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Original Article

## Study on the potential of cold-active lipases from psychrotrophic fungi for detergent formulation



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## ARTICLE INFO

## Article history:

Received 18 July 2017

Received in revised form 6 October 2017

Accepted 30 April 2018

Available online 7 May 2018

## Keywords:

Cold-active lipases

*Penicillium canesense* BPF4*Pseudogymnoascus roseus* BPF6

Detergent lipases

Psychrophilic fungus

Psychrotolerant fungus

## ABSTRACT

Lipases from psychrotrophic fungal isolates BPF4 and BPF6 identified as *Penicillium canesense* and *Pseudogymnoascus roseus* respectively were characterized for their compatibility towards laundry detergent. BPF4 and BPF6 lipases showed maximum activity at pH 11 and 9 respectively and at 40 °C. The residual activities at 20 °C and 4 °C of BPF4 lipase were 35% and 20% and of BPF6 lipase were 70% and 20 °C respectively. Both the enzymes were stable at 4 °C, 20 °C and 40 °C for 2 h losing at the most 20% of activities. Both the enzymes were metalloenzymes with activity enhancement by nearly threefold by Ca<sup>2+</sup>. Contrary to BPF6 lipase, BPF4 enzyme was not stimulated by EDTA nor inhibited, rather stimulated by SDS and Triton X-100 by 125% and 330% respectively. Both the lipases showed minor to moderate inhibition by NaClO<sub>3</sub> and H<sub>2</sub>O<sub>2</sub>, and exhibited nearly 90% residual activity after 1 h of incubation in selected detergent brands thus indicating potential for their inclusion in detergent formulation thereby facilitating cold-washing as a step towards mitigation of climate change.

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## 1. Introduction

Climate change is one of the most important issues the world is currently facing, and as part of solution it calls for multi-pronged strategy. Application of cold-active enzymes is one of the important steps among them as it is expected to reduce energy consumption [8] and consequently carbon dioxide emissions. Washing cloth at ambient temperature requires heating of water in the washing-machine in colder areas, for instance in USA it accounts for 36% of hot water use [26] producing 34 million tonnes of carbon dioxide per annum to heat [23]. Cold-washing with laundry detergent containing cold-active enzymes is regarded as an effective solution to this issue [1] and thus a target to increase global cold-water washing machine loads from 38 to 53% during 2010–2014 was set [37]. Moreover, in a country like India where people mostly women do washing manually at ambient temperature have to put in much labour with currently available detergent. A detergent containing cold-active enzymes would thus save them from this drudgery [38]. Laundry detergent in current form utilizes huge volume of enzymes, for instance in terms of value for 2013 it was approxi-

mately \$1 billion to be reaching \$1.8 billion by 2018 [5] and in terms of volume of lipases only it was as per 1996 data 1000 tones [10] and so expected to be much more now. A switch-over to cold-washing would thus require a huge amount of cold-active enzymes including lipase.

Lipases (E.C. 3.1.1.3) are ubiquitous enzymes that catalyze hydrolysis and synthesis of acylglycerol and other water insoluble esters. They are industrially very important finding applications in food, pharmaceutical, fine chemical, oil chemical, biodiesel and detergent industries [9]. Following successful performance of proteases, lipases have also been introduced in laundry detergent. During laundering, lipases adsorb onto the fabric surface to form a stable fabric-lipase complex and then hydrolyze oil stains present on the fabric. The complex is resistant to the harsh wash conditions and is retained on the fabric during laundering [41]. Even though, lipases to be detergent compatible should exhibit following attributes: stability at alkaline pH, solubility in water, tolerance to detergents proteases and surfactants, and low substrate specificity [24].

Genecor International pioneered in producing commercial bacterial lipases, namely, Lipomax from *Pseudomonas alcaligenes* and Lumafast from *Pseudomonas mendocina*, that could be used as detergent enzymes in the year 1995 [28]. Recently, detergent industries heavily relied on Lipex and Lipolase (brand name of lipases) from Novozymes for detergents due to their stability in

Peer review under responsibility of National Research Center, Egypt.

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E-mail address: [ss000@rediffmail.com](mailto:ss000@rediffmail.com) (S. Sahay).<https://doi.org/10.1016/j.jgeb.2018.04.006>

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presence of harsher detergent supplements [32]. Another lipase, isolated from *Bacillus licheniformis* was sensitive to chelating agent and required  $\text{Ca}^{+2}$  for its stability in the presence of commercial detergents [24]. Since then, the share of lipases in the global enzyme market has grown considerably and is expected to acquire significance comparable to that of the proteases, which currently represent 25–40% of industrial enzyme sales [24].

