



Temperature dependent lipase production from cold and pH tolerant species of *Penicillium*

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Abstract

The psychrotolerant microorganisms are receiving attention of the scientific community due to their ability to produce biotechnological products. The present study is focused on the diversity of cold and pH tolerant isolates of *Penicillium* spp with respect to their potential to produce cold active lipases. The characterization of the fungal isolates was done using polyphasic approach (morphological and molecular methods). The isolates were found to have tolerance for temperature from 4-35 °C (opt.21-25 °C) and pH 2-14 (opt. 5-7). Lipase production was investigated under the influence of temperature between 5-35 °C. The fungal isolates were found to produce lipase, optimally at different temperatures, up to 25 days of incubation. Maximum lipase production was recorded at 15 and 25 °C temperatures, whereas it was minimum at 5 and 35 °C. Three fungal isolates, designated as GBPI_P98, GBPI_P150 and GBPI_P228, were found to produce optimal lipase at 25 °C whereas seven isolates, GBPI_P8, GBPI_P36, GBPI_P72, GBPI_P101, GBPI_P141, GBPI_P188 and GBPI_P222, showed maximum lipase production at 15 °C. In general, production of biomass showed no relation with the lipase activity. The study will have inference in production of cold active lipase for their versatile uses in biotechnological industries.

Key words – Indian Himalayan region – fungi – *Penicillium* – psychrotolerants – lipase

Introduction

Extremophiles are known to survive in extreme environments mainly in terms of temperature, pH, salt, pressure, etc. Low temperature environments are likely to be colonized by psychrophiles and psychrotolerants. These microorganisms have evolved their cellular machinery at biochemical and molecular level to grow under the temperature stress (Wynn-Williams 1990, Aislabie et al. 2004). The cold adaptive microorganisms are reported to secrete cold shock proteins/enzymes (amylase, lipase, protease, etc.) that retain the stability and activity under low temperature conditions. These cold tolerant products are known to have several biotechnological applications (Cavicchioli et al. 2011).

Lipases (EC 3.1.1.3) are hydrolytic enzymes; they are also the most prominent enzymes produced by microorganisms with an important physiological role towards the hydrolysis of triglycerides into mono & di- glycerides, fatty acids and glycerol. They have the capability to hydrolyze the carboxylic ester bond through which they lead various important reactions such as alcoholysis, esterification and trans-esterification reactions (Houde et al. 2004). Lipases are

secreted by wide range of bacteria and fungi. Extracellular lipases have been studied from a number of fungi representing the group ascomycota and basidiomycota including *Mucor*, *Rhizopus*, *Penicillium*, *Candida*, *Aspergillus* etc. (Singh & Mukhopadhyay 2012, Thota et al. 2012, Abrunhosa et al. 2013, Szczesna-Antzak et al. 2014). Due to the extracellular secretion of lipase enzymes, fungi are used preferably for enzyme production (Sumathy et al. 2012). Lipases are considered as a versatile tool in biotechnology, as they lead to multiple industrial applications in pharmaceutical, agrochemicals, dairy and textile, cosmetic and perfume, detergent and surfactant production (Gupta et al. 2004). The cold active lipases are becoming one of the burning issues of the present research mainly due to their high activity at low temperature relevant to the industries based on detergent, fine chemistry catalysis, and food processing. Due to these unique products the psychrophilic and psychrotolerant microorganisms are being explored widely (Joseph et al. 2008).

Penicillium has been reported as one of the dominant genera of the cold temperature environments and widely known for its potential to produce different useful bioactive compounds and biotechnological products (Nicoletii et al. 2008, Cantrell et al. 2011, Dhakar et al. 2014). The present study is focused on the screening of different *Penicillium* spp from Indian Himalayan Region (IHR) for their potential towards lipase production at a wide range of temperature.

Materials & Methods

Fungal isolates

10 fungal isolates were obtained from the Microbial Culture Collection established in the Microbiology Laboratory of the Institute (GBPNIHESD, Almora). These cultures were originally isolated from the soil samples collected from various locations in high altitudes of IHR including cold desert and glacial sites. Pure cultures were maintained on Potato Dextrose (PD) agar slants and stored at 4 °C. Cultures were revived by sub culturing on PD agar and incubated at 25 °C for 7 days for further experimental use.

Physiological characterization

7 days old fungal cultures were inoculated on PD agar plates and incubated at different temperatures ranging from 5 to 45 °C (with an interval of 10 °C) for temperature tolerance. The pH tolerance was determined by inoculating cultures in PD broth with varying pH, ranging from 1-14. All the observations regarding growth were recorded following 7 days of incubation.

Molecular identification and phylogenetic tree

For identification of fungal isolates, DNA was isolated by the method of Voigt et al. (1999). ITS (Internal Transcribed Spacer) region was amplified using primers (ITS1 and ITS4) given by White et al. (1990) through PCR and sequencing of the amplified product was performed in collaboration with National Centre for Cell Science, Pune, India. BLAST analysis of the resulting sequences was performed for identification of fungal isolates. Phylogenetic tree of the given fungal isolates was reconstructed by Neighbour Joining method with bootstrap value of 1000 using MEGA v6.0 (Tamura et al. 2013).

