

Spore germination of fungi belonging to *Aspergillus* species under deep-sea conditions

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Abstract

Wind-blown dry fungal spores and mycelial fragments from the nearest landmass or terrestrial runoff may find their way to the deep sea by hitching a ride on other sinking detrital particles. Once in the deep, they are affected by elevated hydrostatic pressure, low temperature and low nutrients. We have examined effects of these on germination of spores from a few deep-sea *Aspergillus* isolates. Spores from most of the fungi germinated under elevated hydrostatic pressure at 30°C. The ambient temperature of the deep sea, ~4-5°C, was found to inhibit spore germination totally. Sediment extracts prepared in seawater promoted spore germination as did additions of dimethylsulfoxide and sucrose, but only at 30°C/200 bar pressure and not at 4-5°C. Heat shock of 15 min at 50°C helped in breaking the dormancy of the spores and induced germination at 5°C at 1 bar pressure but not at 200 bar. More than 90% of the spores from several deep-sea *Aspergillus* isolates and the terrestrial isolate of *Aspergillus terreus* lost viability within 16-17 days of incubation at 5°C/1 bar. About 2-3% remained viable for more than 3 months at 5°C/1 bar. Mycelial fragments showed growth and biomass production under elevated pressure at 5°C. These results indicate that building biomass under deep-sea conditions from spores is not a viable option for the deep-sea *Aspergillus* isolates. Mycelial fragments, on the other hand, are more likely to grow.

Key words: Fungal spore germination, deep- sea fungi, *Aspergillus*, hydrostatic pressure, sediment extract, Central Indian Basin

1. Introduction

The deep sea is an extreme environment characterized, among other features, by high hydrostatic pressure and a temperature of 1-2°C. Raghukumar et al. (2004) and Damare et al. (2006) have recently demonstrated the presence of fungi in deep-sea sediments from the Central Indian Basin (CIB) from depths of ~ 5000 m. Most of the fungi reported from the deep sea have been the terrestrial fungi, also referred to as geofungi (Roth et al., 1964; Raghukumar et al., 1992; Raghukumar and Raghukumar, 1998; Damare et al., 2006). Several instances of active involvement of geofungi in marine processes have been reported. These include fungi in calcareous shells (Raghukumar et al., 1992), in live and dead corals (Kendrick et al., 1982; Bentis et al., 2000; Golubic et al., 2005), and as pathogens of sea fans (Smith et al., 1996) and plant litter systems (Raghukumar, 2006). Dry spores of terrestrial fungi can easily be transported by wind and reach new habitats such as the marine environment. It has been suggested recently that spores of *Aspergillus sydowii*, blown from Saharan deserts across the Atlantic Ocean during dust storms, reach the Caribbean islands and cause aspergillosis disease in seafans (Shinn et al., 2000). Spores and fungal mycelia may also arrive in oceans through terrestrial run-off and currents and get deposited in the deep sea by sedimentation in association with other particles (Raghukumar and Raghukumar, 1998). It is likely that such fungal spores may go into dormancy, or a few of them may germinate when conditions are favourable.

Studies on fungal spore germination can prove to be the first step in understanding the presence of fungi in deep-sea sediments and the role played by them in the nutrient dynamics of the deep-sea environment. Germination of fungal spores in the deep sea may face several obstacles like the mycostatic effect of seawater (Kirk, 1980), low temperature, elevated hydrostatic pressure and low nutrient conditions. A defining characteristic of spores is their ability to develop into a new individual without fusion with another reproductive cell. Spores thus differ from gametes, which are reproductive cells that must fuse in pairs to give rise to a new individual. Fungal spores are considered the beginning and end of the development cycle (Smith, 1975), as they can remain dormant for several years (Raghukumar *et al.*, 2004) and develop into a vegetative form when favourable conditions are presented to them. Fungal spores are committed to the formation of an actively growing hyphal colony. The first step in this is the spore germination, which can be defined as the sequence of events that converts the resting/dormant spore into a rapidly growing germ tube from which the mycelium is produced by elongation, septum formation and branching (Nielson, 1992; d'Enfert, 1997). Favourable

