Conidial germination of marine-derived fungi under simulated deep-sea conditions in the presence of inducers

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Abstract
The abilities of conidia of four deep-sea fungi, *Aspergillus flavus* NIOCC20, *A. sydowii* CH2, *A. terreus* A4636 and *Penicillium* sp. A4623 and one terrestrial fungus, *A. terreus* MTCC479 to germinate under simulated deep-sea conditions were investigated by exposing the conidia to low temperature (4 °C) and elevated hydrostatic pressures (10, 20, 30, 40, 50 and 60 MPa). The conidial germination was evaluated in presence of chemical inducers, including Thiamine, Sodium pyruvate, Hydrochloric acid, Sulphuric acid and Sucrose, in a basal medium of 1/5th strength of malt extract broth and subjected to heat treatment at 70 °C for 60 min, and incubated for 30 days under simulated deep-sea conditions. The conidia of *Aspergillus flavus, A. sydowii* and *Penicillium* sp. germinated and developed into mycelia under almost all the treatments with and without heat inducement. However, the number of germinated conidia of *A. terreus* A4636 and *A. terreus* MTCC479 was lower than the other three fungal species. This is the first study demonstrating *in vitro* germination of conidia of deep-sea fungi at low temperature with elevated hydrostatic pressures. Further studies are warranted to obtain insights into *in situ* conditions that are favourable for conidial germination and fungal activity in deep-sea niches.

Keywords: chemical-inducers; heat-inducement; high-pressure; low-temperature; viability

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Introduction

The deep-sea ecosystem is characterised by high pressure and predominantly low temperatures (Danovaro et al. 2008). The biotic components surviving therein are capable of enduring these extremities, and show high diversity (Nagano and Nagahama 2012). However, the combined effect of high pressure and low temperature slows down many physiological and metabolic processes (Dover 2000). Biotic components and *in situ* diagenetic processes in the deep-sea sediments play a significant role in organic carbon turnover (Jannasch 1994). Deep-sea fungi can occur as conidia or in mycelial form (Zhang et al. 2014). These conidia germinate to form vegetative mycelia or remain dormant, till the germination is triggered by some events or substances (Gougouli and Koutsoumanis 2012). Thus, insights into the germination of conidia under high pressure and low temperature are crucial in understanding possible roles played by fungi in nutrient dynamics in the deep-sea ecosystem (Raghukumar 2017). Damare et al. (2006) evaluated the germination potential of conidia which were exposed to 20 MPa pressure but at a higher temperature of 30 °C. The conidia reportedly

germinated into mycelia, with mycelia displaying slower growth and abnormalities such as bloating. Damare (2007) reported the presence of both fungal mycelia and conidia without any sign of germination in the deep-sea sediments examined microscopically. Several studies highlighted that germination could be triggered by a variety of physical factors such as heat and chemical additives such as flavonoids, dimethyl sulfoxide and sugars (Cochrane 1974, Ruan et al. 1995, Damare et al. 2006).

Furthermore, subsequent studies by Dango et al. (2011) and Jermy (2010) reported that conidia of deep-sea fungi remain dormant and lose viability at low temperatures. To the best of our knowledge, there is no evidence for conidial germination of deep-sea fungi at low temperature and elevated pressure conditions that exist in the deep-sea. A few reports are available about growth of filamentous fungi as well as yeasts at elevated hydrostatic pressures (Lorenz and Molitoris, 1997, Raghukumar and Raghukumar, 1998, Damare et al. 2006, 2008, Singh et al. 2010). In this study, conidial germination of four fungi isolated from the deep-sea sediments was investigated by simulating deep-sea conditions in the laboratory. A terrestrial isolate of *Aspergillus terreus* was included as a control. Additionally, the effects of heat-treatment and six different chemical inducers on conidial germination of the fungi under high pressure (10, 20, 30, 40, 50 and 60 MPa) and low-temperature (4 °C) conditions were examined.