Lipase production from fungal isolates

35 *Penicillium* isolates were initially screened for zone of hydrolysis on tributyrin agar. 10 *Penicillium* isolates, that exhibited lipase activity in plate assays, were further investigated for quantification of lipase production. Quantitative estimation for lipase production was carried out at four different temperatures viz. (5, 15, 25 and 35 °C). Production of lipase was achieved in lipase production medium (composition in g/l): 3.0g NaNO₃, 0.1g K₂HPO₄, 0.5g MgSO₄.7H₂O, 0.5g KCl, 0.01g FeSO₄.7H₂O, 5.0g yeast extract and 1% olive oil, pH 6.2. Production was carried out in Erlenmeyer flasks containing 50 ml of production media. 5 mm disc of 5 days grown culture of fungus was used for the inoculation, followed with incubation up to 25 days in static conditions, at four different temperatures. The enzyme activity and biomass were determined up to 25th day of incubation at an interval of 5 days.

Lipase activity was measured at 410 nm using 4-nitrophenyl laurate (*p*NPL) as lipase substrate (Pinsirodom & Parkin 2001). All the experiments were performed in triplicates. Lipase activity was expressed in terms of enzyme unit which was defined as the $\mu\text{mol of } p\text{NP}$ released per minute under specified assay conditions.

Biomass determination

For biomass determination, mycelium was harvested after filtration of fungal broth through Whatman No. 1 filter paper. The mycelium was washed under tap water and dried at 55°C until complete dryness and weighed to obtain constant weight.

Statistical analysis

The average value and standard deviation of three replicates was calculated using Microsoft Excel software. One way ANOVA following post hoc Tukeys HSD test was used for comparison of means and measurement of significant difference ($p < 0.05$) in the lipase and biomass production at different temperatures.

Results

Penicillium diversity

Morphological, microscopic, and physiological features of the 10 species of *Penicillium* are presented in Table 1. All the ten *Penicillium* spp. were able to grow from 5 °C to 35 °C, except GBPI_P72 which showed growth up to 30 °C only. Optimum growth temperature for all the species, except GBPI_P72, was observed at 25 °C. In general, pH tolerance range for all the species was found between 2-14, except in case of GBPI_P101 and GBPI_P188 which showed pH tolerance up to 3 pH. The fungal isolates have been deposited in Microbial Culture Collection, National Centre for Cell Science, Pune, India. Identification based on ITS region showed maximum similarity with *Penicillium* spp. The resulting nucleotide sequences have been deposited in the GenBank, NCBI. The identification and nucleotide accession numbers of the fungal isolates are shown in Table 1. The phylogenetic tree of all the ten *Penicillium* spp is shown in Fig. 1.

Quantification of lipase production by Penicillium spp

Seven *Penicillium* spp. (GBPI_P8, GBPI_P36, GBPI_P72, GBPI_P101, GBPI_P141, GBPI_P188, and GBPI_P222) were found to produce maximum lipase enzyme ranging from 5.13 U/ml to 22.82 U/ml at 15 °C, while remaining 3 isolates (GBPI_P98, GBPI_P150, and GBPI_P228) showed maximum lipase enzyme production ranging from 4.02 U/ml to 23.47 U/ml at 25 °C. Production of lipase enzyme and biomass up to 25 days is shown in Table 2. Maximum lipase production by the fungal isolate GBPI_P8 was observed on 10th day of incubation (14.75 U/ml) at 15 °C which was significantly different ($p < 0.05$) from lipase production at other temperatures on same day of incubation, while minimum lipase production (0.17 U/ml) was recorded at 35 °C on 20th day of incubation. Maximum biomass was also observed at 15 °C on 10th (0.30 g) and 15th (0.31 g) day of incubation.

GBPI_P36 produced maximum lipase (11.26 U/ml) on 25th day of incubation at 15 °C, while the production was minimum (0.08 U/ml) at 5 °C on 15th day of incubation. Lipase production increased continuously up to 25th day of incubation at 15 °C. Production of enzyme at 15 °C was significantly higher ($p < 0.05$) than production at other three temperatures after 10th day of incubation. In contrast to lipase production, biomass at 15 °C increased up to 10th day of incubation which decreased afterward up to 25th day. Maximum biomass (0.58g) was recorded at 35 °C on 10th day of incubation, and the values were significantly higher ($p < 0.05$) in comparison to biomass production at other temperatures on the same day of incubation. In case of GBPI_P72, 15

Table 1 Characteristics of *Penicillium* spp along with their identification based on 18s ITS region sequencing.

