

Marine-derived fungi as a source of proteases

Tonima Kamat*, Celina Rodrigues & Chandrakant G. Naik
Biorganic Chemistry Laboratory, National Institute of Oceanography,
Dona Paula, Goa - 403 004, India
*[E-mail: tonima96@yahoo.com]

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Microbial enzymes have continued to assist diverse reactions as biocatalysts. Marine derived microbes offer a prospective resource for such enzymes. In this study thirteen fungi were isolated from marine organisms (soft coral and sponge) collected from Mandapam (Tamil Nadu) coast. The fungal isolates were screened for the protease activity. Fungi *Beauveria brongniartii* and *Acremonium fusidioides* showed remarkable protease activity. Isolation, purification and characterisation of proteases from these fungi may reveal special, significant properties.

[Key words: Marine fungi, proteases, soft corals, sponge]

Enzymes are biocatalysts which assist numerous highly diverse reactions. They have found applications in various domains including therapeutics¹⁻⁷. It has been reported that molecules of marine origin may differ in properties from their terrestrial counterparts^{8,9}. Marine microorganisms (epibionts and endo symbionts), due to the unusual niche in which they are found, are also being explored for therapeutically and industrially important molecules¹⁰⁻¹⁵. Enzymes from these symbionts perhaps have characteristics such as heat-tolerance, cryo-tolerance, pH tolerance, metal tolerance etc.

Proteases are robust enzymes having wide industrial applications in-detergents, leather processing, healthcare, food processing etc.¹⁶⁻¹⁸. They account for nearly 60% of the total worldwide enzyme sales¹⁹. Fungi are well known sources of extra cellular enzymes and proteases from the genus *Aspergillus* have been studied extensively¹. Fungi from marine and estuarine environment have been screened for protease production²⁰. Fungi associated with marine organisms, due to their unusual niche, may be a new source to isolate proteases.

Corals serve as breeding grounds for many marine organisms²¹. Marine sponges harbor abundant diverse microbes. Up to 40% of the biomass in sponges can be constituted by microorganisms²². In the present study, thirteen fungi were isolated from four coral samples and a sponge. These isolates were screened, by rapid plate technique, for the presence of protease activity. This was only a preliminary study to detect

protease activity, if any, of marine-derived fungi in order to identify the potential sources.

Sponge and corals were collected by SCUBA diving, from a depth of 8 to 10 m from the coastal waters of Mandapam, Tamil Nadu (9°16' N; 79°12'E). The samples comprised of a soft coral *Sinularia kavarrattiensis* (from three stations SK1, SK2 and SK3), an unidentified coral and a yellow sponge *Spirastrella inconstans* var. *digitata*. The samples were transported to the laboratory in frozen state under aseptic conditions. From each of the samples, fourteen pieces of approximately 2x2 cm were cut out aseptically. These fourteen pieces of each organism were subjected to two different treatments²³. In the first case seven pieces were vortexed four times, for 20 seconds each, with sterile seawater while in the second case the remaining seven pieces were vortexed with 0.5 % sodium hypochlorite, for 10 seconds and then rinsed by vortexing with sterile seawater for 10 seconds, to remove residual sodium hypochlorite. After these two treatments, each piece was placed on seven different media for the isolation of fungal cultures. Seven media used were: Potato dextrose agar, Czapek dox agar, Corn meal agar, Mycological agar, Starch casein agar, V8 agar and Tomato juice agar (Hi Media, Mumbai). Each agar medium was prepared in seawater with 0.2g/l penicillin added for the inhibition of bacterial growth. Fourteen agar plates per sample of marine organism were incubated at room temperature (28± 2°C) and were observed daily for growth.

Fungal colonies growing in the close vicinity of the sample placed were subcultured on the respective media. Repeated subculturing was done until pure fungal isolates were obtained. All the fungal cultures were identified based on phenotypic characters at Agharkar Research Institute, Pune. The inoculum for spot inoculation was prepared by growing the fungi for 48 hours in 5 ml Potato dextrose broth (Hi Media) prepared in filtered seawater: distilled water (50:50). Screening for protease activity was done using modified González *et al* method²⁴. A basal medium [(NH₄)₂ SO₄ 0.1 % w/v, KH₂ PO₄ 0.1 % w/v, Na₂ HPO₄ 0.09 % w/v, MgSO₄·7H₂O 0.02 % w/v, Yeast extract 0.1 % w/v, Agar 3 % w/v prepared in 50 % v/v filtered seawater] was used with 1 % w/v of skimmed milk. Cultures were spot inoculated on the plate and incubated at 28°C for 3-5 days. The appearance of clearance zone in the medium around the colony indicated protease activity. The zone was measured from the edge of the colony to the edge of clearance zone and recorded in mm.

In all 13 fungal isolates were obtained. Of these, seven were obtained when seawater was used and six were obtained when 0.5 % sodium hypochlorite was used to treat the sample. These fungi were coded, for convenience, according to their source of isolation, treatment of the source, and isolation media. Table 1 gives the details of the fungal cultures isolated.

