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Screening of Psychrotrophic Micro-fungi for Cold Active Extracellular Enzymes Isolated from Jammu City, India

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The psychrotrophic micro-fungi of soil of Jammu city, India, were studied. The fungal isolates were identified by morpho-taxonomically and screened for their ability to grow at low temperatures. Most of the predominant isolates were species of *Mucor sp., Penicillium sp., Rhizopus sp., Aspergillus sp.* and *Fusarium sp.* Isolated micro-fungi were characterized and screened in respective enzymatic agar medium for their degradation capability at 15°C. *Penicillium sp.*, *Fusarium sp.*, *Alternaria sp.* were found to be the maximum producer of cold active lipase, whereas *Aspergillus sp.* and *Microsporum sp.* showed maximum cellulase activity. The findings of this study indicate the possibility that the isolated strains produce novel extracellular enzymes that were active in cold temperature, which has immense application in many industries.

Key words: Psychrotrophic micro-fungi, Cold active enzymes, Jammu city.

Jammu city is located at 32.73° N latitude and 74.87° E longitude. Due to the diverse altitudes, climate and geo-morphological features Jammu city have resulted in the formation of different types of soils and the maximum temperature rarely reaches 37°C and temperatures in the winter months occasionally falling below freezing. The temperature variations during the year lead its climate favorable for diversified growth of psychrotrophs (psychrotolerant) than psychrophiles. On cardinal growth temperature, psychrophiles grow at or below zero and have optimum growth temperature ≤15°C and upper limit of ≤ 20°C. In contrast, psychrotolerants may well grow at mesophillic range with optima 20-25°C or may have upper limits as high as 40°C, whilst retaining the capacity to grow at or close to zero[1]. Soil microorganisms under such conditions experience not only physical stress but also starvation[2]. Cold tolerant mesophiles or psychrotrophs were found rather than psychrophiles isolated from Antarctica[3]. Psychrotrophic fungi can grow at around 0°C as well as grow above 20°C[4]. Many workers found filamentous mesophilic fungi adapted to grow at temperature of 1°C[5, 6, 7].

Microbial groups such as fungi are well recognized to produce a wide variety of chemical structures, several of which are most valuable pharmaceuticals, agrochemicals and industrial products. Cold-active enzymes like amylases, cellulases, lipases, pectinases, and proteases from psychrophilic fungal strains find vast applications in the food, medicine, and detergent industries[8, 9]. To the best of our knowledge, a little work has been done on psychrotrophic micro-fungi in India, and the production of extracellular cold active enzymes. Cold active lipases was reported in *Aspergillus nidulans*[10], *Candida Antarctica*[11, 12, 13, 14], *Candida lipolytica*, *Geotrichum candidum* and *Pencillium roqueforti*[15], *Rhizopus spp.* and *Mucor spp.*[16]; proteases in *Aspergillus ustus*[17]; pectinase in *Cystofilobasidium capitatum*, *C. larimarini*, *Cryptococcus cylindricus*, *C. macerans*, *C. aquaticus* and *Mrakia frigida*[18]; cellulase in *Penicillium cordubense* D28[19]. So, the

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aim of the present study is to estimate the fungal population and enzymatic screening of psychrotrophic micro-fungi isolated from the A-horizon of soils of Jammu City.

MATERIALS AND METHODS

Sample Collection

A total of twenty soil samples were collected from four different places of Jammu city such as garden (GAS), citrus orchards (COS), guava field (GFS), and brinjal field (BFS) in the month of January. The samples taken from each sites for microbiological analysis were placed separately in clean and sterile plastic bags with polar packs. All these samples were brought to the Department of Microbiology, O.U.A.T., BBSR (Odisha) for further study and stored at 4°C. Before use in the study, soil samples were sorted carefully by using sterilized fine forceps in order to remove any stones or plant material.

Moisture content of soil samples

Soil moisture contents was determined by taking 10g of soil from each sample and dried at 60°C for 72 hrs. in oven and calculated[20].

Soil pH determination

Soil pH was determined[21]. 10g soil was weighed into a 50ml size beaker. 20 ml of 0.01M $CaCl_2$ was added to the soil sample. The preparation was allowed to stand for 30min. with occasional stirring before determination of pH by digital pH meter 335 (Systronics, India).