In view of its commercial importance, extensive exploratory works for newer and better sources of detergent compatible lipases have been undertaken [24]. As a result, potential lipases with distinctive characteristics have been reported from the microorganisms belonging to the following genera: *Pseudomonas* [17], *Cryptococcus* [40], *Burkholderia* [42], *Fusarium* [27] and *Candida antarctica* [31]. However, there are very few reports available as to the isolation and characterization of cold-active lipases [2,15,30,31,43] and only some of them were reported to be tolerant to detergent components [43]. There is, however, hardly any report of detergent compatible cold-active lipase from a fungal source.

Furthermore, despite the reports of extensive range of microbial lipases, the use of these enzymes on an industrial scale suffers from a common constraint i.e. the high production-cost invested mainly in media components. This necessitates the search for other sources that can yield these enzymes at reduced cost [25]. While bacteria and yeasts as source of enzymes are generally cultivated in submerged fermentation (SMF), fungal species are preferably cultivated in solid-state fermentation (SSF) [12]. Since, SSF is economical vis-à-vis SMF, a suitable fungal source for cold-active lipase is thus commercially desirable.

Moreover, the laundry detergent contains a variety of harsh chemical components; an ideal enzyme resistant to all of these is hardly available. For instance, a very popular commercial brand of lipase, Lipolase (Novozymes, Denmark) exhibited only 43% of activity after 1 h treatment with 1.0% hypochlorite [24]. It thus calls for further exploration of lipases especially cold-active one which is more compatible with a detergent formulation.

The present work was therefore undertaken to assess the lipases from psychrotrophic fungi for their possible inclusion in detergent formulation.

## 2. Materials and methods

### 2.1. Isolation and maintenance of fungus

The fungal isolates were isolated from soil samples collected from sites of Baramullah (Altitude-2730M; Latitude-34° 34' 0 N; Longitude-74° 45' 0 E) Jammu & Kashmir, India as described earlier [34]. Briefly, soil sample was serially diluted (up to five dilution) in double distilled water, 0.5 ml of thus diluted soil was spread onto MEA (malt extract agar, pH 5.5) medium and inoculated plate was incubated at 7 °C for seven days. Fungal cultures were picked up, purified and maintained on PDA slant at 4 °C.

### 2.2. Screening for lipase producing fungus

Minimal Czapek-Dox medium containing  $(\text{NH}_4)_2\text{SO}_4$  –5 g,  $\text{KH}_2\text{PO}_4$  –1 g,  $\text{MgSO}_4$  –500 mg,  $\text{CaCl}_2$  –100 mg,  $\text{NaCl}$  –100 mg, supplemented with tween-80–30 g as sole carbon source in 1000 ml of distilled water (pH 6.5) was used for screening the lipase producing fungal isolate. The inoculated plates were incubated at 4 °C and growth was monitored for 5 days [34]. Fungal isolates capable of utilizing tween-80 as sole carbon source were deemed to be lipase positive ones. Lipase positive isolates were further screened using olive oil (1% v/v) agar and palm oil (0.5% v/v) agar with phenol red at pH 9, halo zones seen were considered as positive for lipase production.

### 2.3. Taxonomic characterization of fungal isolates

Amplification of the ITS region (ITS1, ITS2, and 5.8 S rRNA Gene) was performed using the primers ITS1 and ITS4 and the conditions described in literature [6]. Primer ITS1: 5'-TCCGTAGGT GAACCTGCGG-3' Primer ITS4: 5'-TCCTCCGCTT ATTGATATGC-3'. The ~600 bp PCR product was purified by gel elution and used for sequencing. Both strands of the rDNA region amplified by PCR were sequenced by automated DNA sequencer – 3037xl DNA analyzer from Applied Biosystems using BigDye® Terminator v3.1 cycle sequencing Kit (Applied Biosystems). For sequencing same primers ITS1 and ITS4 were used. The sequences obtained for upper and lower strands were manually aligned before performing the analysis. Sequences were compared to the non-redundant NCBI database by using BLASTN, with the default settings to find the most similar sequence and were sorted by the E score. A representative sequence of 10 most similar neighbors was aligned using MAFFT tool in CLUSTAL W2 for multiple alignments with the default settings. The multiple-alignment file was then used to create a neighbor-joining phylogram applying MEGA6 software [39].

### 2.4. Enzyme production medium

For enzyme production, minimal medium (Czapek Dox broth) supplemented with Glucose –10 g, as carbon source and olive oil-1 ml as inducer was used. Spores of the selected fungus were harvested from 7-days old slant cultures by suspension in sterile distilled water containing 0.01% tween-80. The spore suspension containing about  $10^6$ – $10^7$  spores/ml was used to inoculate the medium and the culture was incubated at 20 °C on refrigerated orbital shaker at 120 rpm.

