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Research Article

Cold-active Moulds from Jammu and Kashmir, India as Potential Source of Cold-active Enzymes

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Abstract

Cold-active moulds have been isolated from the soil of ten selected sites of Jammu and Kashmir (India) in the winter season. Most of them turned out to be psychrotolerant except BPF-5 and BPF-6 which showed defective growth above 20^{o}C, and thus were identified as psychrophilic moulds. BPF-5 was also found to form sexual structure at 4^{o}C, while BPF-6 formed melanaceous filaments in old culture. The isolate BPF-5 has been identified as *Truncatella angustata* and BPF-6 as *Psudogymnoascus* sp. Among psychrotolerant moulds, the species of *Cladosporium* and *Penicillium* were found to be dominant taxa in terms of frequency and number of species while *Rhizomucor* sp., to be the most prolific mould under *in vitro* culture. Many of them formed adaptive structures and pigment. All of these isolates were able to utilize starch, cellulose, casein and tween-80 while many of them were able to use pectin and carboxy methyl cellulose (CMC) as sole carbon source at 4^{o}C suggesting that they might be important sources of cold-adapted enzymes and other biomolecules. Although α-amylase from all the isolates showed residual cold-activity, that from BPF-6 exhibited the highest one suggesting it to be further explored for biotechnological applications.

Keywords: Cold-adapted moulds; Baramulla-soil; Psychrophilic moulds; Psychrotolerant moulds

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Introduction

A variety of microbes inhabit extreme environments such as high temperature, pH, pressure, salt concentration and low temperature, pH, nutrient concentration and

water availability and also conditions having high levels of radiation, harmful heavy metals and toxic compounds [1]. The realm of cold-active organisms cover psychrophiles that grow at or below zero (0°C) and have an optimum growth temperature ≤ 15°C and an upper limit of ≤ 20°C [2] while psychrotolerant which can also grow close to zero but have optima and upper limit above these temperatures, and may grow well at mesophilic temperature with optimal growth above 30°C, hence they could be considered as being cold-tolerant mesophiles [3]. The physiological and ecological mechanisms that help fungi to overcome and survive cold environment conditions have been discussed [4, 5].

Modern biotechnology requires polymeric molecules, which function at extreme physico-chemical parameters. Psychrophilic and psychrotolerant microbes and their unique cold shock and cold acclimatization protein and enzymes have a host of biotechnological applications [6]. These include cold-water detergents, food-additives and flavor modifying agents, biosensors and environmental bioremediation [7]. Cold-active enzymes with unique molecular adaptabilities [8] have opened up potential newer areas of applications [9, 10]. Extremophiles are also supposed to act as reservoirs of bioactive molecules capable of remedying human maladies [4]. Over the last decades, the cold regions of the world such as Antarctica or Deep Ocean have been investigated mainly for the presence and exploitation of psychrophilic bacteria and archaea, occasionally for algae and more rarely for fungi [11-13]. Apart from these, there has been interest in understanding the cold-active metabolism [14], low temperature host-pathogen interaction [15-16] and ectomycorhizic development [17], and a general life-style in cold-climate [18].

By far six psychrophilic fungi *Geomyces pannorum* [19], *Humicola marinii* [20], *Thelebolus microsporus* [21], *Typhula ishikariensis* [22], *Antarctomyces psychrotrophicus* [23] and *Geomyces destructans* [24] have been isolated from cold areas. There has been a lot of interest in the cold-adapted biomolecules obtained from these moulds [23].

The state Jammu and Kashmir lies between 32^{o} 15' to 37^{o} 05' North latitude and 72^{o}35' to 83^{o} 20' East longitude. It is the northern most state of India comprising three distinct Climatic regions viz. Arctic cold desert areas of Ladakh, temperate Kashmir valley and sub-tropical region of Jammu. There is a sharp rise of altitude from 1000 feet to 28250 feet above the sea level within State's four degree of latitude. The annual rainfall also varies from region to region with 92.6 mm in Leh, 650.5 mm in Srinagar and 1115.9 mm in Jammu. A large part of the State forms part of the Himalayan mountains. The State is geologically constituted of rocks varying from the oldest period of the earth's history to the youngest present day river and lake deposits. The soil is loamy and there is little clay content in them. It is poor in lime but with a high content of Magnesia and there is sufficient organic matter and nitrogen content in the alluvium. The soil and climate thus were hypothesized to be suitable for evolution of cold-active industrially important moulds. The aim of this study was to isolate psychrophilic and psychrotolerant moulds, active in winter season at 0-4^{o}C from soil at different locations

of Jammu and Kashmir as source of a representative cold-active α-amylase.

