

## Endolithic fungi from deep sea calcareous substrata: isolation and laboratory studies

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**Abstract-** Fungi were isolated from calcareous animal shells at 100 - 860m depth in the Bay of Bengal. All the species isolated were terrestrial ones, *Aspergillus restrictus* Smith being the most common. The behaviour of this fungus at various hydrostatic pressures was examined. Conidia germinated on Czapek-Dox medium with 3 and 20% sucrose and on shells in seawater at hydrostatic pressures up to 300 atm., the percentage of germination being highest in CDA medium with 20% sucrose than in normal CDA and on shells. Conidia of an isolate of *Aspergillus niger* van Tieghem, obtained from intertidal calcareous shells, on the other hand, did not germinate above 1 atm. Upto 512 µg calcium was leached out upon growth of *A. restrictus* on 1 g of calcareous shell substrata at 100 atm. in 25 days. This fungus was not capable of acid production on nutrient media in the presence of various carbon sources, nor did it produce proteases. The role of fungi in calcareous substrata in the sea is discussed with reference to our findings.

### INTRODUCTION

Calcareous animal shells comprise one of the several substrata for colonization by fungi in the sea (KOHLMEYER and KOHLMEYER, 1979; RAGHUKUMAR, 1990). This ecological niche of microorganisms has been termed lithobiontic (GOLUBIC *et al.*, 1981; CAMPION-ALSUMARD, 1989). This term was originally intended only for rocky materials, but is now also used for calcareous substrata of biological origin. Endolithic fungi, namely those which grow within such calcareous substrata have been reported by numerous workers. Among these are fungi from calcareous substrata from intertidal beaches (PORTER and ZEBROWSKI, 1937; CAVALIERE and ALBERTE, 1970; KOHLMEYER, 1969), ascomycetous fungi from coral rocks (KOHLMEYER and VOLKMANN-KOHLMEYER, 1987; 1989) and from live corals (KENDRICK *et al.*, 1981). LAUCKNER (1983) has listed several endolithic parasitic fungi. Thus, an unidentified lower marine fungus has been shown to be associated with black line disease of star corals (RAMOS-FLORES, 1983). RAGHUKUMAR and RAGHUKUMAR (1991) have reported a species of *Scolecobasidium* causing necrotic patches on massive corals near the Andaman islands in the Bay of Bengal. The black wart disease of oyster shells is caused by the fungus *Ostracoblabe implexa* Bornet et Flahault (ALDERMAN, 1976; RAGHUKUMAR and LANDE, 1988).

Endolithic fungi may not be rare in the deep-sea habitats, as several workers have reported extensive fungal borings of calcareous sediments of biological origin from various parts of the world (PERKINS and HALSEY, 1971; ROONEY and PERKINS, 1972; PERKINS and TSENTAS, 1976). In spite of these numerous reports, few experimental studies have been carried out in order to understand the role of these fungi in mineralization of calcium carbonate.

In the present study, we have aimed to isolate and quantify fungi from calcareous substrata in various depths. Such fungi can either be indigenous deep-sea forms capable of growing on shells in the deep sea or "aliens" from terrestrial habitats, merely subsisting

as dormant spores. In order to study this, we have carried out experiments on tolerance of an isolate to various hydrostatic pressures and also its capability to mineralize calcium carbonate.

## MATERIALS AND METHODS

*Isolation of fungi* : Calcareous animal shells were dredged from various depths in the Bay of Bengal during a cruise with the research vessel Nand Rachit in January 1990. The area covered was 80-90° E and 10-20° N. Collected material was immediately transferred to sterile bags. A part of the sample was preserved in formalin. Areas of 5 shell pieces from each site were measured on a graph paper. These pieces were then surface sterilised using 0.5 % sodium hypochlorite (NEWELL and FELL, 1982), suspended in sterile seawater and homogenised in a sterilised pestle and mortar covered with a sterile plastic bag. Aliquots of 0.1 ml sample were then plated on cornmeal agar incorporated with 50 mg streptomycin and 10,000 units of penicillin per 100 ml medium. Five replicates were maintained for each sample. The whole operation was carried out in an inoculation chamber on shipboard. Aerial contamination was checked with a Petri dish containing medium exposed for 1 min in the same working area. Plates were incubated at 28° C in the dark. Five surface sterilised shell pieces of each sample were also incubated in a liquid nutrient medium consisting of 1% yeast extract, 1% peptone and antibiotics as above. Formalin-preserved samples were partially decalcified with 5% EDTA, stained with lactophenol cotton blue and examined under a light microscope.