### 2.5. Enzyme preparation

The extracellular enzyme was extracted by centrifuging the broth at 10,000 rpm at 4 °C. The supernatant was saturated with increasing concentration of ammonium sulphate followed by centrifugation at 10,000 rpm to separate pellet from supernatant. After saturation, the presence of enzymes was tested by assaying them in supernatant and pellet till there was no trace of enzyme activity in the supernatant. The pellets showing the appropriate enzyme activity were pooled and resuspended in 10 mM phosphate buffer, pH 7.2 and allowed to stand overnight at 4 °C. The enzyme was then dialyzed against 40% sucrose solution to get concentrated enzyme free from salt and metal ions.

### 2.6. Enzyme purification

The concentrated enzyme solution is loaded onto a DEAE Cellulose column (1 cm dia × 3 cm length) pre-equilibrated with 10 mM sodium phosphate buffer, pH 7.2. The bound fractions were eluted with linear gradient of sodium chloride (0.1–1 M) in the same buffer. The fractions size of 1 ml were collected and assayed for the presence of protein at 280 nm. The protein-containing fractions were assayed for lipase activity; lipase-positive fractions were pooled and dialyzed against 40% sucrose solution as above.

### 2.7. SDS-PAGE

To test the purity of the lipases, SDS-PAGE of purified enzymes was carried out according to the published protocol [19]. The enzyme in the gel was stained with Coomassie Brilliant Blue R-250.

## 2.8. Lipase assay

Lipase activity assay was performed using *p*-nitrophenyl palmitate (*p*-NPP) as described earlier [22] with some modifications. 100  $\mu$ l purified enzyme was added to 3.9 ml of 0.1 mmol/L Tris–HCl buffer (pH 9.0) containing 20  $\mu$ mol of *p*-NPP as the substrate. The activity of the lipase samples was determined spectrophotometrically at 405 nm at 20 °C. One unit of lipase activity was defined as the amount of lipase releasing 1  $\mu$ mol *p*-nitrophenol per min.

## 2.9. Lipase characterization

The effect of pH on enzyme activity was studied by incubating the enzyme with *p*-NPP substrate, prepared in different buffers in the pH range 4–10. The buffers used were, citrate-phosphate (pH 3–7), sodium phosphate (pH 7–8) and glycine–NaOH (pH 9–11). At pH 3, psyllium-gelled medium [33] was used instead of agar-gelled one since the latter could not solidify.

The temperature optima for the enzyme were determined in the range of 4–70 °C, at pH 9 (BPF6 lipase) and pH 11 (BPF4 lipase), as above. The enzymes stability at different temperatures was studied by incubating the enzyme at their optimum pHs (i.e. BPF6 lipase at pH 9 and BPF4 lipase at pH 11) and at different temperatures for 4 h, followed by the activity estimation at 20 °C.

The effect of metal ions or EDTA was determined by incubating enzymes in 10 mM glycine–NaOH buffer pH 9 (BPF6 lipase) and pH 11 (BPF4 lipase) in presence of 10 mM solution of metal salts or EDTA for 15 min followed by estimation of residual activity of the enzymes at 20 °C.

## 2.10. Effect of detergent components

The effect of important detergent components such as surfactants (SDS, tween-80 and triton X-100), oxidizing (H<sub>2</sub>O<sub>2</sub>) and bleaching (NaClO<sub>3</sub>) agents was studied by incubating enzyme (50  $\mu$ l) with 950  $\mu$ l of 1% of these reagents in 10 mM glycine–NaOH buffer (pH 9 for BPF6 lipase) (pH 11.0 for BPF4 lipase) for 1 h and 2 h at 20 °C [27] before assaying the enzyme as compared to the control without any additive.

## 2.11. Effect of laundry detergents

The detergent brands used were Tide® and Ariel® (Procter and Gamble Home Products Ltd) and wheel and Surf Excel® (Hindustan Unilever Limited–Mumbai, India). They were diluted in double distilled water to a final concentration of 7 mg/ml to simulate washing conditions and heated at 100 °C for 15 min to inactivate the enzymes that could be part of their formulation [29]. The enzyme was added to the reaction mix at a concentration of 1 mg/ml and incubated at 50 °C for 12 h. Aliquots (0.5 mL) were taken at different time intervals and the residual activity was determined at 20 °C and 4 °C and, compared with the control sample incubated at 50 °C without any detergent [4,29].

## 2.12. Quality control

All the experiments were conducted in triplicates and the results were expressed as mean  $\pm$  SE.