Materials and Methods

Samples collection

Soil samples were collected from undisturbed forest areas of ten different sites viz., Kishtwar, Doda, Rajouri (Jammu division), Anantnag, Rajouri, Pulwama, Budgam, Srinagar, Baramulla (Kashmir valley division) and Leh (Ladakh division) in triplicates. The temperature and pH of the soil samples were recorded at the collecting sites. The samples were packed in sterile plastic bags and maintained at 4°C using ice box during transportation.

Isolation of fungi

One g each soil sample was suspended in 10 ml of sterile distilled water, which was serially diluted to 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5} dilutions and 0.5 ml of each dilution was spread on MEA (malt extract agar) medium, pH 5.5 supplemented with chloramphenicol at the concentration of 0.1 mg/ml. The plates were incubated at 7°C for up to 2 weeks. Moulds growing on the agar plates were transferred by sub-culturing from hyphal tips, colonies or spores to fresh growth medium for getting pure culture

Identification of moulds

The isolated moulds were identified down to the generic level on the basis of macromorphological and micromorphological characteristics using suitable media, slide cultures and the most updated keys for identifications [25-28]. The isolate BPF-4 and BPF-5 have also been characterized on the basis of ITS1, ITS2 and 5.8s DNA sequences. BLASTN was used to search ten closely related sequences in the GenBank database. ClustalW was used to do pairwise alignment and UPGMA in MEGA5 software was used to carry out phylogenetic analysis.

Growth rate

Growth rate of the isolates was calculated in terms of growth of colony in diameter in mm/day after centrally point-inoculating each of the cultures on 2% MEA plates and incubated at 4, 25, 37^{O}C. The diameters were measured for 5 days [29]. The experiment was set up in triplicates and mean values of these readings was recorded as growth in diameter of the colony.

Effect of pH

The pH of minimal Czapek-Dox medium containing (g/l) glucose 20.0; sodium nitrate 3.0; magnesium sulfate 0.5; potassium chloride 0.5; iron(III)sulfate 0.01; di-potassium hydrogen phosphate 1.0; agar, 20.0; pH 6.5, was used. The pH of medium was maintained at 3, 6 and 9 with 0.1N HCl and NaOH and each was inoculated with all the fungal isolates. The plates were incubated at 20^{o}C and growth was monitored.

Carbon utilization

Minimal Czapek-Dox medium (pH 6.5) without glucose was supplemented with casein, pectin, tween-80, lactose, starch, CMC (carboxy methy cellulose), NAG (N-acetyl glucosamine) or cellulose as sole carbon source (30 g/l), and was used for inoculating the fungal isolates. The plates were incubated at 4^{o}C and growth was monitored for 5 days. The growth was

expressed as positive (+) if there was a growth, negative (-) if there was no growth at all, and delayed (D) if there was only a trace of growth in fourth or fifth day.

Enzyme production and extraction

The enzyme (α-amylase) was produced by transferring 1 ml culture (density 10^6 l^{-1}) from Potato dextrose broth to 100-ml conical flask containing 20 ml of the fungal production medium (minimal Czapek Dox medium minus agar containing glucose as carbon source) supplemented with 0.1 g/l starch as inducer. The growth was carried out at 15°C on a rotatory shaker at 150 rpm. At different time intervals, 5 ml of the samples were withdrawn and mycelia were spun down at 5000 rpm for 10 min. The supernatant was saturated with $(NH_4)_2$ SO_4 to 50 % level and precipitated proteins were collected by centrifugation at 10000 rpm for 10 min. After dialysis against 100 mM phosphate buffer (pH 7.0) at 4°C, protein was resuspended in the same buffer and was used as source of enzyme.

Protein content

Protein concentration was measured according to [30] using bovine serum albumin as standard and was stored at -20°C.