Materials and methods

Fungal isolates

Four deep-sea fungi used in this study were isolated by Damare et al. (2006) from sub-seafloor sediments (0–5 cm sections of sediment core) of the Central Indian Ocean Basin (CIOB) from the water column depths of 4500–5500 m by dilution plating, particle plating and pressure incubation methods. The agar plates with sediment samples were incubated for 20–30 days. Dilution plating allowed the isolation of slow-growing fungi with this duration of incubation. The pressure incubation method (Damare et al. 2006) followed in this study resulted in the isolation of barotolerant fungi. Of the 181 isolates obtained, *Aspergillus terreus* A4636, *A. flavus* NIOCC20, *A. sydowii* CH2 and *Penicillium* sp. A4623 were selected for further processing based on their high abundance in the deep-sea sediments (Damare 2007). *Aspergillus* and *Penicillium* species are recognised as globally distributed fungal taxa abundant in the deep-sea sediments (Singh et al. 2010, Xu et al. 2014, Zhang et al. 2014, Jones et al. 2015). *Aspergillus terreus* MTCC479 / #IM2, of terrestrial origin, was obtained from the Microbial Type Culture Collection (MTCC), Chandigarh, India and included in the study as a control.

Preparation of conidial suspension

Fungal cultures were grown on one-fifth strength malt extract agar (MEA, HiMedia M137, India) for 1–2 days at 30 °C until visible sporulation occurred. The agar plates were then flooded with 0.1% Tween 80 (HiMedia RM159, India) to harvest conidia. The conidial suspensions were serially diluted with 0.1% Tween 80 solution (prepared in seawater collected from the CIOB), and the concentrations of these conidial suspensions were determined by a haemocytometer. To determine the conidial count, 100 µL suspension was spread on a haemocytometer. The conidia in each of the 25 squares were counted, and the average counts from 10 fields of 25 squares were recorded. The conidial suspensions were vortexed to reduce their clumping before enumeration and during inoculation. The conidial suspensions with 30–50 × 10^6 conidia mL^{-1} were kept in the ice and used as inocula for all the further experiments involving pressure incubations.

Conidial germination experiment

For each culture, a total of 96 plastic pouches were prepared for pressure incubations. These 96 pouches were grouped into eight sets of 12 pouches each. The final volume of total suspension in each pouch was 1 mL. In each pouch with 1/5th strength malt extract broth (MEB, HiMedia, M255, India) a final volume of conidial suspension at a concentration of 30–50 × 10^6 conidia mL^{-1} was transferred and subjected to various chemical inducer-treatments and brief heat treatment. The chemical inducers (filtered through 0.22 µm pore sized filters) and their final concentrations used in this study were: Hydrochloric acid (Qualigen 29147, India) and Sulphuric acid (Qualigen Q29997, India) at 0.02 N (Lewis et al. 1991), Sodium pyruvate (Fluka 15990, India) at 0.1 M (Laere et al. 1982), Thiamine (HiMedia CMS182, India) at 0.1 mg L^{-1} (Siqueira et al. 1982) and Sucrose (SD Fine chemicals, 20274, India) at 4 g L^{-1}.

Details of the contents (1 mL volume) of the pouches for each set (of 12 pouches) are as follows: conidia (final concentration in pouch was 30–50 × 10^5 conidia mL^{-1}) and 1/5th strength MEB were common to all the pouches. Thiamine was added to pouch 1 and pouch 7. Sodium pyruvate was added to pouch 2 and pouch 8. Sucrose was added to pouch 3 and pouch 9. Sulphuric acid was added to pouch 4 and pouch 10. Hydrochloric acid was added to pouch 5 and pouch 11. No chemical inducers were added in pouch 6 and pouch 12 (controls).