| SN | Isolate | Colony colour, microscopy, temperature range (optimum), pH range (optimum) | Identification | Accession no. | |
|----|------------|---|-----------------------------------|---------------|------------|
| | | | | Culture | Nucleotide |
| 1 | GBPI_P8 | White, yellow pigment, branched conidiophore, 5-35° (25 °C opt), 2-14 pH (5-7 opt) | <i>Penicillium cordubense</i> | MCC 1123 | KP712867 |
| 2 | GBPI_P36 | Greyish-green, branched conidiophore, 5-35 °C (25 °C opt), 2-14 pH (5-7 opt) | <i>Penicillium</i> sp. | - | KT826696 |
| 3 | GBPI_P72* | Dark green, branched conidiophore, 4-30 °C (21 °C opt), 2-14 pH (5-7 opt) | <i>Penicillium</i> sp. | MCC1054 | KC634218 |
| 4 | GBPI_P98* | Greenish grey, branched conidiophore, 4-35 °C (opt 25 °C), 2-14 pH (5-7 opt) | <i>Penicillium restrictum</i> | MCC1058 | KC634222 |
| 5 | GBPI_P101* | Greyish, branched conidiophore, 4-35 °C (opt 25 °C), 3-14 pH (5-7 opt) | <i>Penicillium raistrickii</i> | MCC1059 | KC634223 |
| 6 | GBPI_P141 | Light green with white margins, branched conidiophore, 5-35 °C (25 °C opt), 2-14 pH (5-7 opt) | <i>Penicillium polonicum</i> | - | KT826697 |
| 7 | GBPI_P150* | Green, branched conidiophore, 5-35 °C (25 °C), 2-14 pH (5-7 opt) | <i>Penicillium commune</i> | MCC1060 | KC634224 |
| 8 | GBPI_P188* | Dark green, branched conidiophore, 5-35 °C (25°C), 3-14 pH (6-9 opt) | <i>Penicillium jensenii</i> | MCC1063 | KC634228 |
| 9 | GBPI_P222 | Greyish with white margins, branched conidiophores, 5-35 °C (25 °C opt), 2-14 pH (5-7 opt) | <i>Penicillium aurantiovirens</i> | MCC1125 | KM458838 |
| 10 | GBPI_P228 | Dark green with white margins, branched conidiophore, 5-35 °C (25 °C opt), 2-14 pH (5-7 opt) | <i>Penicillium</i> sp. | - | KT826698 |

*Dhakar et al. 2014

°C was the optimum temperature for production of lipase with maximum production (5.22 U/ml) on 15th day of incubation, which decreased with the increasing incubation for longer duration. Lipase production at 5 °C and 35 °C was observed minimum with no significant difference except on 15th day of incubation. Biomass production at 5 °C as well as at 35 °C was recorded very less due to restricted growth. Similar to enzyme production, biomass was produced maximum (0.89 g) at 15 °C on 15th day of incubation.

GBPI_P98 produced statistically significant ($p < 0.05$) higher lipase at 25 °C and biomass at 15 °C, both being maximum (23.50 U/ml and 0.55 g) on 15th day of incubation. Biomass and lipase both were observed to be produced in very low quantity at 5 °C as well as 35 °C. GBPI_P101 and GBPI_P141, both showed maximum lipase at 15 °C on 15th (5.13 U/ml) and 20th (7.62 U/ml) day of incubation, respectively, production being significantly higher in comparison to production at other temperature on the same day of incubation. Biomass production by GBPI_P101 at 15 °C (0.51 g) was not significantly different ($p < 0.05$) for production at 25 °C (0.49 g) on 20th day of incubation. GBPI_141 showed restricted growth at 5 °C as well as 35 °C with maximal biomass recorded on 15th day of incubation at 25 °C.

Maximal lipase produced by GBPI_P150 was recorded at 25 °C on 20th day of incubation with an activity of 4.02 U/ml; maximal production at 15 °C was 2.56 U/ml on 10th day of incubation. Biomass was also produced maximum on 20th day of incubation at 25 °C, with production being significantly different ($p < 0.05$) from 15 °C. GBPI_P188 and GBPI_P222, both



Fig. 1 – Phylogenetic tree of ten *Penicillium* spp reconstructed by Neighbour Joining method (bootstrap value = 1000).

produced higher lipases at 15 °C with activity being higher on 15th day (16.26 U/ml) and 20th day (22.82 U/ml) of incubation, respectively. Among all *Penicillium* spp studied, GBPI_P222 was the maximal producer of lipase. Production by both the isolates was observed significantly higher ($p < 0.05$) at 15 °C than other temperatures on each day of incubation. Both the isolates showed very less growth at 5 °C and 35 °C. GBPI_P228 was a weak lipase producer, with maximal activity recorded at 25 °C (5.22 U/ml) on 20th day of incubation. Again, restricted growth was observed at 5 °C as well as 35 °C, while maximum biomass was recorded at 15 °C on 10th day of incubation.

Comparison in lipase production

The *Penicillium* isolates were screened for their potential to produce lipase at wide range of temperature. The optimum temperature for lipase production was found to be 25 °C for three (GBPI_P98, GBPI_P150, and GBPI_P228) of the isolates, whereas 15 °C was optimum for the rest seven isolates (GBPI_P8, GBPI_P36, GBPI_P72, GBPI_P101, GBPI_P141, GBPI_P188, and GBPI_P222) (Fig. 2). It was noticed that, in general, the biomass did not possess any relation with the enzyme production. However, in some cases (for example GBPI_P8 and GBPI_P222 at 15 °C and 25 °C, respectively), the lipase production was estimated to be maximum along with the maximum biomass production.