Assay of α-amylase activity at various temperatures

To 1 ml pre-cooled at 15°C of 1% starch solution, 1 ml of properly diluted enzyme (kept at -20°C) was added; the reaction-mix was incubated at 15°C for 30 min. A 0.3 ml aliquot of this reaction-mix was transferred to new test tube, and 0.3ml of 3,5-dinitrosalicylic acid reagent was added to it. The solution was boiled for 5min, cooled down to room temperature and then 2.7ml of distilled water was added to it. Absorbance was measured at 540 nm using UV-Vis spectrophotometer. One unit of amylase activity was defined as the amount of enzyme that released 1µM of reducing sugar equivalent to glucose per min under the assay condition [31]. The experiments were performed in three sets of duplicate cultures, and the mean values of soluble glucose equivalent released by enzyme were determined. The α-amylase activity was also assayed at different temperatures (4°C, 15°C, 20°C, 25°C, 30°C, 40°C) in 100 mM sodium phosphate buffer (pH 7) to determine the residual cold-activity.

Results and Discussion

Isolation of cold-adapted moulds

In all, 10 different morphological isolates were obtained from various soil samples which were active at 0-4°C. While the plates were examined twice every week at regular intervals of 3–5 days from the first day of incubation, the first sign of fungal growth was visible only after 20 days. In general, all the moulds recovered were slow-growing. All fungal isolates were obtained in pure cultures by single conidial transfer onto MEA plate.

From the data (**Table 1**), it could be shown that the relative frequency of different isolates varies greatly ranging from 10 (BPF-2) to 100% (BPF-10). The species diversity of psychrophilic moulds was only 20% while that of psychrotolerant was 80% indicating a natural selection in favour of those which tolerate more. The predominance of psychrotolerants is also in conformity with the climate of Jammu and Kashmir that has both below and above 20°C temperature regimes. A relationship of

temperature characteristics of microbial community with that of habitat has earlier been reported [32-34].

Table 1 Fungi isolated from soil of ten (1-10)* different sites of Jammu & Kashmir (India).

Fungal isolates	1	2	3	4	5	6	7	8	9	10	% frequency
Curvularia sp BPF1	+	+	+	+	+	-	+	+	+	+	90.0
Cladosporium sp BPF2	-	+	+	+	+	-	+	+	+	+	80.0
Cladosporium sp BPF3	+	-	+	+	-	+	+	+	+	+	80.0
P. canescens BPF4#	+	-	+	-	+	-	+	-	+	-	50.0
Truncatella angustata BPF5#	+	+	-	+	+	+	-	+	+	+	80.0
Pseudogymnoascus sp BPF6	-	-	-	-	-	-	+	-	+	-	20.0
Cladosprium sp BPF7	+	+	-	-	-	+	+	-	-	+	50.0
Penicillium sp BPF8	-	+	+	+	+	+	+	+	+	+	90.0
Penicillium sp. BPF9	+	+	-	-	-	-	-	-	+	-	30.0
Rhizomucor sp BPF10	+	+	+	+	+	+	+	+	+	+	100.0

*1-Kishtwar, 2-Doda, 3-Rajouri, 4-Anantnag, 5-Rajouri, 6-Pulwama, 7-Budgam, 8-Srinagar, 9-Baramulla and 10-Leh; # fungal strains characterized genetically.

Identification of the moulds

The isolated moulds (**Fig. 1**) were identified on the bases of macromorphological and micromorphological characteristics. The BPF-1 showed rapidly growing colony at 25°C, which was olive brown on surface and reverse and wooly in texture. The hyphae were septate, branched and brown in colour, some of them acting as conidiophores which were simple or branched bearing characteristic conidia. The conidia were brown, multiseptate with a central cell larger and darker than other cells (**Fig. 3 A**), only few (5-6%) of them were curved. It was therefore identified as Curvularia sp.

Figure 1 Colonies of F4 (BPF-4), F5(BPF-5), F6 (BPF-6), F7 (BPF-7), F8 (BPF-8) and F9 (BPF-9) grown on PDA at 4oC for 10 days.