While preparing the pouches, care was taken not to introduce air bubbles as the pouches could rupture during de-pressurisation, if air bubbles were present. Similar to the earlier studies by Damare et al. (2008), Dijksterhuis (2007), and Peay et al. (2009), heat treatment as germination trigger was provided for 60 min at 70 °C. Pouches 1–6 were placed at 70 °C for 60 min. Pouches 7–12, without heat treatment, served as the control group for heat-treated pouches. After the heat treatment, for each isolate, seven sets of bags, each bag with 1–12 pouches, were incubated at 0.1, 10, 20, 30, 40, 50 and 60 MPa elevated hydrostatic pressures at 4 °C for 30 days. Pressure cultivation chambers (Tsurumi & Seiki Co., Japan) were used for incubation at elevated pressures. The last set of bags having 1–12 pouches were incubated at 0.1 MPa at room temperature (30 °C) to check for their viability and germination potential. This study was carried out in a total of 480 plastic pouches (12 treatments × 8 pressure conditions × 5 isolates) for 30 days for all five isolates. As the deep-sea fungi employed in this study were isolated from a maximum depth of 5500 m, pressures of 50 MPa and 60 MPa were included to check whether the conidia could germinate during the 30-day incubation at 4 °C at elevated hydrostatic pressures akin to those at 5500 m.

The pouches were cut open at the end of 30-day incubation, and the content was mixed with calcofluor to stain the conidia for visualisation (Raghukumar and Raghukumar 1998). The suspension was observed under an ultraviolet light filter (excitation wavelength 330–385 nm and barrier filter BA 420) using an epifluorescence microscope (BX51 Olympus, Japan). The total and the germinated numbers of conidia were counted. Three subsamples from each pouch were counted to obtain a reliable average value. Each counting was carried out by pipetting 100 µL from the harvested contents from the plastic pouches.

Statistical analysis

Factorial analysis of variance (ANOVA) was performed to find out the statistically significant difference between treatments of inducers. Newman-Keul's test (McHugh 2011) was performed with probabilities for post hoc tests using Statistica (release 6.0, Statsoft Inc., Tulsa OK, USA).

Results

Germination of conidia after 30-day incubation

The per cent germination of conidia of different isolates examined in this study at 10, 20, 30, 40 and 0.1 MPa pressures at 4 °C suggests 40 MPa as the upper limit for conidial germination (Table 1). There was no apparent difference between the germinating conidia exposed to elevated hydrostatic pressure and those exposed to 0.1 MPa at 4 °C and 30 °C (Figures 1 and 2). However, mycelia developed from conidia under high hydrostatic pressures displayed morphological abnormality such as bloating (Figure 3).

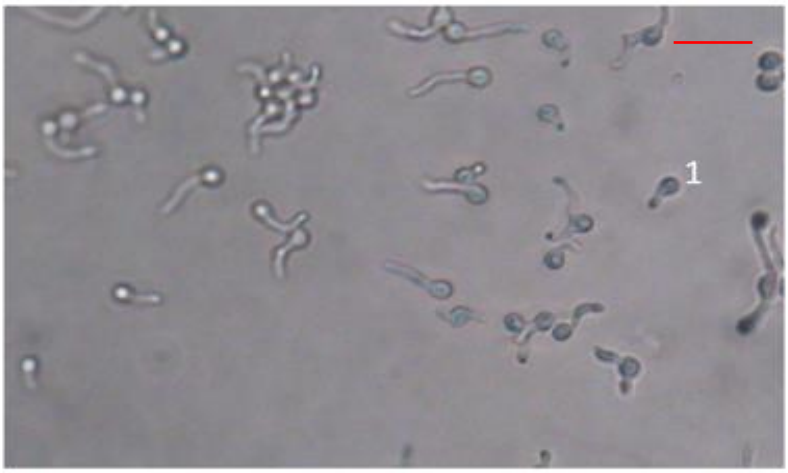

Figure 1: Conidia of *Aspergillus terreus* A4636 showing 100% germination at 40 MPa, heat treated, in Hydrochloric acid and basal medium as viewed at 40x magnification [1 represents germinating conidium with germinating tube protruding from the conidium]. Bar is equal to 10 µm.