## 3. Results

### 3.1. Isolation of lipase producing psychrotrophic fungi

Ten fungal isolates showing faster growth at 4 °C and ability to utilize various substrates including tween-80 as sole carbon source

at this temperature were selected earlier [34]. Further screening of all the lipase positive isolates using olive oil (1% v/v) agar and palm oil (0.5% v/v) agar, two large halo zone forming isolates BPF4 (Psychrotolerant) and BPF6 (psychrophilic) were selected as producer of lipase at cold temperature.

### 3.2. Identification of fungal isolates

The fungal isolate BPF4 was identified as *Penicillium canescens* on the basis of macro and microscopic characteristics. The colony was compact with raised center, margin entire and colour was white although the old colony showed greenish sectors. The growth rate of colony was moderate (Fig. S 'A'). The mycelium was thin-walled, penicilli mono- or biverticillate, phialides ampulliform, conidia subspheroidal, thin walled and borne in loose chains. For molecular identification, the ITS region of BPF4 was sequenced and the sequence was compared with those available in the NCBI database. The phylogenetic tree based on the sequence of BPF4 along with those from ten the most closely related strains obtained from NCBI database showed 99–100% similarity to type and many of the strains of *Penicillium canescens* (Fig. 1a), therefore, the isolate was so named. The nucleotide sequence has been deposited in the GenBank database under accession number KF247215.

Likewise, the colony of BPF6 was pinkish white (Fig. S 'B1'); the reverse of the colony was not yellow nor did it produce yellow secretion. The conidia were smooth and spherical. The fungus produced ascospores which were covered with net like wall (Fig. S 'B2') so that asci could be seen from outside. The peridial hyphae were rough-walled, red to red-brown, bearing short, subhyaline, thin and rough-walled, unbranched appendages. Ascospores smooth and lacking a longitudinal rim. Therefore, the fungal strain BPF6 was identified as *Pseudogymnoascus roseus*. Dendrogram obtained as above involving ITS regions of this fungal isolate and those of its closely related strains in the NCBI database showed 98–100% similarity with *Pseudogymnoascus roseus* and some more species

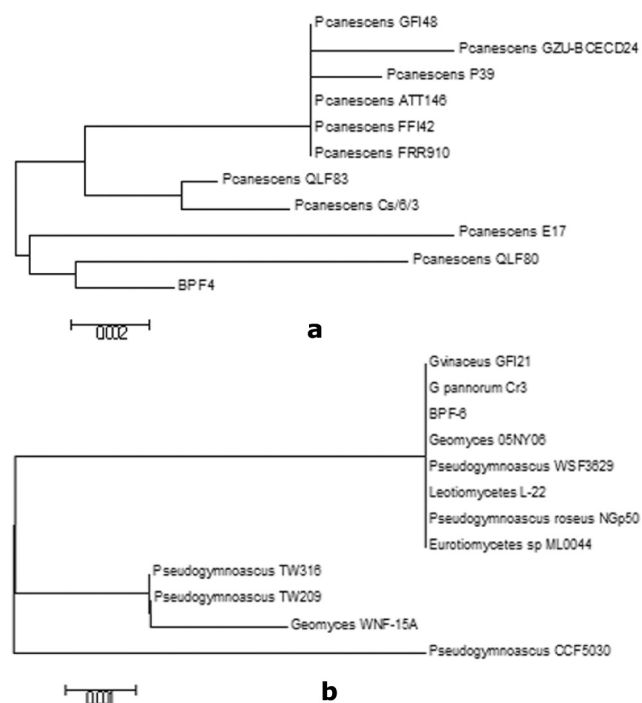


Fig. 1. Phylogenetic relationship with 10 closely related isolates retrieved from Genbank on the basis of ITS sequences of fungal isolates (a) BPF4 and (b) BPF6.

(Fig. 1b). Combining both morphological and molecular data, this isolate was so named. The nucleotide sequence has been deposited in the GenBank database under accession number KX462167.

### 3.3. Purification of lipase

In case of both BPF4 and BPF6, the maximum activity was found in the pellet obtained by 80% ammonium sulphate saturation, a second activity pellet was also found with 60% ammonium sulphate saturation. The pellets were pooled and dissolved in buffer and further purified by chromatographic purification. The pooled fractions were dialyzed and tested for its purity on SDS-PAGE gel. Both the enzymes showed single band (Fig. S 'C').

### 3.4. Characterization of lipase

Both BPF4 and BPF6 lipases showed maximum activity at 40 °C, but while BPF6 lipase retained 70% activity at 20 °C and 30% at 4 °C BPF4 lipase showed 35% and 20% residual activity at corresponding temperatures. Although both the enzymes showed drastic loss of activity at 50 °C as was expected of cold-active enzymes, BPF6 lipases showed more residual activities at lower temperatures (Fig. 2).

The BPF6 lipases showed maximal activity at pH9, which was reduced to 90% at pH 11. BPF4 lipase on the other hand showed maximum activity at pH11 that was gradually reduced to 70% at pH6 (Fig. 3).