Table 2 Lipase activity and biomass production by *Pencillium* spp at different temperatures.

| S.N. | Isolate code | Temp (°C) | Lipase Activity (Unit/ml) | | | | | Biomass (g) | | | | |
|------|--------------|-----------|---------------------------|-------------------------|-------------------------|-------------------------|-------------------------|-------------------------|------------------------|------------------------|-------------------------|-------------------------|
| | | | 5 days | 10 days | 15 days | 20 days | 25 days | 5 days | 10 days | 15 days | 20 days | 25 days |
| 1 | GBPI_P8 | 5°C | 0.89±0.18 ^b | 0.96±0.36 ^b | 0.62±0.04 ^b | 0.91±0.15 ^b | 2.11±0.44 ^b | ND | ND | ND | ND | ND |
| | | 15°C | 2.49±0.11 ^a | 14.75±0.64 ^a | 9.15±0.54 ^a | 5.92±0.90 ^a | 6.63±0.92 ^a | 0.14±0.01 ^b | 0.30±0.02 ^a | 0.31±0.01 ^a | 0.27±0.01 ^a | 0.23±0.02 ^a |
| | | 25°C | 1.05±0.22 ^b | 1.03±0.08 ^b | 0.11±0.96 ^b | 1.15±0.072 ^b | 2.16±0.51 ^b | 0.20±0.02 ^a | 0.22±0.02 ^b | 0.20±0.03 ^b | 0.17±0.01 ^b | 0.13±0.02 ^b |
| | | 35°C | 0.22±0.07 ^c | 1.03±0.04 ^b | 0.72±0.07 ^b | 0.17±0.041 ^b | 1.94±0.26 ^b | 0.03±0.02 ^c | 0.05±0.03 ^c | 0.06±0.01 ^c | 0.04±0.02 ^c | 0.02±0.002 ^c |
| 2 | GBPI_P36 | 5 °C | 1.08±0.07 ^b | 1.92±0.11 ^c | 0.08±0.21 ^c | 1.82±0.11 ^b | 4.83±0.11 ^b | 0.01±0.00 ^d | 0.05±0.01 ^d | 0.10±0.02 ^c | 0.13±0.03 ^c | 0.12±0.01 ^b |
| | | 15 °C | 3.92±0.08 ^a | 6.18±0.19 ^a | 1.74±0.22 ^a | 1.98±0.11 ^b | 11.26±0.58 ^a | 0.29±0.02 ^b | 0.50±0.00 ^b | 0.45±0.01 ^a | 0.30±0.02 ^{ab} | 0.28±0.02 ^a |
| | | 25 °C | 3.78±0.04 ^a | 2.23±0.07 ^a | 0.79±0.12 ^b | 2.85±0.08 ^a | 0.65±0.14 ^c | 0.21c±0.02 ^c | 0.31±0.02 ^c | 0.37±0.05 ^b | 0.36±0.03 ^a | 0.24±0.04 ^a |
| | | 35 °C | 0.89±0.04 ^b | 0.21±0.04 ^{ab} | 0.31±0.04 ^c | 0.55±0.04 ^c | 0.67±0.04 ^c | 0.41±0.03 ^a | 0.58±0.00 ^a | 0.47±0.00 ^a | 0.29±0.01 ^b | 0.29±0.02 ^a |
| 3 | GBPI_P72 | 5 °C | 0.77±0.04 ^c | 1.3±0.08 ^b | 1.72±0.14 ^c | 0.29±0.07 ^b | 0.26±0.15 ^{ab} | ND | ND | 0.04±0.01 ^c | 0.26±0.03 ^c | 0.23±0.02 ^c |
| | | 15 °C | 1.70±0.08 ^a | 2.13±0.08 ^a | 5.22±0.52 ^a | 1.87±0.47 ^a | 1.08±0.26 ^a | 0.37±0.02 ^a | 0.58±0.05 ^a | 0.89±0.04 ^a | 0.47±0.02 ^a | 0.38±0.03 ^a |
| | | 25 °C | 1.13±0.04 ^b | 1.92±0.11 ^a | 3.98±0.11 ^b | 0.72±0.14 ^b | 0.34±0.11 ^b | 0.34±0.02 ^b | 0.37±0.02 ^b | 0.60±0.02 ^b | 0.35±0.01 ^b | 0.30±0.01 ^b |
| | | 35 °C | 0.81±0.04 ^c | 1.33±0.04 ^b | 2.59±0.33 ^b | 0.43±0.14 ^b | 0.26±0.15 ^b | ND | ND | ND | ND | ND |