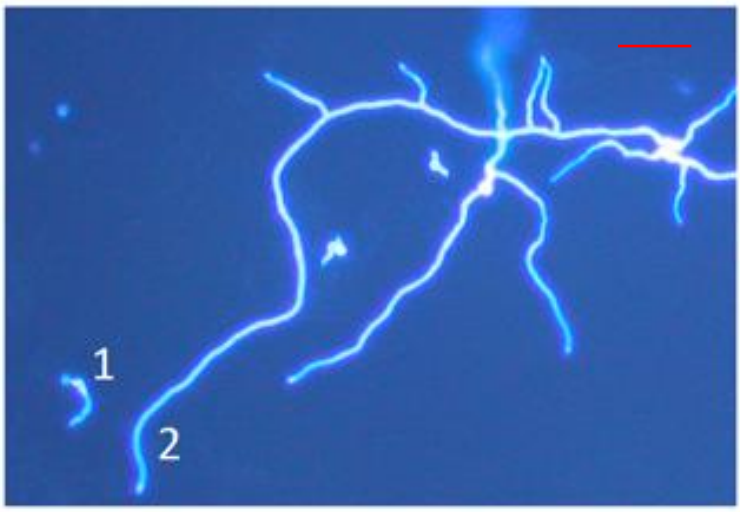

Figure 2: Conidia of *Aspergillus terreus* MTCC479 grown under normal conditions of temperature (30 °C) and pressure (0.1 MPa), stained with calcofluor, showing mycelia and germinating conidia (40x magnification) [1 represents germinating conidium with germinating tube protruding from the conidium; 2 represents growing fungal mycelium]. Bar is equal to 10 µm.

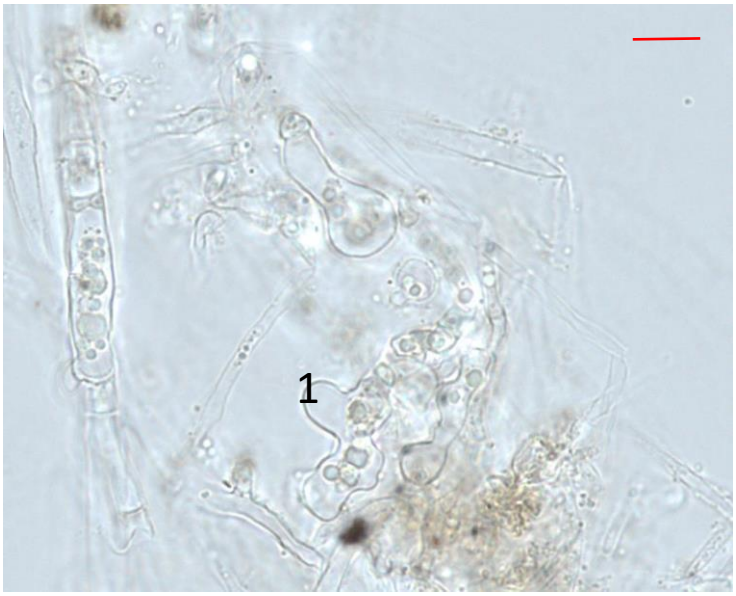

Figure 3: Bloated mycelium of *Penicillium* sp. A4623, developed by incubating conidia of *Penicillium* sp. A4623 for 30-days at 10 MPa, in control set (absence of both physical and chemical inducers) (100x oil immersion lens magnification) [1 represents bloated mycelium]. Bar is equal to 5 µm.

Conidial germination at 0.1 MPa and 30 °C

Conidia of all the isolates germinated at 0.1 MPa at room temperature (30 °C) in less than 24 h and developed a thick mycelial mat after 30-day incubation. *Aspergillus flavus* NIOCC20 developed hyphae within 12 h of incubation. *Aspergillus sydowii* CH2 and *Penicillium* sp. A4623 developed hyphae within 16 h of incubation. Both isolates of *A. terreus* developed hyphae by the end of 24 h incubation.

Conidial germination at 0.1 MPa and 4 °C

Conidia in pouches incubated at 4 °C showed germination from 12th day and developed thin mycelial filaments by the end of 30-day incubation.