The BPF4 enzyme showed reasonable stability at 4 °C, 20 °C and 40 °C for 2 h, the maximum loss of activity was only 20% at 40 °C. The BPF6 enzyme did show stability at all temperatures tested showing more than 90% of residual activity after 1 h (Fig. 4).

When stability was tested at 50 °C for different periods, BPF4 enzyme retained 100% activity for 1 h, afterwards there was erosion in activity and finally in 4 h 40% of activity was eroded. BPF6 enzyme continued to show a residual activity of nearly 90% till 4 h at 50 °C, thus indicating that the enzyme is stable (Fig. 5).

### 3.5. Effect of metal ions and other detergent supplements

The BPF4 lipases showed 300% activity in presence of  $\text{Ca}^{2+}$  followed by 280% in presence of  $\text{Fe}^{3+}$ . EDTA and  $\text{Sn}^{2+}$  had no effect on the activity. In case of BPF6 enzyme, the presence of metals enhanced the activity, maximum enhancement was shown by  $\text{Ca}^{2+}$  (280%). The presence of EDTA which chelates metals surprisingly did enhance the activity (183%). While in case of BPF4 lipase, SDS (by 125%) and Triton X-100 (by 330%) enhanced the activity, Tween-80 and  $\text{H}_2\text{O}_2$  inhibited the activity nearly by 20%; in case of BPF6 lipase, all these detergent components caused inhibition of activity (Table 1). BPF4 lipase was only slightly inhibited (by mere 2% in 1 h) by  $\text{NaClO}_3$ , the inhibition of BPF6 enzyme was however more (28%). By contrast, inhibition by  $\text{H}_2\text{O}_2$  was more (24%) for BPF4 enzyme, while it was less (14%) for BPF6 enzyme (Table 1).

### 3.6. Effect of laundry detergents

Both the lipases were found to exhibit nearly 90% residual activity after 1 h of incubation in various detergent brands (Fig. 6), BPF4 lipase though performed slightly better.

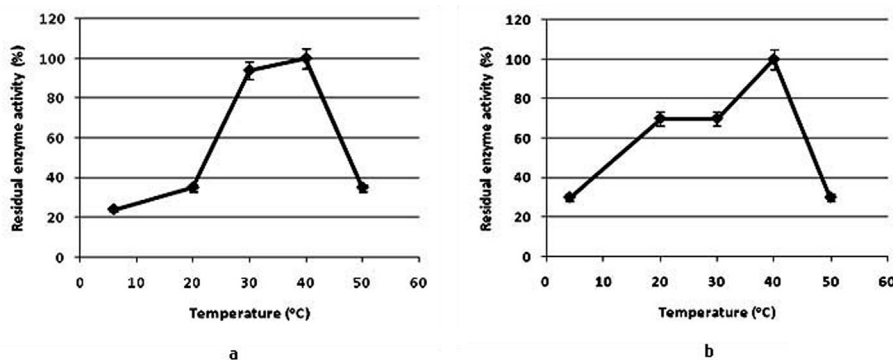


Fig. 2. Effect of temperature on the activity of (a) BPF4 lipase and (b) BPF6 lipase.

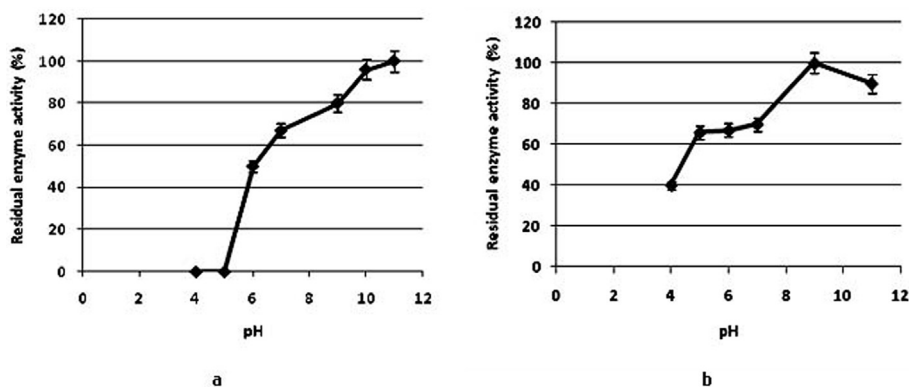
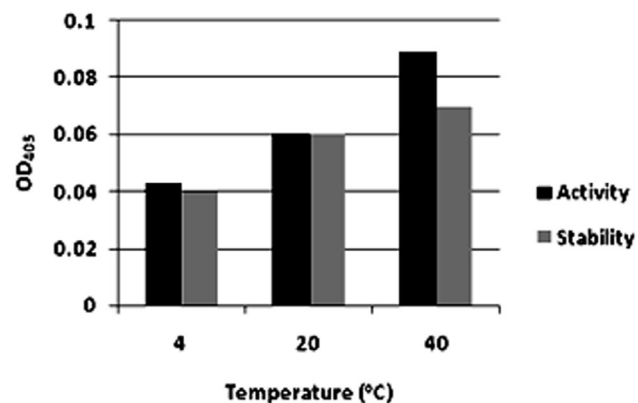
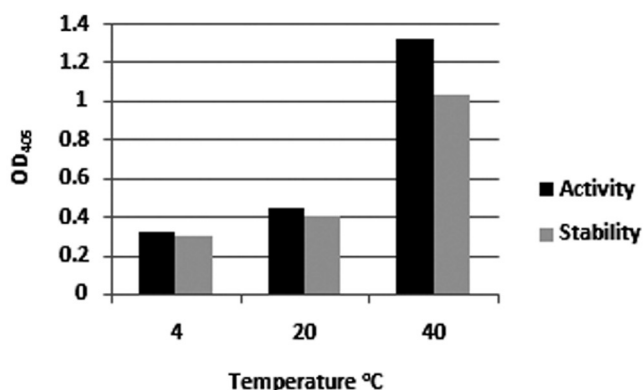