environmental factors are required for activation of spore germination, and these may vary between spore types, both within a species and between species (Wicklow and Carroll 1992). However, water, oxygen and carbon sources are universally required for activating spore germination. Additional factors are required, in particular low-molecular-weight nutrients like sugars, amino acids and inorganic salts, albeit at low concentrations (Carlisle and Watkinson, 1994; Griffin, 1994). A spore is defined as having germinated if the germ tube is at least as long as the width of the spore. Because of its importance in the life cycle of fungi, spore germination has received considerable attention. An extensive literature exists on the physiological requirements and metabolic changes that occur during germination (Schmidt and Brody, 1975; 1976; Van Etten *et al.*, 1983; Brambl, 1985). In the present study, we have compared the effect of the extreme conditions found in the deep sea, pressure, temperature and nutrients, on the germination of spores of the deep-sea *Aspergillus* isolates and their viability. Finally, we have tried to induce germination of spores using various triggering factors such as sugars, dimethylsulfoxide (DMSO) and heat shock, the latter two of which have been reported to protect yeast cells against the damage caused by hydrostatic pressure (Komatsu *et al.*, 1991).

2. Materials and Methods

2.1 Fungal cultures

Aspergillus species (A 4634, NIOCC 20, A 6137 and A 617) obtained from the deep-sea sediments from the Central Indian Basin from depths of ~5000 m (Damare *et al.*, 2006) and for comparison a terrestrial *Aspergillus terreus* (MTCC 479) obtained from Microbial Type Culture Collection (Chandigarh, India), were used.

2.2 Media

Sediment extract was prepared using sediments collected from the Central Indian Basin. Sediment (30 % (w/v)) was suspended in sterile seawater containing antibiotics (penicillin - 40,000 U and streptomycin - 0.1 g). To this, 1% EDTA was added and the contents were mixed on a shaker overnight. The contents were filtered sequentially through GF/F (Whatman, USA) and 0.22 μm nitrocellulose membranes (Millipore, USA) under sterile conditions. This sediment extract (SE) was used as one of the growth media. Malt extract broth (MEB, HiMedia Laboratories, India) prepared in seawater was used for the comparative studies.

2.3 Fungal spore suspension

The cultures were inoculated onto malt extract agar (MEA) plates and incubated at 1 bar pressure and 30°C until sporulating fungal colonies appeared (within 3-5 days). Spores were collected from the surface of such fungal colonies by gently scraping with a loop and suspended in 10 ml sterile seawater containing 2 % (v/v) Tween 80. This suspension was mixed vigorously by vortexing for 15-20 min. The spore suspension stock was diluted in sterile seawater to obtain a concentration of $5-7 \times 10^4$ spores ml^{-1} . The spores were enumerated by direct counting using a Neubauer chamber.

2.4 Germination

Microscopic examinations for germination of spores were conducted after incubation for 20 days. A rectangular cover slip (22 x 50 mm) was placed onto a drop of spore suspension (25 μl) and the whole area screened microscopically at 400x for germination of spores. A spore was considered germinated when the germ tube length was equal to or greater than one-half the spore diameter (Bosch et al., 1995). Germinated spores were counted and recorded as a percentage of the total spore number.

2.5 Germination at various hydrostatic pressures and temperatures

Spores of a few deep-sea *Aspergillus* isolates, and one terrestrial *Aspergillus* isolate, were tested for their ability to germinate under various hydrostatic pressures and temperatures. The deep-sea cultures used were

A 4634 (*Aspergillus terreus*), NIOCC 20 (*Aspergillus ustus*), A 6137 (*Aspergillus* sp.) and A 617 (*Aspergillus* sp.); and the terrestrial culture MTCC 479 (*Aspergillus terreus*) was obtained from a culture collection centre (Microbial Type Culture Collection, Chandigarh, India).