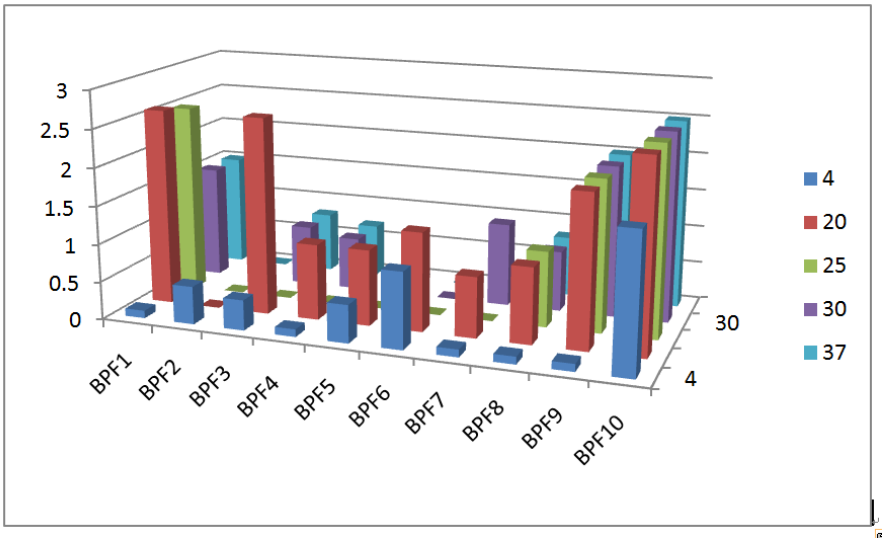

Figure 2 Effect of temperature on the colony-growth (growth in diameter in mm/day) of the cold-adapted moulds.

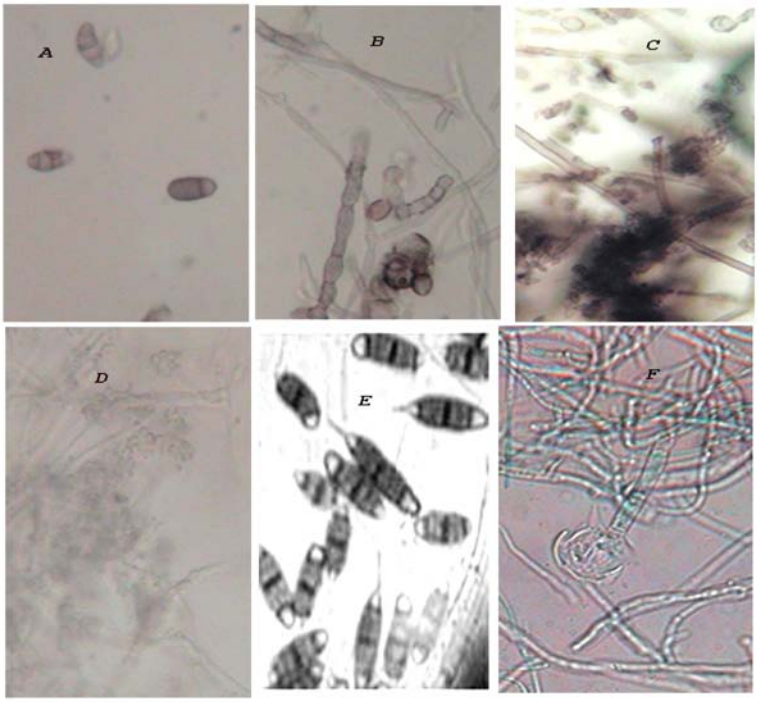

Figure 3 Characteristic microscopic structures of mycelia / propagules of A (BPF-1), B (BPF-2), C (BPF-3), D (BPF4), E (conidia of BPF-5) and F (fusing gametangia of BPF-5).

The isolates BPF-2, BPF-3 and BPF-7 showed similar velvety colony texture and brownish black pigment on surface and reverse of petridishes, the growth rate of BPF-2 and BPF-7 was very low and there was no growth beyond 30°C. BPF-3 was faster in growth and showed residual growth up to 37°C. BPF-7 showed multiple gemmae (**Fig. 4 C**). All these

isolates (**Figs. 3B, 3C and 4C**) showed brown and septate conidophores bearing brown and bicellular conidia in delicate chains. They were identified as the species of *Cladosporium*.