Fig. 3. Effect of pH on the activity of (a) BPF4 lipase and (b) BPF6 lipase.



a

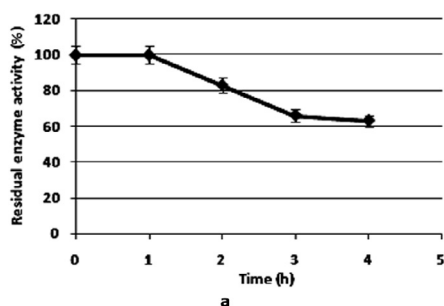


b

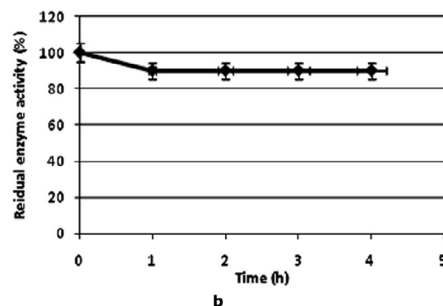
Fig. 4. Effect of temperature (4°C, 20 °C and 40 °C) on the activity and stability of (a) BPF4 lipase and (b) BPF6 lipase.

#### 4. Discussion

Cold-active enzymes are believed to have potential to mitigate the climate change not only by bringing down the processing temperature but also by producing these enzymes themselves at lower temperature applying psychrotrophs [35]. Cold-active microbial lipase has been bioprospected mainly from polar regions [30], deep sea water [43], Himalayan glacier [14] and refrigerated foods [15]. For the first time this was explored from the Himalayan soil. Two stage screening technique as applied during this work had also been applied earlier [29–30]. Among microbial sources for cold-active lipases, bacteria have been the organism of choice although occasionally fungi and yeasts have also been investigated for this purpose [11,15].



a



b

Fig. 5. Effect of higher temperature (50 °C) on the stability of (a) BPF4 lipase and (b) BPF6 lipase.

Table 1

Percentage residual activities of enzymes in presence of metals and selected detergent supplements.

Metal/detergent supplements	BPF4	BPF6
Control	100	100
Ca <sup>+2</sup>	300 (±23.67)	280 (±22.74)
Fe <sup>+3</sup>	280 (±26.43)	210 (±28.86)
Cu <sup>+2</sup>	110 (±16.23)	120 (±12.49)
Sn <sup>+2</sup>	100 (±7.21)	230 (±19.34)
EDTA	100 (±12.21)	183 (±16.53)
SDS	125 (±9.12)	55 (±14.20)
Triton X-100	330 (±21.45)	50 (±4.32)
Tween-20	80 (±9.21)	67 (±6.43)
HClO <sub>3</sub>	98 (±8.34)	72 (±6.44)
H <sub>2</sub> O <sub>2</sub>	62 (±12.22)	56 (±7.98)

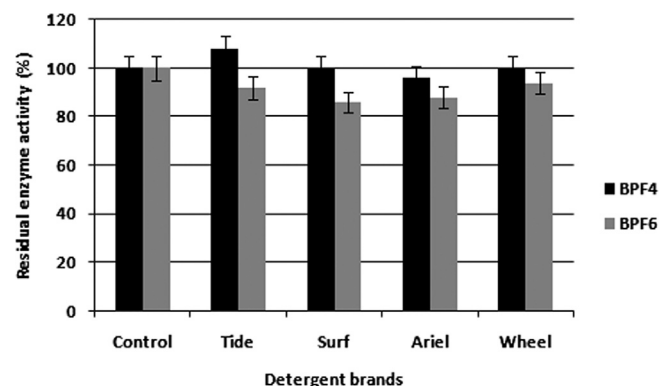


Fig. 6. Effect of selected laundry detergent brands on the activity of (a) BPF4 lipase and (b) BPF6 lipase.