Spores of all the cultures were appropriately diluted so as to get the optimum concentration of spores ($5-7 \times 10^4$ spores ml^{-1}) and inoculated in SE and MEB in sterile plastic pouches as described in detail by Damare et al. (2006). These were then incubated at 1, 100, 200, 300 and 500 bar pressure at 30° and 5°C in a deep-sea culture vessel (Tsurumi & Seiki Co. Japan). After an incubation period of 20 days, the spores were stained with an optical brightener, Calcofluor (Sigma Chemicals, USA), as described by Damare et al. (2006), and examined under an epifluorescence microscope (Olympus BX 60, Japan) for germination.

2.6 Viability of spores at low temperature

Appropriately diluted spores from each culture in seawater containing 1% Tween 80 were incubated at 5°C for 90 days and tested for their viability. At the end of each incubation period the spore suspension were transferred to 30°C and incubated further for 24 hours (time taken for germination at 30°C and 1 bar pressure was 24 h) and examined for germination.

2.7 Effect of nutrient concentration on spore germination

The sediment extract as described above was diluted with seawater to obtain 1:10, 1:20, 1:40, 1:60, 1:80, and 1:100 dilutions. Appropriate concentrations of spores of *Aspergillus* isolates were inoculated in each sediment extract and incubated at 200 bar / 30°C, 200 bar / 5°C, 1 bar / 30°C and 1 bar / 5°C. The spores were examined for germination microscopically after an incubation period of 20 days.

2.8 Effect of additives on spore germination

Different additives such as dimethylsulfoxide (DMSO), which offers protection to vegetative cells against pressure and low temperature (Komatsu et al., 1991), and sugars (glucose and sucrose) were tested for possible effects on germination of spores. Spores of appropriate concentration were inoculated separately in MEB with DMSO (1% (v/v)) and sugars (10 and 20 % (w/v)). The suspensions were incubated at 200 bar / 30°C, 200 bar / 5°C, 1 bar / 30°C and 1 bar / 5°C. The spores were examined microscopically for germination after 20 days.

2.9 Effect of heat shock on spore germination

Heat, if provided in a controlled manner in the form of a shock, is known to break the dormancy of the spores and induce germination and vegetative growth (Garraway and Evans, 1984). Spore of appropriate concentrations were suspended in MEB and exposed to 50, 60, 80 and 100°C for 15 min. for determining the temperature of non-lethal heat shock. After heat shock, the spores were incubated at 1 bar / 30°C and examined for germination after 24 hours. Spores not exposed to heat treatment were used as the control.

2.10 Combined effect of heat shock and additives on spore germination

After determining the appropriate non-lethal temperature for heat shock and the concentration of additives, their combined effect on spore germination under different conditions

of hydrostatic pressure and temperature was examined. Spore suspensions of appropriate dilutions made in MEB (with 20% sucrose (w/v)) and exposed to a heat shock of 50°C for 15 min. These were then incubated under different conditions of hydrostatic pressure and temperature of 200 bar / 30°C, 200 bar / 5°C, 1 bar / 30°C and 1 bar / 5°C. The spores were examined for germination after 20 days of incubation.

3. Results

Spores of all the 5 deep-sea and terrestrial *Aspergillus* isolates tested germinated at 30°C under all the hydrostatic pressures examined (Table 1). However, the % germination decreased gradually with the increasing pressure. The trend in reduction of germination was similar for each of the 5 cultures, with NIOCC 20 showing a higher percentage of germinated spores than the other four cultures (Table 1). Percentage germination of spores in different isolates under various hydrostatic pressures was highly significant (at >0.1 % level) as demonstrated by 2-way analysis of variance (Table 1). The spores failed to germinate at a low temperature of 5°C, even at 1 bar pressure during an incubation time of 20 days.

The viability of the *Aspergillus* spores decreased over a period of time at 5°C. The deep-sea cultures A4634 and NIOCC 20 showed 100 % viability up to 7 days. In A617 and MTCC479 viability has declined by more than 80 % by the day 6 (Fig 1). There was a drop of 80 % in viability of spores of NIOCC 20 and A 4634 by the 9th day. The viability decreased gradually to 4 and 2 %, respectively, in these 2 cultures on the day 16. The other two cultures also showed similar loss in viability by the 16th day (1.7 and 1.8 %). The percentage viability of spores recorded on the 16th day remained unchanged even after 3 months of incubation at 5°C.