Isolation of psychrotrophic fungi

For the isolation of psychrotrophic fungi, soil samples were spread on sterilized Czapek Dox Agar with streptomycin sulfate (0.015%) using serial dilution technique and incubated at 15±1°C. After 7 days of incubation the isolated strains were counted and CFU/gm of soil sample was calculated. The isolated fungi was then revived again on Czapek Dox Agar slants and maintained at 4°C.

Identification of Isolates

Pure cultures of isolated fungi were identified on the basis of their micro- and macro-morphology[22-29]. Colonies of isolated fungi were cultivated on Sabouraud's dextrose agar at corresponding isolated temperature for 7 days. The different morphological characteristics were evaluated i.e. colony growth (length and width), texture of aerial mycelium, colony color, presence of wrinkles and furrows, pigment production etc. Micro-morphological identification was done by lacto-phenol cotton blue and observed under phase contrast microscope (LAS EZ version 1.5.0) both at 40X and 100X.

Characterization of fungi

The isolated fungi was grown on different agar media (Sabouraud dextrose agar, Czapek Dox Agar, Potato dextrose agar and Malt extract agar) and investigated for their accurate identification and characterization. Besides these all isolates were investigated for varied temperature, high pH, and high salt tolerance capacity.

Screening for extracellular cold active enzymes

The isolated strains were spot inoculated on respective pseudo selective agar for screening of cold active enzymes at 15°C. After five days of incubation the plates were assayed by different methods and zones of clearing around the colonies were measured in mm. as the difference between the diameter of the halo and the fungal colony. The investigated cold active enzymes were protease, lipase, amylase, cellulase, gelatinase, and pectinase. Each test was done in triplicates.

Statistical analysis

All the data were analyzed by statistical methods like correlation coefficient and T- test for significant variations. Distribution percentage was calculated by total number of species found per total number of samples multiplied with 100. The diversity of species was studied in terms of species richness and relative abundance of the species. Relative dominance (d) was measured by calculating the Berger- Parker dominance[30]. d = n / N; Where n = no of individuals in a species, N = S = total no of individual (d>0.1 dominant genera, d <0.05 rare and between 0.1-0.05 were general genera). Simpsons Diversity index (D) is a simple mathematical measure that characterizes species diversity in a community. It is calculated using the following formula:

$$\sum_{i=1}^{S} \frac{n_i(n_i-1)}{N(N-1)}$$

Where, n = No. of individuals in each species; N = Total no. of individuals.

The 'D' assumes value lies between 0-1.

D = 0 indicates maximum diversity while, D = 1 represents the least diversity.

RESULTS AND DISCUSSION

A total of twenty soil samples collected from different places of Jammu city were investigated for physico-chemical and mycological study (Table1). The pH values of sampling sites were near to the neutral. Soil moisture content varied with sampling sites. Garden soil showed maximum (5.1×10^3CFU/gm) psychrotrophic fungal load at 15°C than other soil samples. Soil pH, organic content and moisture affect largely on fungal diversity[31, 32]. Soil physico-chemical parameters were found to influence the fungal distributions and population variation at various levels of significance. There is a negative but significant correlation (r = -0.46) between the soil pH and fungal load (CFU/g) at 0.05 level, which implies that when the soil pH is increased there is a corresponding decrease in the psychrotrophic fungal load in soil. There was a positive but insignificant correlation (r= 0.28) between soil pH and soil moisture and a positive but insignificant correlation among the soil moisture with the fungal load (r= -0.04). Present result is contradictory with the results of the investigators who studied the correlation among the different soil physico-chemical parameters with fungal load of eastern Himalaya and it was reported that fungal distribution showed negative but insignificant correlation with soil moisture content (r= -0.107) and soil pH (r= 0.065) showed positive but insignificant correlation with the fungal distribution[33].