Conidial germination at elevated pressures and 4 °C

At 4 °C, over 50% of conidia of both deep-sea and terrestrial isolates of *A. terreus* (A4636 and MTCC479) showed germination in ten of the pressure pouches. At 40 MPa, the conidia of deep-sea isolate, *A. terreus* A4636 germinated in the pouches: Hydrochloric acid (heat treated), Sucrose and Sulphuric acid (without heat treatment) induced conditions. Similarly, at 30 MPa, those conidia treated with Sodium pyruvate (without heat-treatment) germinated.

The conidia of terrestrial isolate, *A. terreus* MTCC479 germinated at 10 MPa and 40 MPa, in the pouch treated with heat + Hydrochloric acid and only at 30 MPa in the pouch exposed to heat + Sulphuric acid treatment, and in the pouch containing Sucrose and Sulphuric acid not exposed to heat-treatment. At 40 MPa, conidial germination was observed in the pouch containing Sulphuric acid without heat-treatment. The germination percentage (< 25%) was found to be low in other pouches for both *A. terreus* A4636 and MTCC479 (Table 1).

On the contrary, at 4 °C, most of the conidia of *A. flavus* NIOCC20, *Penicillium* sp. A4623 and *A. sydowii* CH2 germinated under all four incubation pressures, except for a few conditions allowing ~33% germination (Table 1). There were no germinated conidia in three

cases: in control pouch (MEB) with conidial suspension not exposed to heat at 20, 30 and 40 MPa (Table 1).

Mycelia were observed in most of the conidial suspensions of *A. flavus* NIOCC20, *Penicillium* sp. A4623 and *A. sydowii* CH2, without heat treatment at 10, 20 and 30 MPa at 4 °C at the end of 30-day incubation. Conidia of *A. sydowii* CH2 developed mycelia in all the six treatments (Table 1) only at 10 MPa. Conidia of *A. flavus* NIOCC20 developed mycelia in Sodium pyruvate and Sucrose at 20 MPa. Conidia of *Penicillium* sp. A4623 developed mycelia in Thiamine, Sodium pyruvate, Sucrose and in control pouches (MEB) at 10 MPa, 20 and 30 MPa and in Hydrochloric acid only at 30 MPa.

Table 1: The per cent germination of conidia in the presence of the inducers at different hydrostatic pressures after 30 days of incubation at 4 °C

			Per cent germination of conidia					
Pressure (MPa)	**Heat treatment (70 °C, 1h)**	**Fungal isolate**	**Thiamine (0.1 mg L^{-1})**	**Pyruvate (0.1 M)**	**Sucrose (4 g L^{-1})**	**H_2SO_4 (0.02 N)**	**HCl (0.02 N)**	**Control**
10	Yes	***Aspergillus terreus* A4636**	--	--	--	--	--	--
		***A. terreus* MTCC479**	25±1	--	--	--	100	--
		***A. flavus* NIOCC20**	100	100	100	100	100	100
		***Penicillium* sp. A4623**	100	100	100	100	100	100
		***A. sydowii* CH2**	100	100	100	100	100	100
	No	***A. terreus* A4636**	9±10.4	6±12.0	--	--	--	--
		***A. terreus* MTCC479**	--	--	--	--	--	--
		***A. flavus* NIOCC20**	100	100	100	100	100	--
		***Penicillium* sp. A4623**	M*	M	M	100	100	M
		***A. sydowii* CH2**	M	M	M	M	M	M
20	Yes	***A. terreus* A4636**	--	--	--	--	--	--
		***A. terreus* MTCC479**	--	--	9±3.5	--	--	--
		***A. flavus* NIOCC20**	100	100	100	100	100	100
		***Penicillium* sp. A4623**	100	100	100	100	100	100
		***A. sydowii* CH2**	100	100	100	100	100	100
	No	***A. terreus* A4636**	--	--	--	--	--	--
		***A. terreus* MTCC479**	--	--	--	--	--	--
		***A. flavus* NIOCC20**	13.69±4.2	M	M	100	100	100
		***Penicillium* sp. A4623**	M	M	M	100	100	100
		***A. sydowii* CH2**	100	100	100	100	100	--
30	Yes	***A. terreus* A4636**	--	--	--	--	--	--
		***A. terreus* MTCC479**	--	--	--	100	--	--
		***A. flavus* NIOCC20**	100	21±0.57	100	--	100	100
		***Penicillium* sp. A4623**	30±2	100	--	100	100	100
		***A. sydowii* CH2**	100	100	30±2.5	100	100	100
	No	***A. terreus* A4636**	--	50±5.8	--	--	--	--
		***A. terreus* MTCC479**	--	--	100	50±7.8	--	--
		***A. flavus* NIOCC20**	100	100	100	100	100	100
		***Penicillium* sp. A4623**	M	M	M	100	M	100