There was a very gradual decrease in the % germination of spores with decreasing nutrient concentration (Table 2). Two-way ANOVA revealed significant differences in % germination of fungal spores under various pressures and concentrations of sediment extract indicating a strong influence of these two factors on spore germination (Table 2). The spores failed to germinate at low temperature (5°C). The total organic carbon (TOC) of the sediment extract, was 218 mg ml⁻¹, higher than that of 1/5 strength MEB (58 mg ml⁻¹) used in all the experiments.

Addition of DMSO exerted a positive effect on the germination of spores of 3 out of the 4 cultures tested, both at 1 bar and 200 bar pressure, but it failed to induce germination at low temperature (Table 3). The increase in % germination was more prominent at 200 bar than at 1

bar, indicating that DMSO offers protection against elevated hydrostatic pressure (with the exception of *Aspergillus* strain A 617). Glucose and sucrose also showed a positive effect on the spore germination of all the cultures under elevated pressure at 30°C, but no germination was seen at 5°C.

Heat shock of 50°C did not affect germination of the spores at 1 bar pressure, while a temperature of 60°C and above proved lethal, inhibiting the spore germination of all the cultures. In the combination of sucrose at 20 % concentration and heat shock of 50°C for 15 min, the spores of all the cultures germinated at 1 bar / 5°C but not at 200 bar/ 5°C (Table 4).

4. Discussion

Spores of *Aspergillus* spp., common terrestrial fungi, were used in the present study since this was the most dominant fungus of the cultivable mycelial fungi isolated from deep sea-sediments (Damare et al., 2006). Many terrestrial species of mycelial fungi, including *Aspergillus* spp., have been isolated by conventional culture techniques (Roth et al., 1964; Takami 1999; Raghukumar et al., 2004). Recently, Takishita et al. (2007) showed the presence of *Aspergillus niger* and several other terrestrial fungi in the rDNA libraries of sediments from a deep-sea methane cold seep. Thus, the presence of ubiquitous *Aspergillus* in the deep sea has been established by both molecular and by culture-dependent techniques. However, it is possible that so far obligate, deep-sea marine fungi have not been isolated by culture methods. Sequence data of small-subunit ribosomal DNA (SSU rDNA) obtained from various deep-sea environmental samples have shown the presence of novel lineages belonging to fungi (López-García et al., 2001; Stoeck and Epstein, 2003; Stoeck et al., 2003). Alternative methods may have to be devised to bring such indigenous obligate marine deep-sea fungi into culture.

Spores of several terrestrial fungi, such as *Aspergillus*, are disseminated by air. Such terrestrial fungi sporulate only when exposed to air and not in submerged cultures (Vézina et al., 1965). On the other hand, aquatic fungi often sporulate under submerged conditions (Sridhar and Bärlocher, 1997). *Aspergillus* being a typical terrestrial fungus did not sporulate under deep-sea conditions (personal observation). Since such terrestrial fungi are not likely to sporulate and also since germination of their spores under deep-sea conditions is very limited, it is possible that such fungi that have developed adaptations to grow under deep-sea conditions can propagate only by mycelial fragments.

In the present study, the viability and germination of spores was not inhibited by elevated hydrostatic pressure as much as by the low temperature of 4-5°C. The isolates from the deep-sea and the terrestrial cultures showed principally a similar response to the two conditions studied. Zaunstöck and Molitoris (1995) have also demonstrated that low temperatures and increasing pressure retard germination of fungal spores. However, this inhibition was not an irreversible process. Germination of fungal spores at elevated hydrostatic pressures at 30°C is a possibility in the vicinity of hydrothermal vent areas, where such conditions are likely to exist.

