Research & Reviews in Biotechnology & Biosciences
Website: www.biotechjournal.in
Volume-8, Issue No: 1, Year: 2021 (January-June)
DOI: http://doi.org/10.5281/zenodo.5118371

ISSN No: 2321-8681
Research Paper
PP: 1-5
Peer Reviewed Journal

Multienzyme Producing Marine Fungi from the Mangalore Seacoast

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Article History

Received: 15/02/2021
Accepted: 08/04/2021

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ABSTRACT

Mangalore is one of the prominent cities in South Canara, Karnataka situated in costal region. Marine environment has diverse species including microbe. Marine microorganisms make up about 70% of the biomass in the ocean. The beach sand harbor it's own flora such as bacteria, fungi, algae, viruses and protozoans etc. These microbes are known for their enzymes and metabolic by products useful for various purposes. Potential resources of these marine microbes can be tapped for wider applications in bio industries. In the present study seaweed samples were collected from the NITK beach, Suratkal and sand samples were collected from NITK, Someshwar and Tannirbhavi beaches of Dakshina Kannada District Mangalore, India.

The marine fungi were isolated from these samples and the they were screened for enzymes like amylases, cellulases, chitinase and protease production. The fungi isolated using specific marine agar from seaweed and sand samples were identified based on colony morphology and microscopic characteristics. *Aspergillus niger, Aspergillus fumigatus, Aspergillus nidulans* were about 75% and *Penicillium chrysogenum* was 25% of the total isolates. In the screening, Aspergillus species were the prevalent multi-enzyme producer comprising amylases, Cellulases, chitinase and proteases whereas *Penicillium* isolates showed predominant proteolytic activity, moderate chitinase, minimal amylase and no chitinase activity. Thus marine isolates of Aspergillus and Penicillium can be leveraged for the large scale production of amylase, cellulose and protease on suitable strain development, considering it's wider applications.

Key words- ***Marine fungi, Aspergillus, Penicillium, sea weed, enzymes***

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INTRODUCTION

Oceans cover about 70% of the earth's surface and contain 97% of our planet's water. Marine habitats represent the largest reservoir of biodiversity of the planet. The microbial diversity of marine origin has developed cellular machinery to thrive well even in extreme conditions. Various enzymes have been characterized from seawater and different marine sediments. The main marine enzymes reported so far includes lipases, proteases, laccases, and polysaccharases. The polysaccharide-degrading enzymes from marine microbes have gained global attention due to novel industrial applications. (1)

Carbohydrates are the important biomolecules for life, for storage, cell recognition, cell adhesion, immunity and other biological functions. Chitin (β-(1–4)-poly-N-acetyl-D-glucosamine is the second most abundant polysaccharide and can be found as essential component in different living structures.(2) Chitin-degrading enzymes are a heterogeneous group of enzymes belonging to glycoside hydrolyses, which are able to catalyse the cleavage of β-(1–4) glycosidic linkage in chitin. These enzymes are found in large numbers of organisms including algae, fungi, bacteria, arthropods, insects, higher plants, and crustaceans.(3)

Chitinolytic microbes have been found in various marine sources such as water, sediment, and digestive tracts of fish.(4) There is a large amount of chitin available in the seawater due to continuous shedding of marine zooplankton. Available reports states that a wide variety of microorganisms including *Aspergillus*, *Pseudomonas*, *Chromobacterium*, *Clostridium*, *Flavobacterium*, *Penicillium*, *Rhizopus*, *Enterobacter*, *Klebsiella*, and *Streptomycetes* can produce chitinase.(5)

Cellulases have a wide range of biotechnological applications including from the manufacture of paper and textiles, the formulation of laundry detergents and, more recently, in the development of biofuel production processes from renewable cellulosic and lignocellulosic plant material. For bulk fermentation processes, the relatively high cost of cellulases is a significant economic impediment to the development of cellulosic biofuels.

Amylases are enzymes that break the complex sugars into simple sugar. Due to advancement of marine science and biotechnological research, a large number of microorganisms in the marine habitats are known to produce amylase. A novel α-amylase from marine actinobacteria Streptomyces sp. was isolated.(6) Bacillus species collected from decomposing mangrove leaves exhibited maximum amylase activity and minimum or no activity for cellulose.(7)

Proteins are degraded by numerous hydrolytic enzymes called proteases. The fungi belonging to genera *Penicillium* and *Aspergillus* are known to produce proteases of commercial and biotechnological importance. (8)

Marine bio-enzyme research has been extensively done mainly by Canada, Spain, Finland and Russia and other countries. (9) Present study is focused on dominant fungal species present in the coastal marine regions of Mangalore and screened the isolated fungi for enzymes such as amylase, cellulase, chitinase and proteases. Enzymatic

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screening is the precursor step in the development of novel enzymes of commercial and industrial interest.