		***A. sydowii* CH2**	100	100	100	100	100	--
40	Yes	***A. terreus* A4636**	--	--	--	--	100	--
		***A. terreus* MTCC479**	--	11±9.1	--	25±8.1	50±10.0	--
		***A. flavus* NIOCC20**	100	100	100	100	100	100
		***Penicillium* sp. A4623**	100	100	100	100	100	18±1
		***A. sydowii* CH2**	100	100	100	100	100	100
	No	***A. terreus* A4636**	1±4.7	--	100	50±24.5	--	--
		***A. terreus* MTCC479**	--	--	--	100	--	--
		***A. flavus* NIOCC20**	100	100	100	100	100	100
		***Penicillium* sp. A4623**	100	100	100	100	100	100
		***A. sydowii* CH2**	100	100	100	100	100	--
Pressure (MPa)	**Heat treatment (70 °C, 1h)**	**Fungal isolate**	**Thiamine (0.1 mg L^{-1})**	**Pyruvate (0.1 M)**	**Sucrose (4 g L^{-1})**	**H_2SO_4 (0.02N)**	**HCl (0.02N)**	**Control**
0.1	Yes	***A. terreus* A4636**	H**	H	H	H	H	H
		***A. terreus* MTCC479**	H	H	H	H	H	H
		***A. flavus* NIOCC20**	H	H	H	H	H	H
		***Penicillium* sp. A4623**	H	H	H	H	H	H
		***A. sydowii* CH2**	H	H	H	H	H	H
	No	***A. terreus* A4636**	H	H	H	H	H	H
		***A. terreus* MTCC479**	H	H	H	H	H	H
		***A. flavus* NIOCC20**	H	H	H	H	H	H
		***Penicillium* sp. A4623**	H	H	H	H	H	H
		***A. sydowii* CH2**	H	H	H	H	H	H

*M indicates production of visible slender mycelia and 100% germination
**H indicates production of hyphae

Heat treatment did not always have a positive impact on the germination of conidia. With respect to the chemical inducers used in this study, no inducer was found to be stimulant for germination under all hyper pressure conditions tested (Table 1).

Results of statistical analysis

Results of factorial ANOVA with Newman-Keul's test (Table 2) showed that there is no statistically significant effect of any inducer on the conidial germination.

Discussion

Fungal conidia are unique in the sense that they can remain dormant till right conditions for their germination become available. One needs to understand the conditions that could trigger their germination under deep-sea conditions. The present study investigated the germination potential of conidia of four marine-derived and one terrestrial fungal isolates under simulated deep-sea conditions. The four isolates of deep-sea fungi used in this study were the inhabitants of sub-seafloor sediments of the CIOB (Damare et al. 2006, Jones et al. 2015). The conidial germination was evaluated in the presence of physical and chemical inducers. Our findings are in agreement with Jesenska et al. (1993), who reported that heat is not essential for conidial germination. However, heat treatment increased the germination of conidia in case of *A. terreus* MTCC479 at 10, 20 and 40 MPa in the presence of chemical inducers (Table 1). Surprisingly, the conidia of deep-sea isolate *Aspergillus terreus* A4636

showed less germination as compared to that of the conidia of its terrestrial counterpart (Table 1). The chemical additives/ inducers and heat factor were provided in laboratory set-up to check their effect on conidia and germination. Interestingly, deep-sea niches such as hydrothermal vents, marine snow and sinking dead materials are likely to contain such additives (Haymon 2005, Tivey 2007, Smith et al. 2015, Bilenker et al. 2016). Generation of inorganic acids can be more rapid during deep sea mining as compared to terrestrial mining (Bilenker et al. 2016).