*Experiments with elevated hydrostatic pressures* : An isolate of *Aspergillus restrictus* Smith, isolated from 650 m depth and *A. niger* van Tieghem, isolated from calcareous shells in the intertidal beaches of Goa were used for the experiments. Since the former species is known to be osmotolerant, we employed Czapek-Dox agar medium with various sugar concentrations besides calcareous shells in unenriched seawater for our experiments. Conidial suspensions of the fungi were obtained by flooding the colonies grown on Czapek-Dox agar (CDA) with sterile seawater and decanting into a sterile flask containing sterile glass beads. The flask was shaken on a shaker for 30 min to get a homogeneous suspension of spores. The following experiments were carried out: (1) Using a glass spreader, a 0.1 ml aliquot of the conidial suspension was spread on a thin layer (2-3 mm) of CDA with 3 and 20% sucrose and on plain seawater agar. Discs of 5 mm diam were cut from the plates and introduced into sterile, disposable syringes filled with seawater and closed with rubber septa at both ends. The syringes were then suspended in a deep - sea bacteria cultivator (Tsurumi Seiki Co., Japan) filled with distilled water and pressurised to the desired hydrostatic pressures. The setup was left undisturbed for 3-4 days. At the end of the experiment, the agar discs were stained with lactophenol cotton blue and the percentage germination of conidia microscopically estimated. (2) Pieces of shells were sterilised in seawater and dipped in a conidial suspension prepared as above for 15 min. They were then transferred to sterile syringes and subjected to different hydrostatic pressures. The shells were removed after 7 days, stained with lactophenol cotton blue and the percentage germination of conidia on the surface was estimated microscopically. Three replicate syringes, each with 5 discs/shell pieces were maintained for all the experiments. Germinating conidia were counted from a total of 20-25 microscopic fields.

*Acid production by the test fungi* : CDA with 3 and 20% sucrose, containing the indicator dye Phenol Red was used to test for acid production by the test fungi. A change from red to yellow in the medium was indicative of acid production and a further deepening of red colour indicative of an alkaline reaction. Acid production under elevated hydrostatic pressures was studied using agar discs.

*Dissolution of calcium from shells:* Sterilised shell pieces inoculated with dense conidial suspensions were subjected to pressures of 1 and 100 atm. and incubated for 20-25 d at room temperature. Controls consisted of uninoculated shell pieces under the same conditions. The calcium content of the seawater medium was estimated after 25 d using a titrimetric method (CULKIN and COX, 1968).