The protective effect of heat shock against hydrostatic pressure in yeast cells was demonstrated by increased cell viability (Iwahashi et al., 1991). Such an induced barotolerance was similar to the thermotolerance induced by heat shock in yeast cells (Iwahashi et al., 1991). We also observed germination of spores at 5°C after a heat shock treatment. However, this shock was not effective enough against the combined effects of low temperature and elevated pressure. On the contrary, addition of DMSO gave protection against elevated pressure, but not against low temperature. In yeast cells (*Saccharomyces cerevisiae*), DMSO treatment also offered protection against damage caused by hydrostatic pressure (Komatsu et al., 1991). However, the concentration of DMSO used by us was much higher than what is reported to be present in oceanic waters (Simó et al., 2000). Thus it appears that spores of *Aspergillus* need a lot of induction (physical and chemical) to germinate under deep-sea conditions. We do not know at present whether this is a general phenomenon or is applicable only to deep-sea *Aspergillus* isolates in particular. Further studies on the response of spores of other mycelial fungi to deep-sea conditions will throw light on this.

As hypothesized earlier (Raghukumar and Raghukumar, 1998; Damare et al., 2006), wind - blown fungal spores and those arriving with terrestrial run-off may slowly sink into the deep sea. The present results indicate that about 90% of these lose their viability within 15 days (at 5°C) and the rest retain their viability even up to 3 months (in the present studies) and probably for longer periods as reported by Raghukumar et al. (2004). Such spores are capable of germinating into new mycelia when presented with favourable conditions. We do not know what these "favourable conditions" are which determine the germination of the remaining percentage of *Aspergillus* spores. These results further indicate that the viable fungal cultures recovered from deep-sea sediments may not always originate from spores as most of the spores do not remain viable for a long period under these conditions. Thus, it appears that low temperature causes irreversible damage to spores of *Aspergillus* isolates used in our present

studies. Such recovered fungi may be derived from mycelial fragments, which show active growth (Damare et al., 2006).

Although low nutrient concentrations as such do not prevent spore germination under atmospheric pressure, they substantially affect germination in combination with elevated hydrostatic pressure (Table 2). Low temperature, acting as an additional stress factor, totally inhibits this process. Irrespective of their terrestrial or marine origin, mycelial inocula of fungi produced substantial growth at both 30° and 5°C under elevated hydrostatic pressure (Damare et al., 2006). These authors demonstrated that fungal mycelial fragments are more tolerant to elevated hydrostatic pressure at low temperature than fungal spores.

The fungistatic effect of unfavourable environmental conditions is a well-known phenomenon (Steiner and Lockwood, 1969; Lockwood, 1992) and is more pronounced on spores than mycelial fragments (Ivanova and Marfenina, 2001). Thus in conclusion, mycelial fragments have better chances of propagation under deep-sea conditions than do spores. Further, as observed by Ivanova and Marfenina (2001), we suspect that the critical size of mycelial fragments may also influence the survival of fungal species under stressful environmental conditions.

Mycelial fragments, in the active metabolic state, may adapt to the stressful conditions of the deep- sea by producing cold shock or pressure shock proteins. Exposure of both prokaryotic and eukaryotic cells to a variety of physiological stresses results in the rapid synthesis of a specific class of proteins. This phenomenon is known as “stress response”, and the newly formed transient proteins are termed “stress proteins” (Welch et al., 1993). These proteins play a significant role in cells in manifestation of adaptations and may serve as a defense mechanism against a variety of stress conditions. Therefore, a suitable approach to understanding the survival and growth of mycelia in deep sea would be to look for stress proteins in fungi grown at different hydrostatic pressures and low temperatures.

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Legends to the figure

Fig. 1. Viability of *Aspergillus* spores (in percentage) of deep-sea (# NIOCC 20 \triangle , # A4634 \square , # A617 \bullet) and terrestrial (\circ # MTCC 479) isolates at 5°C and 1 bar pressure.

