran	0.052
3.	Coffee husk	0.053
4.	Glycerol	0.050
5.	Urea	0.053

Fig.1 Asparaginase production in *Trichoderma viride* using Carbon and Nitrogen Sources

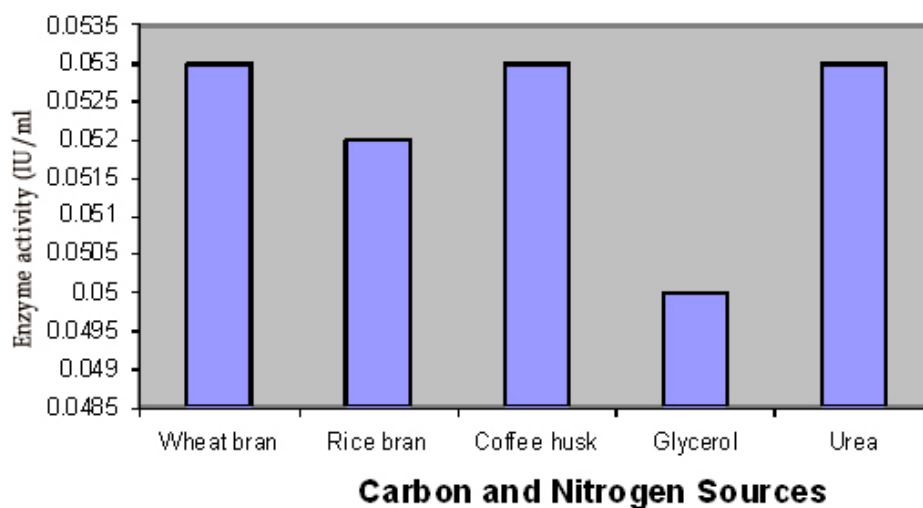


Table.3 Chitinase production in *Trichoderma viride* using Carbon and Nitrogen Sources

S.No.	Carbon and Nitrogen Sources	Enzyme activity (IU/ml)
1.	Wheat bran	0.004
2.	Rice bran	0.004
3.	Coffee husk	0.049
4.	Glycerol	0.005
5.	Urea	0.041

Fig.2 Chitinase production in *Trichoderma viride* using Carbon and Nitrogen Sources

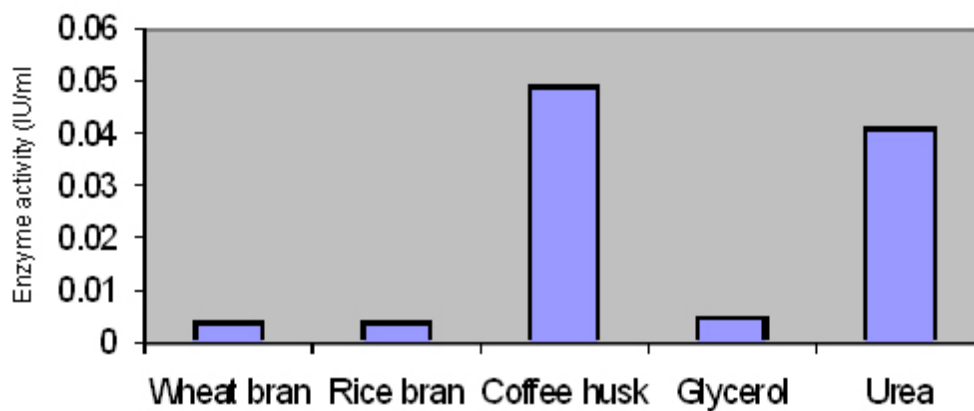
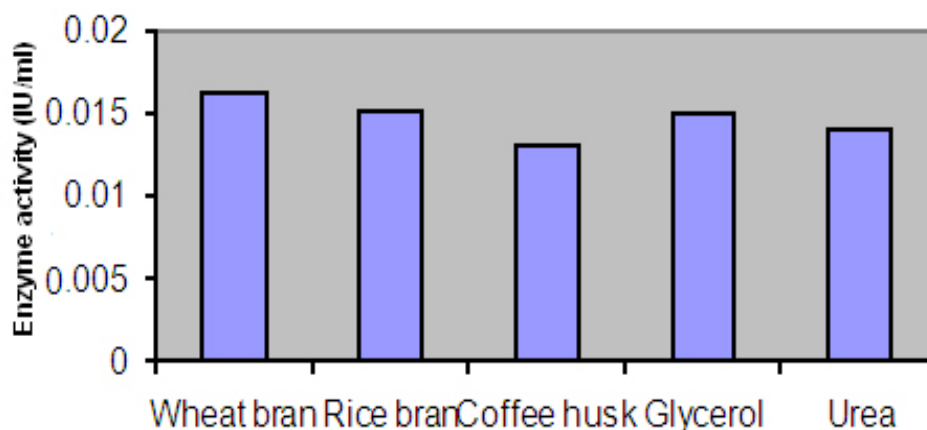


Table.4 Xylanase production in *Trichoderma viride* using Carbon and Nitrogen Sources

S.No.	Carbon and Nitrogen Sources	Enzyme activity (IU/ml)
1.	Wheat bran	0.0162
2.	Rice bran	0.0152
3.	Coffee husk	0.013
4.	Glycerol	0.015
5.	Urea	0.014

Fig.3 Xylanase production in *Trichoderma viride* using Carbon and Nitrogen Sources



fungi *Aspergillus tamari* and *Aspergillus terreus* (De mourasaris *et al.*, 2004) and *A. oryzae* (Beer *et al.*, 2006). L-Asparaginase catalyzes the hydrolysis of L-asparagine into L-aspartate and ammonia. The eucaryotic microorganisms like Yeast and filamentous fungi have a potential for asparaginase production (Wade *et al.*, 1971; Wiame *et al.*, 1985 and Pinheiro *et al.*, 2001).

Although a plethora of chitinolytic enzymes have been detected and purified from various *Trichoderma* sp. (Lorito *et al.*, 1998). Many studies have proved the potential of *Trichoderma* sp. as biological agents antagonistic to several soil borne plant pathogens. The production of lytic enzymes influenced by the composition of the culture media (Bruce *et al.*, 1995). Maximum chitinase activity peak (0.049 IU/ml) was obtained at 62 hrs from the media containing coffee husk. In the medium containing urea, the production level of chitinase was 0.041 IU/ml. Minimum chitinase production showed when the culture grow at glycerol (0.005 IU/ml), rice bran (0.005 IU/ml) and wheat bran (0.004 IU/ml) containing medium (Table: 3) (Fig:2).

Filamentous fungi are attracting greater attention than bacteria as potential sources of plant cell wall hydrolyzing enzymes such as xylanases because they secrete high levels of the enzymes into the culture medium. (Berry and Paterson, 1990). Xylan rich neutral substrates such as sawdust corn cob Bennett wheat bran sugar beet Pulp and sugarcane pulp or baggase have been used to stimulate xylanase production by different organisms Wheat bran has the greatest prospect to serve as low cost substrate for xylanase production using the wild strain

of *P. chrysogenum* PC 501. The present results revealed that, the maximum xylanase production (0.016 IU/ml) was produced by *T. viride* grown on medium containing wheat bran as a substrate. The other substrates produced moderate to minimum level of xylanase (Table: 4) (Fig:3).

Although, the present work gives an idea on the bio diversity of fungi and the enzyme producing ability, further study is essential to the separation and purification of enzymes. The fungal isolates have great potential as enzyme producers for their application in industries. This study can be further revealed that the enzymes are wide range of application in the Pharmaceuticals food, feed and leather Industries.

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