The chemical inducers evaluated in this study are also known to play a role in cell-cycle. For example, Thiamine is reported as a vital player in carbohydrate breakdown (Lonsdale 2006) and in triggering spore germination by initiating pyruvic acid breakdown (Mkrtchyan et al. 2016). The absence of trigger for the start of the pyruvate cycle was probably the only rate-limiting factor (Laere et al. 1982) for a dormant spore to initiate development. Acids soften the fungal cell wall (Rao 2011) and allow the diffusion of nutrients between spore and the external environment. Sucrose helped in initial swelling (Wooley and Thoene 2004) of spore for the uptake of nutrients to initiate germination. However, in all these studies as well as in the current study, the inducers were provided freely, as a single-component additive for the experimental setup; whereas in deep-sea, these inducers might occur as minerals and in combination with other compounds and/ or elements. While none of the additives tested in this study was a trigger for germination of conidia, a combination of different compounds/ elements can play a role in triggering conidial germination in deep-sea. The present study reveals that the conidia can germinate irrespective of the presence of the additives (Table 1).

Table 2: Results of factorial ANOVA (Univariate tests of significance with Sigma - restricted parameterization) with Newman-Keuls test

Isolate	Final F and p-value for two varied parameters (inducer and pressure), in all incubated conditions	
	F-value (critical)	p-value
***Aspergillus terreus* A4636**	3.78703	0.000018
***A. terreus* MTCC479**	7.24944	< 0.000000
***A. flavus* NIOCC20**	4.704	0.000001
***Penicillium* sp. A4623**	20.667	< 0.000000
***A. sydowii* CH2**	2.500	0.003634

Null hypothesis: No inducer plays pivotal role in triggering germination
At p <0.01 the above null hypothesis can be accepted.
All F values mentioned here are lesser than F statistic, hence null hypothesis is accepted.

Fungal conidia require certain inducers for the germination, apart from the moisture (Florian 1991). The exogenous dormancy of conidia can be broken by the conditions favourable for germination. The endogenous dormancy of spores is disrupted mostly by ageing or by physiological shock which may allow nutrient entry or by endogenous inhibitors that leach out of the spore (Moore et al. 2011). In addition to adequate wetting of conidia, both inorganic and organic inducers and, heat in some instances, might be responsible for the molecular intake leading to germination. Certain enzymes or pathway-intermediates (Laere 1986), heat-treatment, the medium of growth, external pressure and pH of the medium (Dijksterhuis 2007) are known to break spore dormancy and initiate germination and development of germ tube (Feofilova et al. 2012). The role of inducers in conidial germination has not been documented conclusively (Florian 1991). Results of this study

indicate that the deep-sea fungi probably require no additional inducers apart from hydration for conidial germination. The conidia germinated not only at standard conditions of room temperature and atmospheric pressure but also under elevated hydrostatic pressures and low temperature, both in the presence and absence of the inducers tested (Table 1).

When conidia of *A. terreus* A4636 and MTCC479 were cultivated in the laboratory under simulated deep-sea conditions, the percentage of germinating conidia was low as compared to that of the other three isolates *A. flavus* NIOCC20, *Penicillium* sp. A4623 and *A. sydowii* CH2 (Table 1). *Aspergillus terreus* is a mesophilic fungus (Chidi et al. 2008), and prolonged low temperatures severely hamper germination potential of conidia of *A. terreus*. This study reveals that conidial germination under simulated deep sea conditions is not affected by the exogenous factors like heat and chemical inducers and also provides insights into germination potential of fungal conidia in deep-sea conditions.

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Statement on conflict of interest
The authors declare that there is no conflict of interest.

Author contribution
The work was conceived by the corresponding author and all the experimental work was executed by the first author. The MS was written by both the authors.

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