The isolate BPF-4, BPF-8 and BPF-9 exhibited compact and elevated colonies, initially white but gradually becoming yellow-green (BPF-4), blue-green (BPF-8) or green (BPF-9). While BPF-9 showed rapid rate of growth, the other two showed low to moderate rate of growth. The colony-texture of all these isolates were velvety, the hyphae were hyaline and septate, the conidiophores were branched, the philides were bunched as brush bearing at the tips conidia which were single-celled, round/ovoid, hyaline and in chains. These isolates (**Figs. 3D, 4D and 4E**) were identified as different species of *Penicillium*. One of the *Penicillium* isolates i.e. BPF-4 (**Fig. 3D**) was identified on the basis of ITS sequence characteristic and was found to be P. canesanse and the sequence was submitted to GenBank under accession number KF247214.

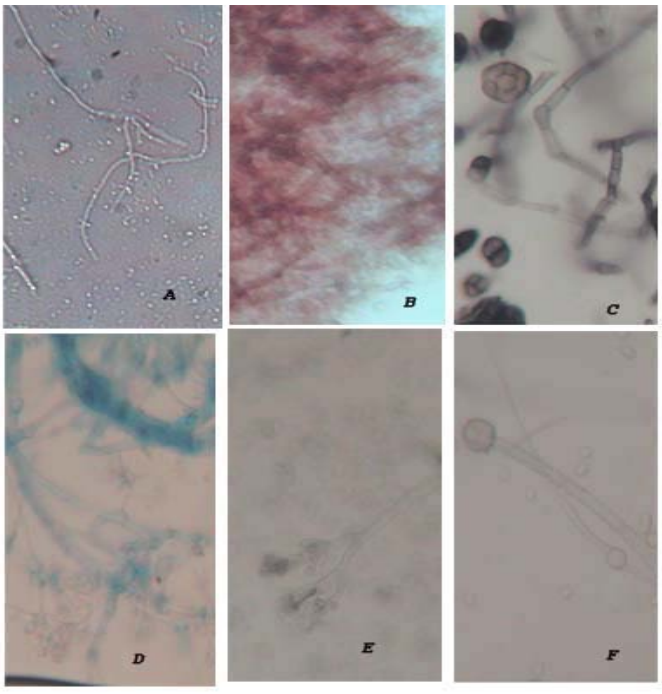

Figure 4 Characteristic microscopic structures of mycelia / propagules of A (mycelia of BPF-6), B (characteristic asci of BPF-6), C (BPF-7), D (BPF-8), E (BPF-9) and F (BPF-10).

BPF-5 showed lose cottony and dull white to brown colony with black acervuli (<1 mm in diameter) that were mostly found near the center of the PDA plates. The conidia were with dark median cells, two-to-five (mostly two) dark brown transverse septa, with branched apical appendages though some of them were unbranched (**Fig. 3E**). Based on these morphological characteristics, the isolates were identified as *Truncatella angustata* (Pers.) Hughes [35]. The isolate was also identified on the basis of ITS sequence homologies with ten closely related isolates from GenBank database [10]. The sequence was submitted to GenBank under accession number JNO38391.

The colony of BPF-6 was light pinkish and compact, mycelia were slender and branched

(Fig. 4A), yellow on the reverse, ascocarp redish-orange and with smooth, peridial hyphae and long, branched appendages (**Fig. 4B**). On the basis of these characters it was identified as *Pseudogymnoascus* sp. [27].

The isolate BPF-10 showed dull white and profusely branched mycelia with stolon, raised aerial sporangiphores, black globose sporangia (**Fig. 4F**). Based on these morphological features, the isolate was identified as *Rhizomucor* sp. All these isolates were submitted to departmental culture collection.