On the basis of morphological and molecular analysis, BPF4 and BPF6 were identified as *Penicillium canescens* and *Pseudogymnoascus roseus* respectively. Both isolates BPF4 and BPF6 yielded alkaline lipases satisfying the basic requirements for their inclusion in detergent formulation.

The optimum temperature for both BPF4 and BPF6 enzymes activity was found to be 40 °C; the residual activity at 20 °C and 4 °C however differed greatly. When compared to a mesophilic lipase from *Fusarium oxysporium* that showed 20% activity at 10–20 °C [27], BPF4 and BPF6 lipases certainly showed higher activity at lower temperatures and thus these are cold-active lipases. BPF6 which is psychrophilic isolate, as expected, yielded more robust cold-active lipases exhibiting higher residual activity at lower temperature. However, as opposed to general belief of labile nature of cold-active enzyme, BPF6 lipase was found to be highly tolerant to higher temperature (50 °C) and even more tolerant than BPF4 lipase at this temperature. The finding thus suggests that a generalization of principal as to an evolutionary process is not possible

and necessitates the exploratory work to be undertaken continuously to find a bio-molecule with novel combinations of features for specific applications.

Although, both the lipases showed higher activity at lower temperature as per the definition of cold-active enzyme, their optimum temperature (40 °C) was found to be same as those of many of mesophilic fungal lipases [27]. For example optimum temperature of lipase from *Candida rugosa* [3] and *Mucor meichei* [21] were reported to be 40 °C. Possibly, this is due to preference of this enzyme (lipase) to act on the substrates when the latter (especially saturated fats) is in molten state. Among cold-active alkaline lipases, those reported from intertidal flat metagenome [18] and marine bacterium *Janibacter* sp. called MAJ1 [43] showed optimum activity at 30 °C, but their overall activity was found to be very low. The cold active enzyme MAJ1 was reported to display about 50% of the optimal activity at 5 °C but it acted only on mono- and diacylglycerols, and did not show activity to triacylglycerols [43]. Another cold-active alkaline lipase from deep-sea area of Edison Seamount showed an optimal temperature at 25 °C and displayed more than 50% activity at 5 °C, but it was highly sensitive to EDTA and 0.1% (v/v) and 1% (v/v) nonionic and ionic detergents respectively [13].

When studied stability at 4 °C, 20 °C and 40 °C, BPF4 lipase retained more than 80% residual activity after 2 h while BPF6 enzyme retained more than 90% of residual activity after 1 h. Even at 50 °C, BPF4 enzyme retained 100% and 60% activity after 1 h and 4 h suggesting both the enzymes as reasonably thermostable. Although more stable alkaline lipases suitable for detergents have been reported earlier [24], they are mesophilic. Cold-active enzymes have inherent property of instability at higher temperature, so the stability in respect of these enzymes can be studied with reference to another cold-enzyme only. For examples, BPF4 and BPF6 lipases are reasonable stable if they are compared with earlier studied cold-active lipases such as metagenomic lipase that unfolds rapidly above 25 °C [18], MAJ1 (optimum temperature 30 °C) that loses activity rapidly at 40 °C [43] and lipases from *Candida antarctica* (optimum temperature 40 °C) that lose activity abruptly at 50 °C.

A detergent compatible lipase is required to be active in alkaline pH range. The optimum pH of both the enzymes was found in the alkaline range, BPF4 lipase has it at pH11 while BPF6 at pH9 and thus both these enzymes qualify for their inclusion in detergent formulations. Alkaline lipase has earlier been reported mostly from bacteria [24] and few from fungi eg. *Mucor michei* (optimum pH 8) [21]. As for the cold-active lipase, the optimum activity of the purified MAJ1 has been reported to be at pH 7.0 at 30 °C [43] and that of intertidal metagenome lipase at pH 8.0 [18]. The BPF4 and BPF6 lipases are thus superior in their pH related compatibility with detergent formulations over all other ones reported so far. The fabric washing is generally conducted at alkaline pH because most of the products resulted from oil stain digestion by lipases are more soluble under alkaline conditions [16].