| 4 | GBPI_P98 | 5 °C | 0.22±0.07 ^b | 3.14±0.22 ^c | 0.22±0.07 ^c | 2.31±0.25 ^c | 1.10±0.11 ^c | ND | ND | ND | ND | ND |
| | | 15 °C | 0.38±0.25 ^b | 8.91±0.25 ^a | 14.97±0.27 ^b | 4.74±0.14 ^b | 1.89±0.11 ^{bc} | 0.56±0.02 ^a | 0.51±0.02 ^a | 0.55±0.02 ^a | 0.42±0.02 ^a | 0.28±0.01 ^b |
| | | 25 °C | 2.47±0.57 ^a | 7.5±0.22 ^b | 23.5±0.83 ^a | 18.03±0.80 ^a | 3.90±0.61 ^a | 0.47±0.03 ^b | 0.44±0.02 ^b | 0.40±0.01 ^c | 0.35±0.03 ^b | 0.25±0.01 ^c |
| | | 35 °C | 0.26±0.04 ^b | 2.23±0.47 ^b | 0.86±0.07 ^c | 2.9±0.29 ^c | 2.18±0.32 ^b | 0.51±0.01 ^{ab} | 0.46±0.01 ^b | 0.45±0.02 ^b | 0.36±0.01 ^b | 0.32±0.02 ^a |
| 5 | GBPI_P101 | 5 °C | 0.77±0.15 ^b | 1.08±0.07 ^c | 2.59±0.19 ^b | 1.80±0.07 ^b | 0.86±0.07 ^c | ND | 0.06±0.01 ^b | 0.16±0.02 ^c | 0.46±0.02 ^a | 0.34±0.02 ^b |
| | | 15 °C | 0.77±0.04 ^b | 2.70±0.08 ^a | 5.13±0.11 ^a | 4.38±0.07 ^a | 3.04±0.15 ^a | 0.21±0.03 ^a | 0.33±0.02 ^a | 0.48±0.01 ^a | 0.51±0.01 ^a | 0.44±0.03 ^a |
| | | 25 °C | 1.34±0.08 ^a | 1.84±0.15 ^a | 2.4±0.041 ^b | 1.34±0.08 ^a | 0.55±0.10 ^c | 0.25±0.04 ^a | 0.31±0.04 ^a | 0.39±0.01 ^b | 0.49±0.02 ^{ab} | 0.28±0.03 ^a |
| | | 35 °C | 0.60±0.04 ^b | 1.10±0.04 ^c | 1.24±0.11 ^c | 1.13±0.15 ^c | 0.43±0.07 ^c | ND | 0.03±0.01 ^b | 0.04±0.01 ^d | 0.03±0.01 ^c | 0.03±0.01 ^d |
| 6 | GBPI_P141 | 5 °C | 0.55±0.18 ^c | 1.51±0.07 ^b | 0.69±0.08 ^c | 1.19±0.11 ^d | 0.41±0.15 ^b | ND | ND | ND | ND | ND |
| | | 15 °C | 0.55±0.04 ^c | 1.56±0.15 ^b | 6.42±0.37 ^a | 7.62±0.38 ^a | 3.26±0.67 ^a | 0.03±0.01 ^b | 0.15±0.02 ^a | 0.25±0.03 ^b | 0.15±0.01 ^a | 0.2±0.01 ^a |
| | | 25 °C | 1.10±0.11 ^b | 2.78±0.32 ^a | 3.64±0.22 ^b | 5.34±0.11 ^b | 1.22±0.26 ^b | 0.06±0.01 ^a | 0.16±0.01 ^a | 0.32±0.02 ^a | 0.13±0.01 ^b | 0.15±0.01 ^b |
| | | 35 °C | 1.87±0.19 ^a | 1.15±0.07 ^b | 0.96±0.04 ^c | 3.57±0.48 ^c | 0.89±0.04 ^b | ND | ND | ND | ND | ND |
| 7 | GBPI_P150 | 5 °C | 1.01±0.07 ^d | 2.25±0.04 ^b | 0.42±0.01 ^b | 1.03±0.04 ^c | 1.32±0.04 ^b | ND | ND | ND | ND | ND |
| | | 15 °C | 1.60±0.11 ^b | 2.56±0.04 ^a | 0.53±0.04 ^{ab} | 0.46±0.11 ^d | 1.22±0.04 ^c | 0.13±0.02 ^c | 0.38±0.03 ^c | 0.51±0.01 ^b | 0.45±0.02 ^a | 0.27±0.02 ^c |
| | | 25 °C | 1.32±0.04 ^c | 2.35±0.04 ^b | 0.50±0.07 ^{ab} | 4.02±0.07 ^a | 1.22±0.07 ^b | 0.41±0.01 ^a | 0.51±0.03 ^a | 0.56±0.03 ^a | 0.43±0.03 ^a | 0.35±0.02 ^b |
| | | 35 °C | 1.94±0.07 ^a | 2.61±0.04 ^a | 0.57±0.07 ^a | 1.75±0.04 ^b | 1.51±0.07 ^a | 0.37±0.02 ^a | 0.45±0.01 ^b | 0.41±0.01 ^c | 0.38±0.01 ^b | 0.39±0.01 ^a |