Methodology

1. Collection of samples:

Four sea weed and sand samples were collected on January 15, 2020 at noon from coastal shores of NITK beach, Suratkal at a depth of 1.5 meters. The sea weeds were uprooted and collected in a sterile polythene zip lock bag. Similarly sand samples were also collected from NITK beach, Someshwar beach and Tannirbhavi beach. The samples were processed for microbial isolation.

2. Isolation of marine fungi:

The seaweed samples collected were crushed in a mortar-pestle and 0.1ml was inoculated into the Malt Extract media prepared in filtered raw seawater (FRS). The sand samples were serially diluted and inoculated into Malt Extract media by Pour plate method.

Medium Composition for isolated cultures:

Agar (20 g. L-1) and Malt extract (20 g.L-1) in FRS (1L) and adjusted to pH 8 by addition of 3M KOH. The mixed colonies were sub cultured to obtain pure cultures designated as MF1, MF2, MF3, MF4. (MF= Marine Fungi)

3. Microscopic identification of marine fungi:

Fungal morphology were studied macroscopically by observing colony features (Colour and Texture) and microscopically by staining with Lactophenol cotton blue and observed under compound microscope for the Conidia, Conidiophores and arrangement of spores.

4. Screening for enzyme production by plate assay method:

The isolated marine fungi were screened for Amylase, Cellulase, Chitinase and Protease exo-enzymes.

4.1. Screening for amylase production:

The fungal isolates were screened for amylase production using Starch Agar Media.

(Starch Agar Media Composition: Starch - 20g, Peptone - 5g, Beef Extract- 5g, Agar - 15g, FRS- 1L).The fungal isolates were inoculated on the sterile solid media by line-streak method and incubated at room temperature for 3 days. After incubation, the plates were flooded with iodine solution and observed for clear zones around the fungal colony against the dark-blue starch-iodine complex in the surrounding. A clear zone indicated amylase production and was recorded as a positive result. (10)

4.2. Screening for chitinase production

The fungal cultures were spotted on the selected Colloidal Chitin Agar Media.(Colloidal chitin: 5g/l, KH_2PO_4: 2g/l, $MgSO_4.7H_2O$: 0.3g/l, $(NH_4)_2SO_4$: 1.4g/l, $CaCl_2.2H_2O$: 0.5 g/l, Peptone: 0.5g/l, Urea: 0.3 g/l,$FeSO_4.7H_2O$: 0.005g/l, $MnSO_4.7H_2O$: 0.0016g/l, $ZnSO_4.7H_2O$: 0.0014g/l, $CoCl_2.2H_2O$: 0.002 g/l, Agar: 15g/l, FRS 1L pH: 6.0) and the plates were incubated at 28°C for 5 days. Development of halo zone around the colony was considered as positive for chitinase enzyme production. (11)

Preparation of colloidal chitin:

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Chitin powder derived from crab shell flakes was used to prepare the colloidal solution. (12) Chitin powder (40 g) was dissolved in 200 ml of concentrated HCl and continuously stirred at for one hour. The suspension was added to 1 litre of ice-cold distilled water with rapid stirring and kept overnight at 4°C. The precipitate was collected by centrifugation at 10,000 rpm for 20 min and washed with tap water until the colloidal chitin became neutral (pH 7.0). It was centrifuged again and stored at 4°C until further use.

4.3. Screening for cellulase production

The enzymatic screening for cellulases was based on Carboxymethyl cellulose (CMC) plate assay whereby the agar medium was mixed with Congo Red (CR) dye. The composition of the medium was 1% CMC, 1.5% agar, 0.67% YNB without amino acids, and 0.01% Congo Red. Ten fungal isolates obtained were cultured on CMC-CR agar and incubated at 30°C for 72-96 hours. As growth develops on CMC-CR agar plate, the hydrolysis of CMC releases the bound CR dye. This is revealed by the appearance of pale yellow halo zone surrounding the fungal colony. (13)

4.4. Screening for protease production

Production of proteolytic enzymes by fungal isolates was detected by using the Plate assay method of "Hankin and Anagnostakis", in which gelatine is the protein source of that growth medium. The fungal isolates were spot inoculated in Petri dishes with Nutrient Agar medium supplemented with 1% gelatine (Peptone, 5g; Beef extract, 3g; NaCl, 5g; Agar, 15g; Distilled water, 1 litre, pH 6). Following inoculation, the were incubated at 28 ± 1°C for 3 days. After a week of incubation, gelatine degradation was observed as a clearing zone around the fungal colonies.