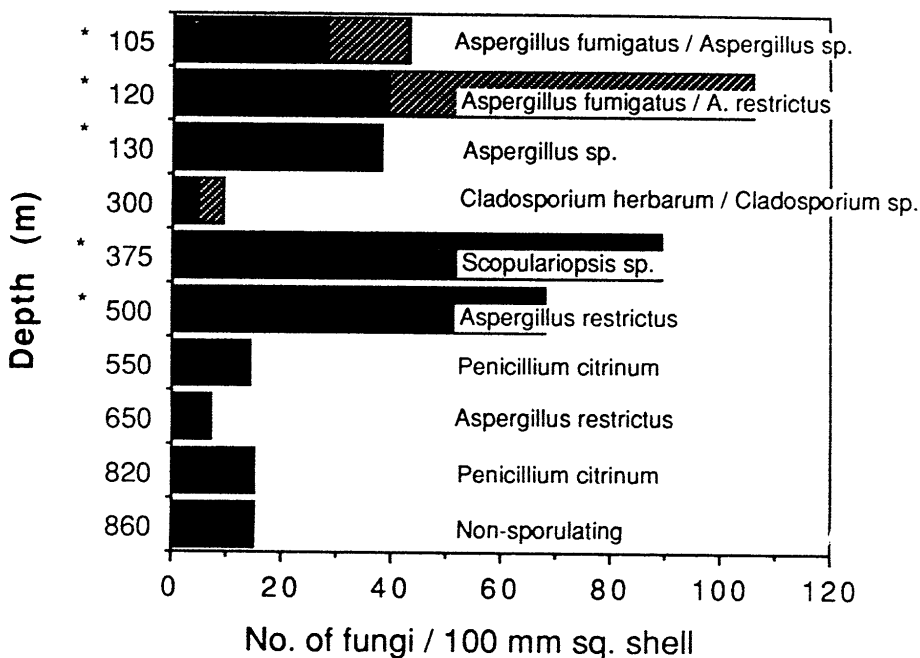


Fig.1. Number and species of fungi in shells collected from various depths. Asterisk indicates coastal stations.

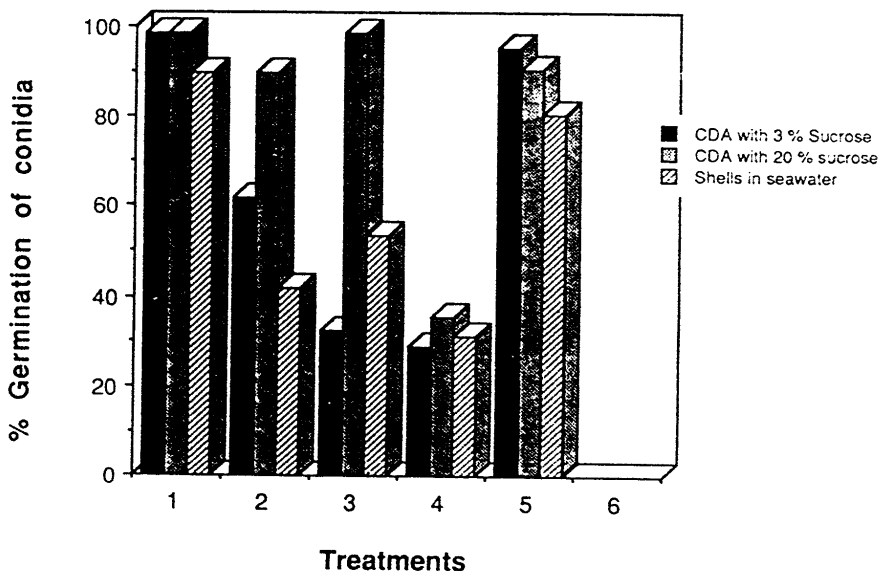


Fig.2. Germination of conidia of *Aspergillus restrictus* and *A. niger* under different treatments. Treatment 1: *A. restrictus* at 1 atm.; Treatment 2: *A. restrictus* at 100 atm.; Treatment 3: *A. restrictus* at 200 atm.; Treatment 4: *A. restrictus* at 300 atm.; Treatment 5: *A. niger* at 1 atm.; Treatment 6: *A. niger* at 100 atm.

## RESULTS

Shells from all the 10 stations sampled, ranging in depth from 105 to 860 m yielded fungi on nutrient agar plates (Fig. 1). All of these, except those from 860 m, were terrestrial hyphomycetes, with *Aspergillus restrictus*, the most frequent (142 of the total 304 colonies belonging to this fungus). This species was isolated from 120,500 and 650 m. All our isolates of this species grew optimally on CDA with 20% sucrose, as is characteristic of this species. A species of *Scopulariopsis* was the next highest in numbers, but this was isolated only from 1 station at a depth of 375m. Shells from coastal stations yielded higher numbers of fungi than those from offshore regions. All the isolates of fungi obtained from 860m, the deepest sample we have examined were nonsporulating and therefore could not be identified.