| S.N. | Isolate code | Temp (°C) | Lipase Activity (Unit/ml) | | | | | Biomass (g) | | | | |
|------|--------------|-----------|---------------------------|-------------------------|-------------------------|-------------------------|-------------------------|-------------------------|------------------------|------------------------|------------------------|------------------------|
| | | | 5 days | 10 days | 15 days | 20 days | 25 days | 5 days | 10 days | 15 days | 20 days | 25 days |
| 8 | GBPI_P188 | 5 °C | 0.09±0.04 ^c | 2.13±0.18 ^c | 1.99±0.08 ^c | 4.45±0.22 ^c | 0.45±0.11 ^c | ND | ND | ND | ND | ND |
| | | 15 °C | 1.70±0.11 ^b | 8.02±0.18 ^a | 16.26±0.11 ^a | 14.32±0.11 ^a | 7.49±0.29 ^a | 0.39±0.01 ^b | 0.45±0.01 ^a | 0.38±0.02 ^a | 0.42±0.01 ^a | 0.35±0.01 ^a |
| | | 25 °C | 4.56±0.18 ^a | 8.05±0.19 ^a | 8.77±0.14 ^b | 5.27±0.41 ^b | 2.79±0.43 ^b | 0.40±0.02 ^b | 0.31±0.01 ^b | 0.25±0.02 ^b | 0.26±0.01 ^c | 0.26±0.01 ^b |
| | | 35 °C | 1.68±0.22 ^b | 4.57±0.25 ^b | 8.86±0.04 ^b | 0.17±0.41 ^d | 0.95±0.43 ^c | 0.45±0.02 ^a | 0.27±0.02 ^c | 0.36±0.01 ^a | 0.28±0.01 ^b | 0.25±0.05 ^b |
| 9 | GBPI_P222 | 5 °C | 0.12±0.04 ^c | 1.41±0.36 ^c | 0.98±0.08 ^c | 5.63±0.15 ^c | 3.66±0.26 ^c | ND | ND | ND | ND | ND |
| | | 15 °C | 5.49±0.29 ^a | 19.47±0.22 ^a | 20.19±0.19 ^a | 22.82±0.53 ^a | 18.75±0.31 ^a | 0.19±0.01 ^b | 0.22±0.01 ^b | 0.21±0.01 ^b | 0.30±0.01 ^a | 0.21±0.01 ^a |
| | | 25 °C | 1.77±0.18 ^b | 8.04±0.50 ^b | 10.37±0.68 ^b | 15.71±0.50 ^b | 10.39±0.25 ^b | 0.25±0.01 ^a | 0.22±0.02 ^a | 0.28±0.01 ^a | 0.31±0.01 ^a | 0.21±0.01 ^a |
| | | 35 °C | 0.55±0.41 ^c | 1.25±0.11 ^c | 1.75±0.11 ^c | 0.91±0.15 ^d | 1.99±0.17 ^d | 0.04±0.01 ^c | 0.04±0.01 ^c | 0.03±0.01 ^c | 0.07±0.01 ^b | 0.07±0.01 ^b |
| 10 | GBPI_P228 | 5 °C | 0.77±0.11 ^c | 0.96±0.04 ^c | 1.51±0.04 ^c | 1.46±0.04 ^c | 0.62±0.04 ^c | ND | ND | ND | ND | ND |
| | | 15 °C | 2.03±0.15 ^b | 2.23±0.07 ^b | 3.07±0.11 ^b | 2.57±0.08 ^b | 1.39±0.18 ^b | 0.17±0.02 ^b | 0.37±0.02 ^a | 0.23±0.01 ^a | 0.30±0.02 ^a | 0.29±0.01 ^a |
| | | 25 °C | 2.42±0.04 ^a | 3.71±0.27 ^a | 3.64±0.04 ^a | 5.22±0.15 ^a | 2.44±0.14 ^a | 0.30±0.021 ^a | 0.28±0.02 ^b | 0.16±0.02 ^b | 0.27±0.02 ^a | 0.22±0.02 ^b |
| | | 35 °C | 0.72±0.08 ^c | 0.59±0.04 ^c | 1.64±0.04 ^c | 0.72±0.08 ^d | 0.09±0.04 ^d | ND | ND | ND | ND | ND |