This zone of gelatinolysis was seen clearly upon flooding the plate with aqueous saturated solution of Mercuric chloride reagent (15g $HgCl_2$ dissolved completely in 20 ml 7M conc. HCl, then raised to 100 ml with sterile distilled water). Mercuric chloride solution reacted with gelatin to produce a white precipitate which made the clearing zone visible. The clearing zone was measured indicative of the extracellular protease activity of the fungal strain. (14)

Results and discussion

A total of 4 salt-tolerant fungi were isolated of which *Aspergillus* was the most dominant genera. Other studies conducted using marine fungi also report the similar results. More than 100 species of fungi including sugar fungi have been enumerated from the coastal dunes of Odisha, the majorities of which belonged to the genus *Aspergillus* followed by *Penicillium* and *Trichoderma*. (15)

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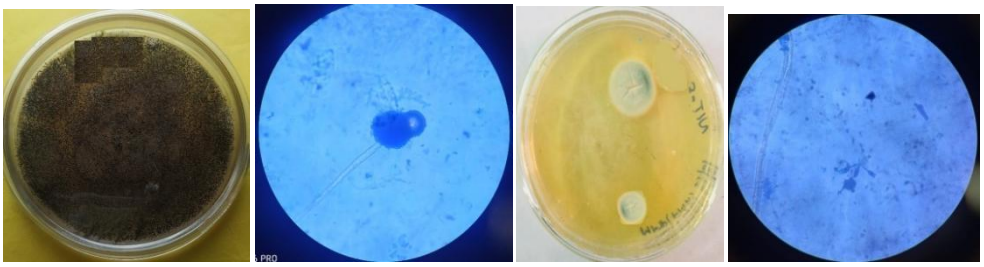
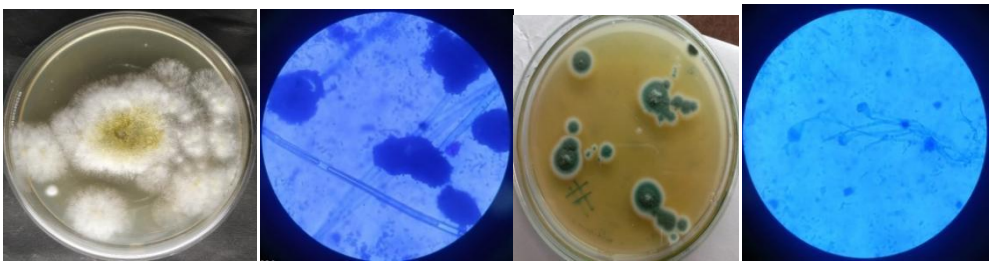
Table 1: Isolation of Marine Fungi

Sl.No	Sample Source/Type	Colony Morphology	Organism identified	Designated code
1	Someshwara Beach - Sand	Raised moldy growth - black spores	*Aspergillus niger*	MF1
2	NITK Suratkal Beach – Sand	Flat colony – Greyish spores	*Penicillium chrysogenum*	MF2
3	NITK Suratkal Beach – Seaweed	Raised moldy growth – yellowish green spores	*Aspergillus nidulans*	MF3
4	Tannirbavi Beach – Sand	Flat growth – dark green spores	*Aspergillus fumigatus*	MF4

(MF = Marine Fungi)



(a)MF1 **(b) MF2**



(c)MF3 **(d)MF4**

Fig-1.Growth pattern and staining of marine fungal isolates , Note-MF- Marine Fungi

Table 2: Enzymatic Screening of the Marine Fungal Isolates

Fungal Isolate	Amylase	Cellulase	Chitinase	Protease
MF1	++	+	+	+
MF2	+	++	-	+++
MF3	+++	+	++	++
MF4	++	++	++	+++

Note-*: - No inhibition, +: Minimum Zone ,++ :Moderate zone ,+++ : Maximum Inhibition*

The growth pattern of marine fungal isolates is iven in figure 1.The enzymatic screening of marine fungal isolates is given in the figures 2,3,4,5. The details of sample collection sites and fungal isolates and their

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enzyme production is given in the table 1 and 2.