Twelve fungal isolates were studied for physiological characterization i.e. salt and pH tolerance, urease test, growth at 10°C and antibiotic resistance study (Table 2). Only five of them were able to tolerate 10% NaCl concentrations but all isolates were able to grow at 9.5 pH. Only F2 and

Table 1. Physico-chemical parameters and psychrotrophic fungal load of different soil samples

Sampling sites	No. of samples	Soil pH[a]	Soil moisture content (%)[a]	Fungal load[a] ($\times10^3$CFU/g)
GAS	1	7.30	16.20	3.43
	2	7.28	21.10	3.65
	3	7.10	20.00	5.10
	4	7.33	30.00	4.10
	5	7.49	17.70	3.02
COS	6	6.80	36.00	4.83
	7	7.10	16.00	-
	8	6.95	16.20	-
	9	7.20	27.77	-
	10	6.70	19.00	4.98
GFS	11	6.77	13.46	4.81
	12	6.94	16.00	-
	13	6.80	17.70	4.71
	14	7.01	23.00	-
	15	6.88	22.00	4.60
BFS	16	7.50	14.10	2.30
	17	7.72	36.00	2.25
	18	7.93	24.60	-
	19	7.55	16.20	-
	20	7.90	28.60	-

[a]Experiments were done in triplicates. (-) No growth

F13 were urease negative. All isolates were able to grow at 10°C but F2 and F3 took maximum (15 days) for visible growth as compared to others. Among the isolates all were resistant to Fluconazole (100%), but 6 (50%) and 3 (25%) fungal isolates were resistant to Amphotericin B and Nystatin respectively. The isolates were identified at genus level i.e. *Rhizopus sp.*, *Penicillium sp.*, *Aspergillus sp.*, *Mucor spp.*, *Fusarium spp.*, *Chaetomium sp.*, *Microsporum sp.*, *Alternaria sp.*, and *Absidia sp.*

From table 3, incidences of psychrotrophic fungi were enumerated from different sites and detected that sampling sites (60%) were found to be positive for psychrotrophic fungi. Maximum incidences of psychrotrophic fungi were found to be present in garden soil (100%) where as minimum was in both brinjal field soil and citrus orchad soil (40%). The number of occurrences of psychrotrophic micro-fungi was more in guava field soil sample (36%). A total of 25 psychrotrophic fungal isolates were obtained which were categorized into 10 genera namely *Absidia sp.* (5%), *Alternaria sp.* (5%), *Aspergillus spp.* (20%), *Chaetomium sp.* (5%), *Coccoides sp.* (5%), *Fusarium spp.* (20%), *Microsporum sp.* (5%), *Mucor spp.* (40%), *Penicillium spp.* (10%), and

Table 2. Characteristics of psychrotrophic micro-fungi

Fungal isolates	Species Identified	High Salt tolerance (10% NaCl)	High pH tolerance (9.5)	Urease test	Growth at 10°C	Antibiosis		
						Fluconazole	Amphotericin β	Nystatin
F1	*Rhizopus sp.*	+	+	+	+	R	S	S
F2	*Penicillium sp.*	+	+	-	+	R	R	R
F3	*Aspergillus sp.*	+	+	+	+	R	R	S
F4	*Mucor sp.*	-	+	+	+	R	S	S
F5	*Fusarium sp.*	+	+	+	+	R	S	S
F6	*Mucor sp.*	-	+	+	+	R	S	S
F7	*Chaetomium sp.*	-	+	+	+	R	R	S
F8	*Fusarium sp.*	-	+	+	+	R	S	S
F9	*Microsporum sp.*	-	+	+	+	R	R	R
F10	*Fusarium sp.*	-	+	+	+	R	R	S
F11	*Alternaria sp.*	+	+	+	+	R	S	S
F12	*Absidia sp.*	-	+	-	+	R	R	R

R- resistant; S- susceptible; + growth; - no growth

Table 3. Occurrence and dominance of psychrotrophic fungi from Jammu city

Soil Samples	GAS	COS	GFS	BFS	Total	Distribution (%)	Relative dominance 'd'
Number of samples	5	5	5	5	20		
Positive samples (%)	100	40	60	40	60		
Fungi recorded	No. of occurrence						
Absidia sp	-	-	1	-	1	5	0.1
Alternaria sp.	-	-	1	-	1	5	0.1
Aspergillus spp.	1	-	3	-	4	20	0.4
Chaetomium sp.	-	-	1	-	1	5	0.1
Coccoides sp.	-	1	-	-	1	5	0.1
Fusarium spp.	1	1	1	1	4	20	0.4
Microsporum sp.	-	-	-	1	1	5	0.1
Mucor spp.	3	2	1	2	8	40	0.8
Penicillium spp.	1	-	1	-	2	10	0.2
Rhizopus spp.	1	1	-	-	2	10	0.2
Total	7	5	9	4	25		