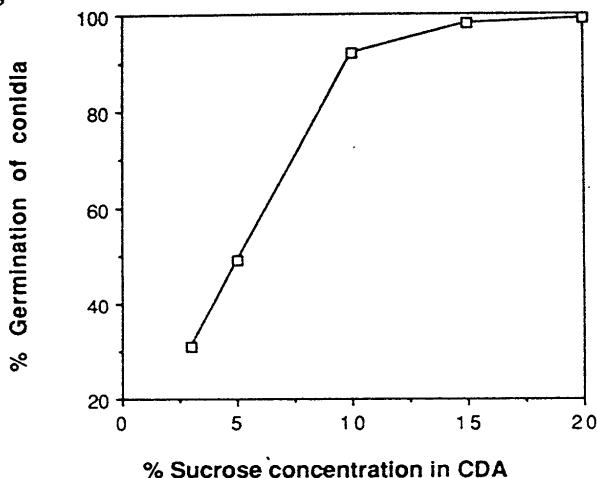


Fig. 3. Percentage germination of conidia of *A. restrictus* in CDA medium with various sucrose concentration at 100 atm.

Table 1. Dissolution of calcium from 1 g shell material.

Treatment	1 atm.	% increase over control	100 atm.	% increase over control
Uninoculated control	230*	0	200*	0
<i>Aspergillus niger</i>	697*	203	206*	3
<i>Aspergillus restrictus</i>	610*	165	512*	156

\*  $\mu\text{g}$  Calcium leached.

Shell pieces incubated in the liquid nutrient medium produced only nonsporulating hyphae. However, these sporulated on transfer to cornmeal agar plates. All were terrestrial hyphomycetes with *A. restrictus* again the most frequent.

Conidia of *A. restrictus* germinated on CDA with 3% and 20% sucrose as well as on the surface of shells up to a hydrostatic pressure of 300 atm. (Fig. 2). Highest percentage of germination at all these pressures occurred in the presence of 20% sucrose. Up to 30% of the conidia germinated on the surface of shells in seawater even at 300 atm. Microscopic examination revealed that conidia on shells could even penetrate the surface

at the end of the 7 d incubation period. Germ tubes of the conidia on shells, however, were stunted compared to those on nutrient media. In contrast to *A.restrictus*, conidia of *A.niger* did not germinate above 1 atm. and at this pressure, the percentage of germination was higher with 3% sucrose than at 20%. When grown on CDA with different concentrations of sucrose at 100atm., *A. restrictus* grew best with 20% sucrose (Fig.3).

Both the test fungi showed considerable growth inside and outside the shell pieces within 5 d after inoculation at 1 atm. pressure. There was an active leaching of calcium from shell pieces under these experimental conditions (Table 1). Dissolution of calcium was much higher in the case of *A.niger* than in *A.restrictus*. At 100 atm., however, no leaching of calcium was observed from shells inoculated with *A.niger*. On the other hand, when shells were inoculated with *A.restrictus* at 100 atm., a 156% increase of calcium was observed in the seawater, compared to the uninoculated controls.

Acid production by the two isolates in nutrient media was examined to check whether leaching of calcium was directly related to this phenomenon (Table 2). At 1 atm., *A.niger* acidified the media in the presence of most of the sugars tested. *A. restrictus*, on the other hand produced only an alkaline reaction. Both the fungi did not acidify the medium at 100 atm. Neither of these fungi also yielded a positive reaction for proteases, either with gelatin or with casein.

Table 2. production of acid in the presence of various carbon sources.

Carbon source	<i>Aspergillus restrictus</i>	<i>Aspergillus niger</i>
Glucose	Alkaline reaction	++
Sucrose	- do -	+++
Fructose	- do -	++
Galactose	- do -	+
Mannose	- do -	+
Maltose	- do -	+
Starch	- do -	+
Cellulose	- do -	+

\* Degree of acidification of growth medium.