Studies on enzymatic screening showed that *Aspergillus* produced multiple enzymes including amylase, cellulase, chitinase and protease whereas *Penicilluim* showed the widest zone of proteolytic activity. This result agrees with the 'Species diversity and enzymatic studies of marine fungi' done by Qu J et. al. in the marine sediments of South China Sea. (16)

Amylolytic activity was identified by several fungal species isolated from soil and *Aspergillus spp*. was found to possess the highest amylase activity using bran wheat bran, rice bran, black gram bran) as carbon source in shake flask cultures of hemophilic strains of *Aspergillus niger*. (17)

Chitin-degrading enzymes have been isolated from several marine microbes including bacteria from different genus such as *Bacillus*,(18) *Vibrio, Pseudoalteromonas*, (19) *Micrococcus,* (20) *Alteromonas*, (21) and some fungi such as *Aspergillus terreus*, (22) and *Aspergillus carneus*.(23) *Trichoderma*,

Aspergillus, *Fusarium*, *Chaetomium*, *Phoma*, *Sporotrichum*, *Penicillium*, etc. These fungi are also able to produce cellulase. (24)

Figure 2: Enzymatic screening of Marine Fungi Isolates for amylase production

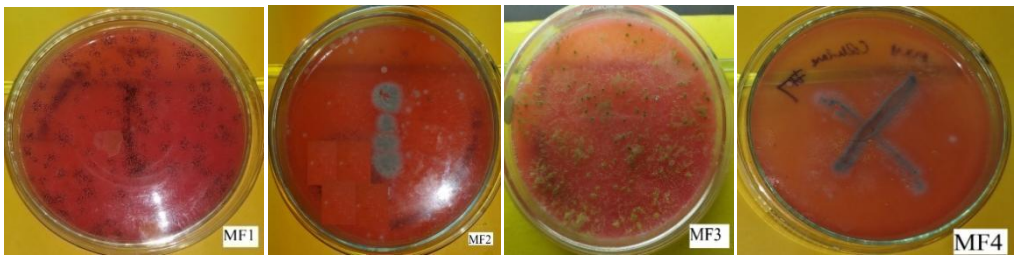

Figure 3: Enzymatic screening of marine fungi isolates for cellulase production

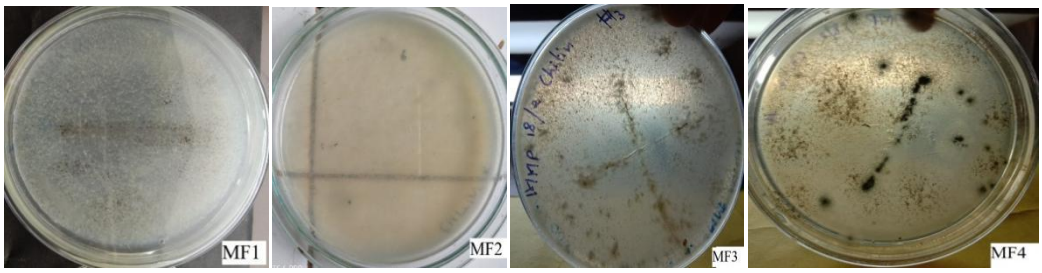

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Figure 4: Enzymatic screening of marine fungi isolates for chitinase production

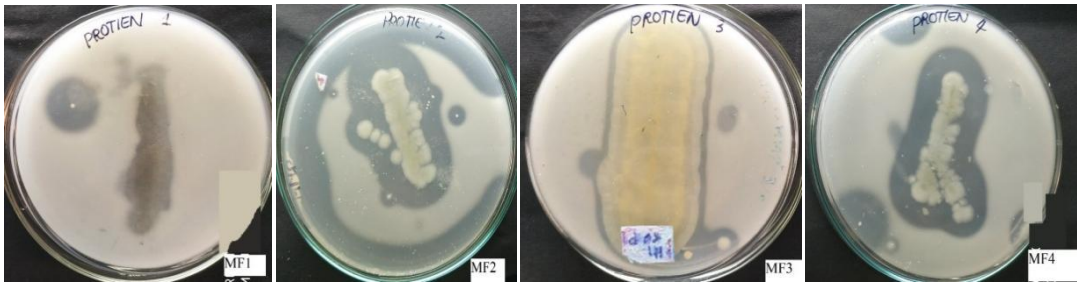

Figure 5: Enzymatic screening of marine fungi isolates for protease production

CONCLUSION:

Our study reports that marine isolates of Aspergillus Spp produces multienzymes like amylase, protease, chitinase and cellulase and Penicillium species produces proteolytic ezymes predominantly. These fungal isolates from Mangalore coast can be used for the large scale enzyme production after specific screening. Further studies can be done for Strain Improvement and optimization of the fungal isolates, including pH, temperature salinity and nutritional supplement. This result can be used for the strain improvement for large scale production of enzymes in industry and other useful applications.

Conflict of interest-There is no conflict interest

Authors contribution- First four authors have done the sample collection, research work,
SCH has supervised and directed the work,BP has provided the needful fascilities, guidance and written the manuscript and submitted.

Acknowledgement-Authors would like to thank University College and Mangalore University,Mangalore.

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