Rhizopus spp. (10%). *Mucor sp.* was found to be dominant genera followed by *Fusarium spp.* and *Aspergillus spp.* (Table 4). It is known and reported that *Mucor sp.* is a cosmopolitan species and has been recorded from various parts of India[33]. The most frequently isolated psychrotrophic fungal species from Antarctica were belonged to the genera *Penicillium, Aspergillus, Cladosporium, Alternaria, Geomyces, Mucor, Rhizopus* and *Lecanicillium*[34, 35]. All were cold tolerant rather than cold loving psychrophiles and present study is in accordance with the above mentioned data.

Table 4 indicates that Simpson's diversity index 'D' was closer to '0' which indicate maximum diversity in all the sampling sites but guava field soil showed maximum species diversity as compared to others (D= 0.08).

All isolates were tested for cold active extracellular enzymes i.e. protease, lipase, amylase, cellulase, gelatinase, and pectinase at 15°C. Among the isolated psychrotrophs, maximum percentage of fungi (100%) showed lipolytic activity than other activities. Cold active protease activity was less among all isolates (41.67%). Cold active gelatinase positive isolates were 66.67% whereas cellulase, amylase and pectinase were found to be positive for 50% of fungal isolates. When halo zone diameter was studied, *Penicillium sp.* (F2), *Fusarium sp.* (F8), *Alternaria sp.* (F11) were found to be the maximum producer of lipase, whereas *Aspergillus sp*. (F3) and *Microsporum sp*. (F9) showed maximum cellulase activity (Table 5).

Cold active enzymes were studied by many workers and reported their production at different temperature with different fungi. Cellulases were obtained from strains of *Aspergillus*[33, 36], *Penicillium cordubense* D28[19]. *Aspergillus terreus* AV49 was investigated for the cellulase production by using groundnut shell at 28°C[37].Filamentous fungi are known to be good lipase producers; examples are *Aspergillus niger*[38], *Fusarium solani*[39], *Rhizopus oligosporus*[40] and members of the genera *Geotrichum, Mucor* and *Penicillium*[41]. Cold active lipases was reported in *Aspergillus nidulans*[10], *Pencillium roqueforti*[15], *Rhizopus sp.* and *Mucor sp.*[16] and proteases in *Aspergillus ustus*[17] Cold active amylolytic, cellulolytic and pectinolytic activity was studied

Table 4. Diversity analysis

Sampling sites	Total no. of colonies isolated 'S'	No of genera identified	Simpson's Diversity Index 'D'
GAS	7	5	0.143
COS	5	4	0.1
GFS	9	7	0.08
BFS	4	3	0.17
Total	25		

Table 5. Cold active extracellular enzymes produced by fungi at 15°C

Fungal isolates	Protease	Lipase	Gelatinase	Cellulase	Amylase	Pectinase
F1	-	+	-	-	-	+
F2	+	+++	-	+++	++	+
F3	++	++	+	+++	+	-
F4	-	+	-	-	-	-
F5	+	++	+	-	-	+
F6	-	+	+	-	-	-
F7	-	++	+	+	+	-
F8	-	+++	+	-	-	++
F9	++	++	+	+++	-	-
F10	-	+	-	+	+	+
F11	++	+++	+	+	+	++
F12	-	+	+	-	+	-

+++ (>10mm diameter); ++ (<10mm diameter); + (<5mm diameter) ; - (no activity)

on *Aspergillus aculeatus, A. flavus* at both 4°C and 20°C[42]. Antarctic fungi have been evaluated for extracellular enzyme activity including cellulase, amylase, and pectinase and fungi studied were *Fusarium lateritium, Aspergillus aculeatus, A. flavus, A. niger, Mucor, Myrothecium and Penicillium*[43]. The present data is in accordance with the above mentioned works.

The present work indicates that psychrotrophic micro fungi exist in the soil of Jammu city. The fungal isolates studied are good producers of many cold active enzymes, which find vast applications in the food, medicine, and detergent industries. Besides, these may be used to facilitate the mineralization of agro-wastes in colder hilly areas across the world, including the Himalayas in India.

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