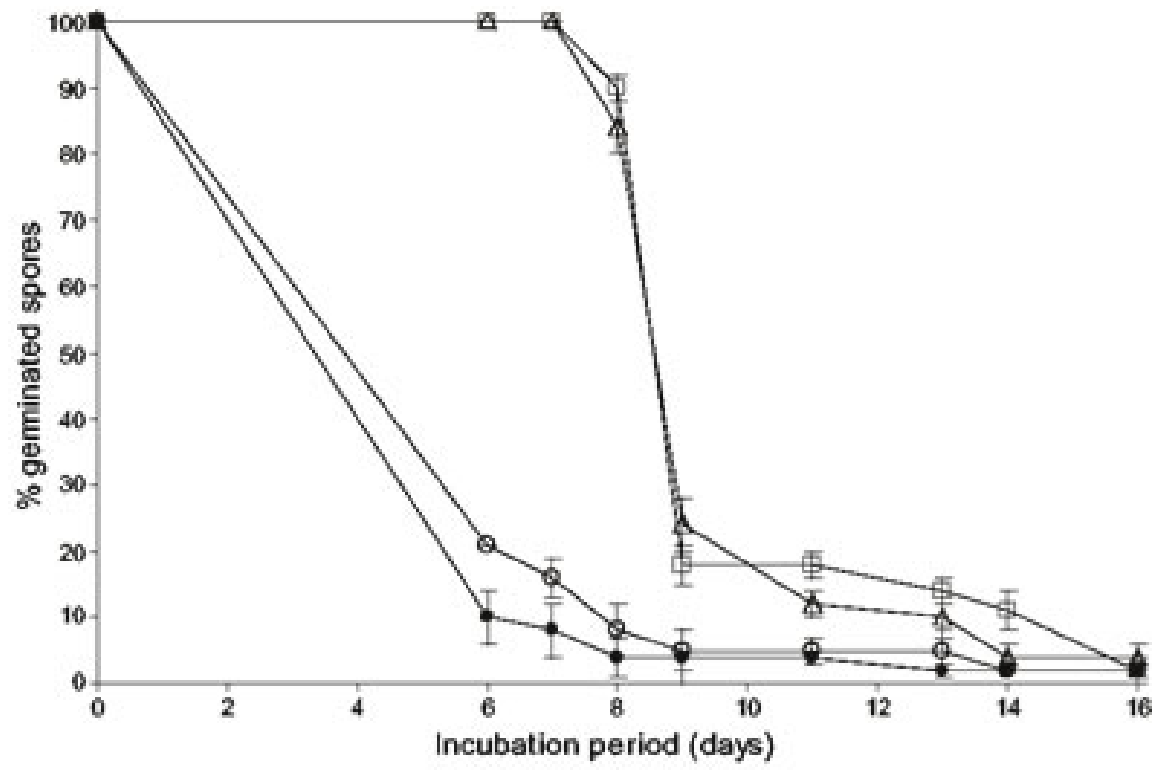


Fig 1

Table 1. Spore germination of deep-sea and terrestrial *Aspergillus* isolates at elevated hydrostatic pressures and 30°C

Incubation pressure (Bar)	Germination of spores (%)				
	A 4634 (deep-sea isolate)	NIOCC 20 (deep-sea isolate)	A 6137 (deep-sea isolate)	A 617 (deep-sea isolate)	MTCC 479 (terrestrial isolate)
1	94	100	89	92	90
100	10	29	16	22	21
200	9	25	20	20	19
300	5	16	12	12	5
500	3	8	5	5	6
2-way ANOVA between incubation pressures			2-way ANOVA between cultures		
F-critical	3.0			3.0	
Observed F	582.25			7.66	
df	4			4	
P-value	4.2 E-17***			0.0012***	

No germination was observed at 5°C at any of the hydrostatic pressures.

F-value higher than F-critical value indicates significant difference between the pressure incubation and different cultures. *** Indicates highly significant differences between incubation pressures and between fungal cultures as analyzed by 2-way ANOVA.