Metal cations, particularly Ca<sup>2+</sup>, play important roles in the structure and function of enzymes, and some of the lipases are strictly calcium dependent [7]. The activity of both BPF4 and BPF6 lipases was found to be enhanced by Ca<sup>2+</sup> markedly and thus they are metalloenzymes. Considering the metalloenzyme activity in presence of Ca<sup>2+</sup> as control, BPF4 lipase was inhibited as much as by 66% but BPF6 lipase was inhibited only by 50% by EDTA. Earlier, cold-adapted lipases from bacterium LipEH166 [18] has been reported to be metalloenzyme whose activities were enhanced by CaCl<sub>2</sub>. The activity of lipases from *Cryptococcus* sp. [40] has however been found not to be affected by EDTA. Most remarkably, the activity of cold-active MAJ1 has been found to be unaffected by Ca<sup>2+</sup> but completely inhibited by EDTA [43]. The stability of a lipase

in EDTA is a pre-requisite for any detergent enzyme since the chelating agent aids in removing ions responsible for water hardness, leading to a proper stain release. Though, both BPF4 and BPF6 are metalloenzymes, the latter exhibits better tolerance to EDTA and so is more suitable as detergent enzyme.

The activity of both BPF4 and BPF6 enzymes was also found to be enhanced by Fe<sup>3+</sup> and Cu<sup>2+</sup>, however, only BPF6 enzyme activity was stimulated by Sn<sup>2+</sup>. Fe<sup>3+</sup> and Cu<sup>2+</sup> have however been regarded as general inhibitors of lipases. These lipases are therefore attractive for detergent purpose.

A detergent lipase must be stable and compatible with anionic surfactants (SDS), nonionic surfactants (Tween 80, Triton X-100), bleaches (NaClO<sub>3</sub>) and oxidizing agents (H<sub>2</sub>O<sub>2</sub>). In case of BPF4 lipase, the activity was enhanced by surfactants SDS and Triton X-100.

In contrast, in case of BPF6 lipase, SDS and Triton X-100 were found to inhibit the activity. Detergent compatible lipases tolerant to SDS such as those of *S. arlettae* [40] and sensitive to SDS such as those of some species of *Pseudomonas* [17] and *Fusarium* [27] have earlier been reported.

There was low impact of nonionic surfactants viz., 1% of Triton X-100 and Tween-80 on BPF4 lipases, rather the enzyme activity was stimulated by 330% in presence of TritonX-100. In contrast, BPF6 lipases showed negative impact of these surfactants, though the inhibition was less than 30%. Tween 20/40/60/80 and Triton X-100/114 have been reported to activate or have no effect on the lipases of *Cryptococcus* sp. [40]. The emulsifying agents enhance the lipolytic activity by decreasing the surface tension of the medium facilitating contact between the substrate and the enzyme active site [16]. On the other hand, many other alkaline lipases were reported to be slightly or partially inhibited by Tween 20/80 [27] or both Tween 80 and Triton X-100 [17].

So far as the effect of H<sub>2</sub>O<sub>2</sub> is concerned, it was found to be inhibitory to both the enzymes although the degree of inhibition was different, 24% for BPF4 enzyme and 14% for BPF6 enzyme. By contrast, BPF4 lipase was only slightly inhibited (by mere 2% in 1 h) by NaClO<sub>3</sub>, the inhibition of BPF6 enzyme was however more (28%). Overall, the enzymes have been found to behave differently in presence of various detergent supplements and in general the inhibition whenever seen was only moderate (less than 30%) and thus their inclusion in the detergent formulations does not seem to be thwartable on this ground.

To obtain further support for their suitability as detergent supplements, the stability of both BPF4 and BPF6 lipases was tested in presence of selected laundry detergents. Both the lipases were found to exhibit reasonable stability viz., more than 90% residual activity after 1 h of incubation in presence of popular laundry detergent brands. This result was better than or almost equal to others reported earlier in cases of *B. smithii* [20,36].

In conclusion, both BPF4 and BPF6 lipases were found to be alkaline metalloenzyme, showing maximum activity at pH 11 and 9 respectively and at the temperature 40 °C. The residual activities at 20 °C and 4 °C of BPF4 lipase were 35% and 20% and of BPF6 lipase were 70% and 20 °C respectively thus they are cold-active lipases. Both the enzymes were stable at 4 °C, 20 °C and 40 °C. While Ca<sup>2+</sup> enhanced activity of both the enzymes, other metals and reagents affected the activity variably. Importantly, both the lipases were found to exhibit nearly 90% residual activity after 1 h of incubation in selected detergent brands proving them to be superior to any of the reported detergent compatible alkaline lipases.

#### Disclosure statement

We declare that there is no conflict of interest.

## Acknowledgements

The work was supported by the Madhya Pradesh Council of Science and Technology (grant number 3902/CST/R&D/2011) to the corresponding author.

## Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at <https://doi.org/10.1016/j.jgeb.2018.04.006>.

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