Values are mean±SD of three replicates. Means with different letters are significantly different ($p < 0.05$) on same day of incubation; ND = Not detected due to restricted growth

Discussion

The fungal isolates were characterized and identified using polyphasic approach. The morphological and molecular methods revealed the identity of fungal isolates as species of *Penicillium*. So far, the morphological approach which has been in use creates difficulty to distinguish between closely related species. The sequence analysis of ribosomal RNA in the molecular studies is authenticated and efficient. The fungal isolates are designated as the species of *Penicillium* on the basis of ITS sequence similarity (between 98-100 %) to the NCBI database. The fungal isolates identified as the species of *Penicillium* are: *P. cordubense*, *P. restrictum*, *P. raistrickii*, *P. polonicum*, *P. commune*, *P. jensenii*, and *P. aurantiovirens*. The ITS and IGS (Inter Generic Sequence) region are reported to have potential for phylogenetic analysis due to its variability (Skouboe et al. 1999, Visagie et al. 2014). ITS region is being considered as a universal DNA barcode marker for fungi (Schoch et al. 2012). Generally, *Penicillium* fungi are known for their growth in different physiological conditions, such as low to high temperature, low water availability, and high salt concentration (Houbraken & Samson 2011).

In the present study, *Penicillium* spp. are reported to have temperature tolerance from 4 - 35 °C and pH tolerance from 2-14. The temperature preference indicated that the fungal isolates are psychrotolerants and the pH tolerance revealed that they have unique characteristics to tolerate a wide range of pH (2-14). The temperature at the study sites, considered for the isolations, has been reported to be at the lower side up to sub zero levels (Pandey et al. 2006, Ghildiyal & Pandey 2008, Rinu & Pandey 2011) and, thereof, was likely to support the occurrence of psychrotolerants. However,

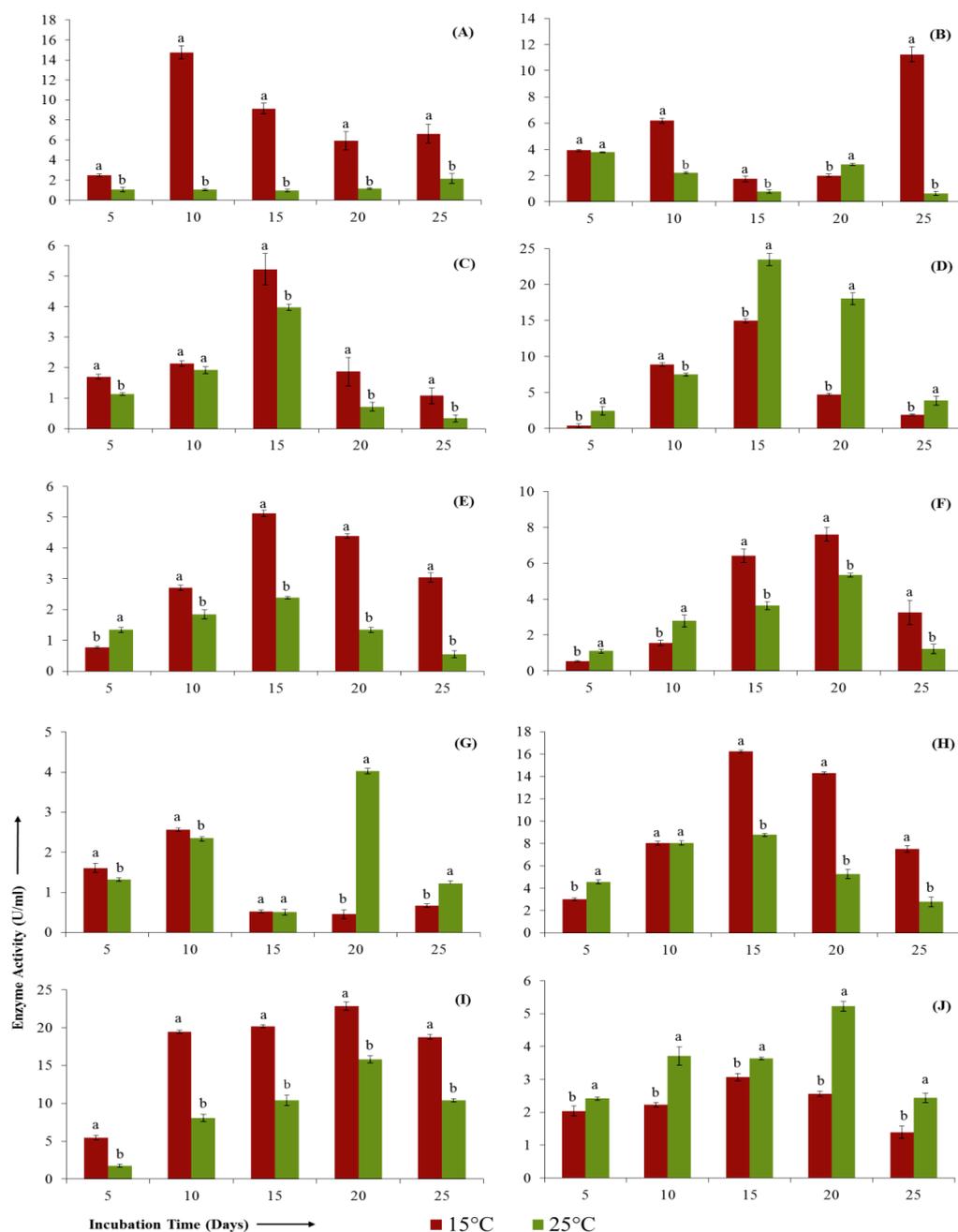


Fig. 2 –Comparison of lipase production by ten *Penicillium* spp at 15 °C and 25 °C. (A) GBPI_P8, (B) GBPI_P36, (C) GBPI_P72, (D) GBPI_P98, (E) GBPI_P101, (F) GBPI_P141, (G) GBPI_P150, (H) GBPI_P188, (I) GBPI_P222, and (J) GBPI_P228. Means with different alphabets on a bar group are significantly different (p<0.05).