Table 2. A comparison of spore germination of deep-sea and terrestrial *Aspergillus* isolates, in different concentrations of sediment extract at 30°C under 1 and 200 bar pressure

Concentration of sediment extract (SE)	% Germination of spores							
	A 4634 (deep-sea isolate)		NIOCC 20 (deep-sea isolate)		A 617 (deep-sea isolate)		MTCC 479 (terrestrial isolate)	
	1 bar / 30°C	200 bar / 30°C	1 bar / 30°C	200 bar / 30°C	1 bar / 30°C	200 bar / 30°C	1 bar / 30°C	200 bar / 30°C
1 (undiluted)	100	36	100	23	100	19	100	28
1 : 10	100	31	100	26	114	20	90	25
1 : 20	93	30	100	19	78	21	82	21
1 : 40	77	35	100	24	77	17	77	20
1 : 60	73	27	96	20	73	10	79	13
1 : 80	65	22	88	10	70	10	67	7
1 : 100	51	12	63	10	60	9	52	9

Two-way ANOVA showing the effect of pressure incubation conditions and the sediment extract (SE) dilutions on spore germination of fungi examined in the study

Two-way ANOVA	A 4634		NIOCC 20		A 617		MTCC 479	
	Incubation conditions	SE dilution	Incubation conditions	SE dilution	Incubation conditions	SE dilution	Incubation conditions	SE dilution
F-critical	5.99	4.28	5.99	4.28	5.99	4.28	5.99	4.28
Observed F	122.22*	4.34*	435.75*	4.29*	133.55*	2.25	307.13*	6.3*
df	1	6	1	6	1	6	1	6
P-value	3.26 E-5	0.0485	7.87 E-7	0.0497	2.52 E-5	0.1735	2.21 E-5	0.021

SE- Sediment extract

No germination was observed at 5°C, either at 1 bar or at 200 bar.

* Observed F-value greater than F-critical indicates significant difference between pressure incubation conditions of 1 bar / 30°C & 200 bar / 30°C and sediment extract at 1:10, 1:20, 1:40, 1:60, 1:80 and 1:100 dilutions.

Table 3. Effect of DMSO on spore germination of deep-sea and terrestrial *Aspergillus* isolates at 30°C under 1 and 200 bar pressure

Incubation conditions	% germination							
	A 4634 (deep-sea isolate)		NIOCC 20 (deep-sea isolate)		A 617 (deep-sea isolate)		MTCC 479 (deep-sea isolate)	
	MEB		MEB		MEB		MEB	
	MEB	+	MEB	+	MEB	+	MEB	+
	DMSO		DMSO		DMSO		DMSO	
200 bar / 30°C	30 (± 5)	36 (± 5)	26 (± 6)	45 (± 4)	23 (± 6)	17 (± 4)	24 (± 4)	30 (± 4)
1 bar / 30°C	94 (± 3)	100	89 (± 7)	94 (± 7)	89 (± 3)	84 (± 2)	88 (± 4)	92 (± 2)

No germination when incubated at 1 bar / 5°C or 200 bar / 5°C.
 Values within brackets indicate standard deviation (SD)

Table 4. Comparison of spore germination of deep-sea and terrestrial *Aspergillus* isolates under various pressure and temperature conditions

Incubation condition	% germination								
	A 4634 (deep-sea isolate)			NIOCC 20 (deep-sea isolate)			MTCC 479 (terrestrial isolate)		
	A	B	C	A	B	C	A	B	C
200 bar / 30°C	14 (\pm 4)	12 (\pm 3)	16 (\pm 4)	6 (\pm 2)	9 (\pm 2)	8 (\pm 3)	13 (\pm 3)	16 (\pm 4)	18 (\pm 4)
200 bar / 5°C	0	0	?	0	0	0	0	0	0
1 bar / 30°C	100	100	100	100	100	100	100	100	100
1 bar / 5°C	0	0	100	0	?	100	0	0	100

A – Malt extract broth (MEB); B – MEB with 20 % sucrose; C – Spores given a heat shock of 50°C for 15 min and then incubated in MEB with 20 % sucrose.
 ? – germination was doubtful, could not be confirmed as per the definition in the text.
 Values within brackets indicate standard deviation (SD)