## DISCUSSION

Different techniques are available to observe and isolate filamentous marine fungi. Substrata may be directly examined under a stereoscopic microscope for the presence of ascocarps or conidia of obligate marine fungi and the fungi may be identified or cultured from these (KOHLMEYER and KOHLMEYER, 1979). KOHLMEYER and VOLKMAN-KOHLMEYER (1987) have reported marine ascomycetes in coral rocks using this method. Our direct observations of decalcified shells from deep-sea samples frequently revealed hyphae-like filaments. Seawater incubation has been used to isolate marine phycomycetes (NEWELL and FELL, 1982). Numerous terrestrial fungi have been reported from marine macrophytic detritus using surface sterilisation and plating on nutrient media (NEWELL and FELL, 1982). We too obtained only terrestrial fungi inspite of using surface-sterilisation techniques (Fig.1). While seawater incubation was not used in this study, incubation in a dilute liquid medium was carried out. This too, however, yielded only terrestrial species. Although terrestrial species of fungi have been isolated from deep-sea sediments (ROTH *et al.*, 1964; HOEHNK, 1969), these have been considered contaminants or aliens in the form of dormant conidia rather than as an active microbial component growing in the form of hyphae (KOHLMEYER and KOHLMEYER, 1979). Extensive fungal borings of calcium carbonate sediments have been described from 210-1450 m in the Bahamian waters (ZEFF and PERKINS, 1979)

and at depths up to 758 m from Carolina shelf sediments (PERKINS and HALSEY, 1971). Fungal infestation of star corals, resulting in black line disease has been reported by RAMOS-FLORES (1983). No isolations were carried out by this author. KENDRICK *et al.* (1982) have isolated a number of terrestrial species of fungi from live corals. RAGHUKUMAR and RAGHUKUMAR (1991) have reported a species of the terrestrial fungus *Scolecobasidium* from diseased massive corals. While the question as to whether terrestrial species of fungi are active in deep-sea calcareous animal shells can be resolved unambiguously only using techniques such as immunofluorescence, we have attempted to examine it using the pressure tolerance of one of the frequently isolated fungi from our experiments.

Conidia of the isolate of *Aspergillus restrictus* studied by us, germinated at 300 atm. even on plain shells immersed in seawater (Fig.2). This fungus can be classified as "barotolerant", namely as one which can tolerate and grow at elevated hydrostatic pressures as well as under normal pressure (ZOBELL and MORITA, 1957). The fact that the conidia of the terrestrial isolate of *A.niger* did not germinate even at 100 atm., suggests that *A.restrictus* is an active component in deep sea shells. Our preliminary experiments have shown that the isolate of *A.restrictus* grows much faster at 7-10<sup>o</sup> C than *A.niger*, suggesting a further adaptation to the deep sea habitat. An additional support for this view comes from the fact that this fungus was capable of substantially leaching out calcium from the shells even at 100 atm. Endolithic cyanobacteria are capable of leaching out calcium from calcareous animal shells (RAGHUKUMAR *et al.*, 1988). One of the modes of dissolving calcium from the shells would be by production of organic acids upon growth. Bacteria involved in the decay of stones are known to acidify the growth medium by changing the pH from 6.8 and 7.4 to 4.9 and 5.2, in the presence of various carbon sources (LEWIS *et al.*, 1988). Biodeterioration of sandstone monuments by production of acids and dissolution of calcium has been recently demonstrated in fungi (PETERSON *et al.*, 1988). *Aspergillus restrictus*, however, was not capable of this under our experimental conditions (Table 2). Nor did it elaborate proteases, which by virtue of removal of the protein matrix in the shells might have caused calcium leaching. We have not examined the involvement of "siderophores", namely ferric ion specific ligands which have been shown to be involved in fungal etching of microfractures in stones (see KRUMBEIN, 1988).

Our studies suggest that terrestrial fungi may colonise and actively grow in the deep sea. A substratum, where they might possibly play an important role is in that of calcareous animal shells. Shell-boring microorganisms are considered to be important as organic eroders of calcium carbonate skeletons and as producers of fine carbonate detritus (PERKINS and HALSEY, 1971; TUDHOPE and RISK, 1985). The role of fungi in these processes deserves to be studied.

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