the wide pH tolerance of the fungal isolates from the soils with normal pH (6.0-6.5) appears to be a remarkable phenomenon for further research. Similar observations on wide pH tolerance have been reported from the organisms including bacteria, actinomycetes and fungi and also the thermophilic organisms from the study area (Malviya et al. 2009, Sharma et al. 2009, Pandey et al. 2015, Dhakar & Pandey 2016). These microorganisms are reported to have some alternative adaptive mechanisms to survive in the low temperature. The presence of cold shock proteins, change in the lipid compositions, anti freeze proteins, accumulation of cryoprotectants (sugars, polyols, etc.) to avoid the stress of desiccation etc. have been reported to support the survival of the respective microorganisms in the colder environment (Robinson 2001). The wide tolerance of pH of the fungal isolates, in the present study, needs further investigations at molecular level. The

phenomenon can be explained by the expression of supportive genes upon the exposure of the extreme conditions (in terms of pH) (Schlichting & Wund 2014). Several characteristics of tolerance to extreme conditions indicate the phenomenon of ecological resilience by the microorganisms and can be further utilized to study physiological mechanisms of the fungal isolates and their applications in biotechnological sector.

In this study, three *Penicillium* isolates produced maximum lipase at 25 °C, which was also the optimum temperature for their growth. Besides, seven isolates produced maximal lipase at 15 °C. This might be due to psychrotolerant nature of the *Penicillium* isolates, causing maximum lipase production at sub-optimal temperature (15 °C). There are several reports in literature on the production of lipase from different fungal genera in the mesophilic range (Lima et al. 2003, Ulkeret al. 2011). *Penicillium expansum* SM3 has been reported to produce lipase optimally at 25 °C (Mohammed et al. 2013), whereas in another species, i.e. *P. citrinum*, the optimal temperature for lipase production was reported to be 37 °C (Pimental et al. 1997). *Aspergillus nidulans* WG312 has been reported to produce maximum lipase at 30 °C (Mayordomo et al. 2000). Lipase production at low temperature (near/below 20°C) has also been studied in a number of fungal species belonging to the genera such as *Alternaria*, *Penicillium*, *Trichoderma*, *Curvularia*, etc (Kakde & Chavan 2011). *Penicillium chrysogenum* has been reported to produce lipase 68 U/ml on the 5th day of incubation at 20 °C (Bancerz et al. 2005). In the present study, three *Penicillium* isolates designated as GBPI_P8, GBPI_P188, and GBPI_P222 can be considered for detailed studies due to their ability to produce higher lipase production at low temperature (15 °C), while GBPI_P98 can be harnessed for its potential to produce lipase at 25 °C.

Production of enzymes depends greatly on the culture conditions. The microorganisms have the tendency to respond against the change in their external environments. The psychrotolerant *Penicillium* isolates, used in the present study, exhibited their preferences for lipase production with respect to the temperature. Most of the psychrotolerant fungal species are likely to be inclined towards low temperature for the production of lipases. Such cold adaptive species have potential to produce enzymes that are different from the mesophilic species in their catalytic nature and are promising to have advantage in several industrial and biotechnological applications (Feller & Gerday 1997).

In industries, lipases possess several applications from processing the fatty compounds to their role as surfactants. Apart from this, the esterification and trans-esterification reactions of lipases are crucially involved in the cosmetics, pharmaceuticals and agricultural sectors (Hasan et al. 2006). Screening and selection of promising *Penicillium* isolates for production of cold active lipases was the focus of the present study. The cold active enzymes are desired as superior alternatives of mesophilic enzymes in many wine and cheese manufacturing industries (Collins et al. 2002). They are involved in different biotechnologically important phenomenon and are receiving attention for improvement through recombinant technology for their catalytic properties (Tutino et al. 2009). Also, cold adapted lipases are known to play role in other biotechnological applications such as protein polymerization, synthesis of optically active esters, improving food texture, etc (Cavicchioli & Siddiqui 2004).

Lipases have also grabbed attention in the fields of bioremediation and biodegradation. They have been proven as strong tools, in form of emulsifiers, for cleaning of waste water released from the oil and grease based factories (Abd El-Gawad 2014). The lipases in the colder regions also contribute in the biogeochemical cycle of carbon. The cold active lipases have been found effective in bioremediation and the degradation of organic pollutants in low temperature environments where other microorganisms lose their activities. Their use in bioremediation with respect to oil polluted places, waste water treatment and in reduction of the toxic compounds (xenobiotic compounds, heavy metals, biopolymers etc.) in low temperature environments has recently been described (Maiangwa et al. 2015).

Microorganisms are capable of producing several bioactive compounds for their survival in various environmental niches including extreme conditions. Psychrotolerant fungi have importance in decomposition cycles in mountain ecosystem and a significant amount of biomass is contributed

by them to the microbial community. These fungi also possess role in biodegradation by degrading high amount of organic matter at low temperatures in lesser time (Margesin & Feller 2010). Several reports on microorganisms (bacteria and fungi) from IHR, in last two decades, have received attention for exploration of the microbial diversity with respect to their potential in plant growth promotion, biocontrol, biodegradation and industrially important enzymes (Pandey et al. 1999, Pandey et al. 2002, Trivedi & Pandey 2007, Trivedi et al. 2007, 2012, Dhakar & Pandey 2013, Pandey et al. 2014, Rinu et al. 2014). Screening and selection of promising microbial species is likely to have found their way for a range of applications in the industries.

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