Effect of pH and temperature

All the fungi were able to grow at neutral and acidic pH (**Table 2**). The absence of alkaliphilic mould in the Baramullah-soil (acidic) is fully in conformity with the fact that natural selection is driven by specific stress in the environment. Variation in growth among isolates was observed when they were cultivated at temperatures between 4^{o}C and 35^{o}C on agar medium (**Fig. 2**). Development of sexual structure BPF-5 (**Fig.3F**) at 4^{o}C confirms that this fungus is fully active at winter temperature in the soil. In the same way, BPF-6 could grow faster and formed spores at 4^{o}C hence both BPF-5 and BPF-6 are fit to be classified as psychrophiles. The psychrotrophic (psychrotolerant) strains were able to grow both at low (4^{o}C) and at medium temperatures with an optimum temperature of 25^{o}C. They formed spores very late and in scarce quantity at 4^{o}C. It seems that moulds do not follow strict rule of psychrophiliy and psychrotrophy (**Fig 2**) as diverse range of growth temperatures and growth optima are seen. For example, BPF-6 cannot grow above 20^{o}C while BPF-5 and BPF-2 cannot grow above 25^{o}C and 30^{o}C respectively. So, it is not possible to fix a ceiling of upper temperature to classify an organism under psychrophile. Earlier, while Morita [2] favoured 20^{o}C to be cardinal temperature to delimit psychrophiles, Nakagawa et al [36] considered the organism showing residual growth at 25^{o}C also to be psychrophile.

Table 2 Utilization of different substrates as sole carbon source by fungal isolates.

Isolates	Casein	Pectin	Tween-80	Lactose	Starch	CMC	Cellulose	NAG
BPF-1	+	-	+	-	+	-	+	-
BPF-2	+	-	+	+	+	-	+	+
BPF-3	+	+	+	+	+	-	+	+
BPF-4	+	D	+	-	+	+	+	+
BPF-5	+	+	+	+	+	+	+	+
BPF-6	+	+	+	+	+	-	+	+
BPF-7	+	-	+	+	+	-	+	+
BPF-8	+	-	+	-	+	-	+	+
BPF-9	+	-	+	+	+	-	+	+
BPF-10	+	-	+	+	+	+	+	+

CMC-carboxy methyl cellulose; NAG-N-acetyl glucosamine.
(+) - indicates normal growth, (-) – no growth, (D) - delayed and weak growth.

Morphological features

The mycelia of many fungal isolates (species of *Cuvularia*, *Cladosporium* and *Pseudogymnoascus*) recovered from the Himalayan soils were found to have abundant intercalary, swollen, thick-walled cells, called chlamydospores (**Fig.5**). Chlamydospores in the fungi are generally considered as dormant resting spores. It is possible that Himalayan fungi in general posses these features as an adaptation to survive in extreme low temperature. Earlier, many of the moulds isolated from Antarctica showed colonies with concentric rings of thinner and denser growth or rings with conidia and sterile mycelium or uneven surface with many irregular convolute wrinkles and deep radial furrows [37]. Such growth characteristics were not shown by any of the Himalayan isolate indicating that either this is not a general characteristic shown by moulds under cold stress or related with more adverse condition which exist in Antarctica-soil and not in Baramullah-soil. Coincidently, many of the genera which were isolated from Antarctica are also represented in the Himalayan soil.

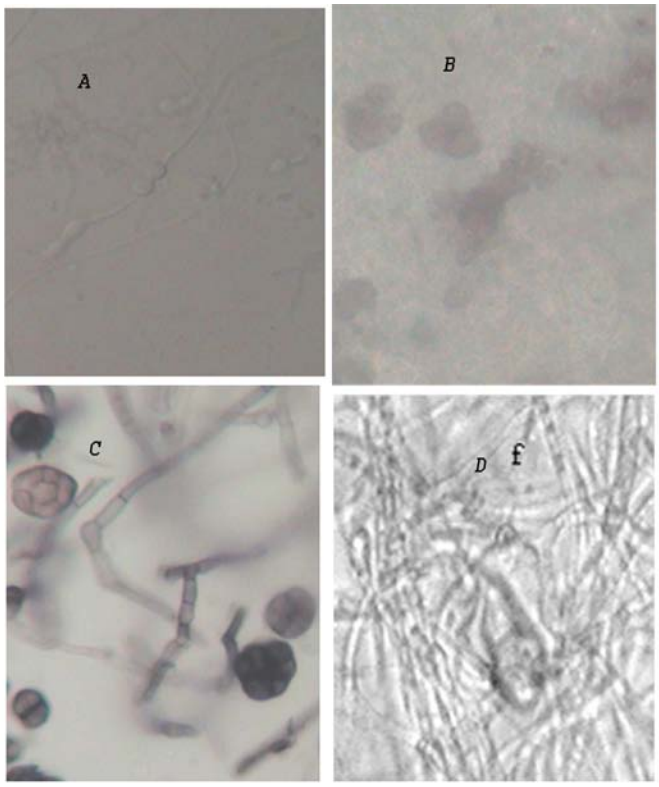

Figure 5 Chlamydospore of A (BPF-8), B (BPF-6), C (BPF-7) and D (BPF5).