Almost all thirteen fungi showed protease activity when observed on the plate. The growth and protease activity as observed is presented in Table 2. Amongst the fungal isolates obtained from five marine organisms *Aspergillus terreus* group was most dominant followed by *Acremonium fusidioides*. The soft coral *Sinularia kavarattiensis* was collected from

three different stations to check for diversity of micro flora present depending on the site of collection. Out of seven isolates obtained from *Sinularia kavarattiensis*, four isolates (from SK1, SK2 and SK3) belonged to the genus *Aspergillus*. Also, *Beauveria brongniartii* was isolated from SK1 and *Acremonium fusidioides* and *Acremonium butyri* were isolated from SK3.

The pieces of sponge and corals used for isolation of fungi were treated using sea water or sodium hypochlorite, so that the micro flora could be isolated without contamination. High numbers of fungi (six) were isolated when Corn meal agar was used as isolation medium. This was followed by Starch casein agar with four isolates. Potato Dextrose agar, Czepex dox agar and V8 agar yielded one isolate each. Mycological agar and Tomato juice agar did not support the growth of any fungi from the marine samples.

Almost all the isolates except *Aspergillus terreus* group showed good growth when tested for protease activity. But the activity was remarkably distinct in three fungi namely *Beauveria brongniartii* isolated on Potato dextrose agar, *Acremonium fusidioides* isolated on Corn meal agar and *Acremonium fusidioides* isolated on Starch casein agar. In these cases, the proteolytic zone indicated that the protease is actively released in the medium by the fungus and therefore it could be considered to be the measure of the availability of free protease, whereas, the size of the colony is indicative of more cell mass only.

Proteases constitute one of the largest groups of industrially important enzymes. In the present study, the marine-derived fungi were screened for protease activity and it was found that they can be a significant

Table 1—Details of the fungal isolates used for screening.

Code	Marine Source	Fungi	Isolation medium	Treatment for isolation
PR1	<i>Sinularia kavarattiensis</i> (SK1)	<i>Beauveria brongniartii</i>	Potato Dextrose Agar	Sodium hypochlorite
PR2	<i>Sinularia kavarattiensis</i> (SK1)	<i>Aspergillus terreus</i> group	Corn meal Agar	Seawater
PR3	<i>Sinularia kavarattiensis</i> (SK1)	<i>Aspergillus terreus</i> group	Starch casein Agar	Seawater
PR4	<i>Sinularia kavarattiensis</i> (SK2)	<i>Aspergillus terreus</i> group	Czepex dox Agar	Seawater
PR5	<i>Sinularia kavarattiensis</i> (SK3)	<i>Aspergillus wentii</i> group	Corn meal Agar	Sodium hypochlorite
PR6	<i>Sinularia kavarattiensis</i> (SK3)	<i>Acremonium fusidioides</i>	Corn meal Agar	Seawater
PR7	<i>Sinularia kavarattiensis</i> (SK3)	<i>Acremonium butyri</i>	Starch casein Agar	Seawater
PR8	Unidentified coral	<i>Aspergillus terreus</i> group	Corn meal Agar	Sodium hypochlorite
PR9	<i>Spirastrella inconstans</i> var. digitata	<i>Aspergillus terreus</i> group	Corn meal Agar	Seawater
PR10	<i>Spirastrella inconstans</i> var. digitata	<i>Acremonium fusidioides</i>	Starch casein Agar	Seawater
PR11	<i>Spirastrella inconstans</i> var. digitata	<i>Acremonium fusidioides</i>	Corn meal Agar	Sodium hypochlorite
PR12	<i>Spirastrella inconstans</i> var. digitata	<i>Acremonium fusidioides</i>	Starch casein Agar	Sodium hypochlorite
PR13	<i>Spirastrella inconstans</i> var. digitata	<i>Aspergillus wentii</i> group	V8 Agar	Sodium hypochlorite

Table 2—Growth and Protease activity of the fungal isolates.

Code	Fungi	Growth on basal medium with 1 % w/v skimmed milk	Activity zone (mm)
PR1	<i>Beauveria brongniartii</i>	Very good	2
PR2	<i>Aspergillus terreus</i> group	Good	
PR3	<i>Aspergillus terreus</i> group	Good	1-2
PR4	<i>Aspergillus terreus</i> group	Good	1
PR5	<i>Aspergillus wentii</i> group	Good	1
PR6	<i>Acremonium fusidioides</i>	Very good	1-2
PR7	<i>Acremonium butyri</i>	Poor	1
PR8	<i>Aspergillus terreus</i> group	Very poor	-
PR9	<i>Aspergillus terreus</i> group	Good	1-2
PR10	<i>Acremonium fusidioides</i>	Very good	1-2
PR11	<i>Acremonium fusidioides</i>	Very good	4
PR12	<i>Acremonium fusidioides</i>	Very good	4
PR13	<i>Aspergillus wentii</i> group	Good	2-3

source of proteases. Optimization of enzyme production and enzyme characterization are the future avenues of research which would reveal the relevance of these proteases in biotechnology.

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