BPF-6 formed red pigment initially (**Fig. 4B**), which was found to have lost later due to repeated subculturing. However, old culture of this psychrophile now formed melanaceous strands. All the species of *Cladosporium* and *Curvularia* formed black pigment (possibly melanin). Melanin has been referred to as "fungal armor" due to the ability of the polymer to protect microorganisms against a broad range of stress factors including low temperatures and

UV-radiation [37]. Melanin was found to be formed by these species earlier also [4]. However, this seems to be a genera-specific ability as no isolate of *Penicillium* nor *Rhizomucor* was found to produce black pigment.

Carbon utilization

The carbon utilization pattern of fungi may indicate their metabolic flexibility and help in assessing them as source of various enzymes. The result of carbon utilization pattern (**Table 2**) suggests that fungal isolates though could use most abundant carbon sources (eg., starch, cellulose, casein and tween 80), only few of them could use pectin and CMC. The result is important as it indicates that the fungal isolates may be explored for the psychrophilic enzymes of immense industrial importance such as amylases, cellulases, proteases, lipase etc [38-39]. Apart from these, the cold-adapted moulds could also be source of other bioactive molecules [4]. Since moulds are found to have post translational machinery and efficient secretary pathway they might be useful as the expression system of those eukaryotic proteins which are not expressed in soluble form at mesophilic temperatures. This is further to note that we have isolated very rare psychrophilic mould *T. angustata* BPF5 which is considered an anamorphic fungus. But this isolate has been found to form sexual structure at 4°C and BPF-6 that forms melanin and that cannot grow above 20°C.

The results showed the frequency of occurrence of different isolates varied from 10 (BPF-2) to 100% (BPF-10 *Rhizomucor* sp) (**Table 1**). While *Cladosporium* and *Penicillium* were the most dominant genera in terms of number of species obtained, *Rhizomucor* sp. showed the most prolific growth at 4°C on petridishes.

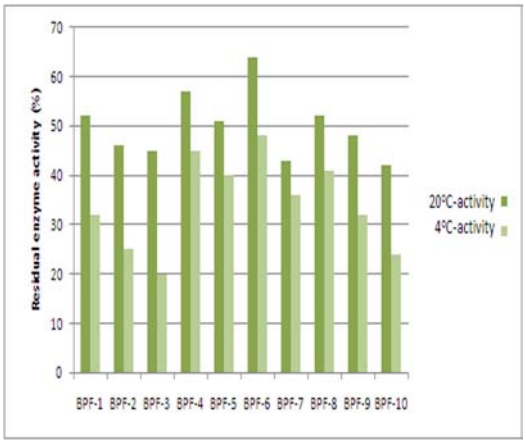

Figure 6 Effect of temperature on the activity of α-amylase isolated from various cold-active isolates

α-amylase activity

The optimum temperature of α-amylase obtained from various fungal strains was found to be 40°C, except that from BPF7 and BPF10 which showed optimum activity at 50°C. The residual cold-activity shown by the α-amylase from various strains is given in (**Fig. 6**). While BPF-6 showed maximum residual cold-activity, BPF-10 exhibited minimum residual cold-activity indicating modulatory-mechanism at the level of catalytic

efficiency (K_{cat}/K_m) of cold-adaptation as reported earlier [40]. This modulation of activity is achieved either by higher turnover numbers (k_{cat}) at the expense of K_m or by optimizing both parameters (increasing k_{cat} and decreasing K_m) [41]. These adaptive strategies of cold-active microbes justify continuous exploration of the most potent microbes from the environment to tap them for biotechnological applications. Further characterization of the enzyme from the isolates especially the most potent isolate BPF-6 is required to find out the most appropriate industry for its application.

Conclusions

The paper highlights the importance of exploration of fungi from temperate climate in order to get cold-active enzymes there from. It also underlines the necessity to explore more and more microbes in order to obtain enzymes with the desirable features. Newer information regarding the life cycle of extremophiles may also be found as in case of *T. angustata* which is regarded as anamorphic fungus, has been found to bear sex organs like structure during this investigation.

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