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ORIGINAL ARTICLE

Purification and characterization of lipase by *Bacillus methylotrophicus* PS3 under submerged fermentation and its application in detergent industry

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KEYWORDS

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Abstract Lipase production bacterial isolate was isolated from soil of service station and identified as *Bacillus methylotrophicus* PS3 by 16SrRNA with accession number |LN999829.1|. Lipase enzyme was purified by sequential methods of ammonium sulfate precipitation and Sephadex G-100 gel column chromatography. The molecular weight of purified enzyme was 31.40 kDa on SDS-PAGE. This purification procedure resulted in 2.90-fold purification of lipase with a 24.10% final yield. The purified lipase presented maximal hydrolytic activity at a temperature of 55 °C, and pH of 7.0. Lipase activity was stimulated by Triton X-100 and SDS with Mg^{2+} and Ca^{2+} metals employ a positive effect and outlast its stable in organic solvent i.e. methanol and ethanol.

1. Introduction

The biosphere is occupied by a wide variety of microorganisms that carry out important functions such as global primary energy, element cycling and they form the largest part of living organisms in the sense of total biomass and species diversity. This diversity of microorganisms is the most common source of genes which can be used in several industrial and research applications [1]. Lipase (triacylglycerol acylhydrolase, EC 3.1.1.3) catalyzes the hydrolysis of the carboxyl ester bonds in triacylglycerols to produce diacylglycerols, monoacylglycerols, fatty acids and glycerol under aqueous conditions and the synthesis of esters in organic solvents [2]. Under the controlled conditions, lipases are able to catalyze a large number of reactions [3]. Lipases of microbial origin are of considerable commercial importance, because of the high versatility and high stability, moreover, the advantage of being readily produced in high yields [4]. Many microbial lipases have been commercially available in free or immobilized form. Numerous species of bacteria (*Bacillus, Pseudomonas, and Burkholderia*), yeasts (*Candida rugosa, Yarrowia lipolytica, and Candida antarctica*) and molds (*Aspergillus, Trichoderma viride*) produce lipases with different enzymological properties and specificities but microbes are known to be more potent lipase

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producer [5]. Lipases have broad variety of industrial applications such as food industry (improvement of flavor) [6], detergent (hydrolysis of oil and fats) [7], pharmaceutical (synthesis of chiral drugs), paper (control of pitch), medicine (triglyceride measurement), cosmetics (exclusion of lipids), wastewater (decomposition and removal oil), leather (elimination of fat from animal skin)[8]. However, the major contributions of microbial lipases are in the detergent formulations. The main reason for the steadily growing interest in lipases is because of their enantio-selective, regio-selective and chemo-selective nature [9]. Therefore in the present study an attempt has been made for the isolation of potential lipase producing bacteria, its purification and characterization and its application as a detergent.

2. Material and methods

2.1. Organism and inoculum preparation

Soil of service station for automobiles was collected from the nearby areas of Solan, Himachal Pradesh, India and stored at 4 °C. The soil samples were enriched by adding 1 % of tributyrin into the sample, kept at 37 °C and incubated for 3 days. The samples were serially diluted with enriched sample on nutrient agar medium containing 1 % tributyrin, kept at 37 °C for 24 h of incubation. The pure cultures obtained were maintained at 4 °C on PDA medium. Different bacterial strains were screened for lipase production using Tributyrin hydrolysis test to screen hyper-lipolytic bacteria [10].The zone forming bacterial strains were further screened for quantitative analysis of lipase using titrimetric method [11]. The genomic DNA was isolated using DNA Kit DNA prep kit (Bangalore Genei, India Pvt. Ltd. make). The 16S rRNA gene was selectively amplified from genomic DNA using PCR with oligonucleotide universal primers. The strain was identified on the basis of 16S rRNA gene sequences using appropriate software (nucleotide BLAST) in "National Centre for Biotechnology Information" resource. The lipase producing bacteria was grown in nutrient agar medium containing 1% tributyrin medium as carbon source and the pH was maintained at 7.0. The culture was incubated at 40 °C for 72 h with 10% inoculum size with divalent ion Ca^{2+}, tween 80 as surfactant with 1% substrate concentration. The culture medium was removed after 12, 24, 36, 48, 60 and 72 h for determining the growth pattern and lipase activity. The growth patterns of bacterial strains were taken at O.D. at 540 nm with a spectrophotometer (Cary, 100 Bio, Varian Co, Australia).

2.2. Assay for lipolytic activity [11]

Lipase activity was determined titrimetrically on the basis of olive oil hydrolysis. One ml of the culture supernatant was added to the reaction mixture containing 1 ml of 0.1 M Tris–HCl buffer (pH 8.0), 2.5 ml of deionized water and 3 ml of olive oil and incubated at 37 °C for 30 min. Both test (in which all the reaction mixture were added with enzyme) and blank (in which all the reaction mixture were added without enzyme) were performed. After 30 min the test solution was transferred to a 50 ml Erlenmeyer flask. 3 ml of 95% ethanol was added to stop the reaction. Liberated fatty acids were titrated against 0.1 M NaOH using phenolphthalein as an indicator. End point was an appearance of pink color. A unit lipase is defined as the amount of enzyme, which releases one micromole fatty acid per min under specified assay conditions.

2.3. Lipase purification

Bacterial culture grown in nutrient medium and 1% tributyrin was centrifuged at 10,000 rpm for 20 min at 4 °C in a refrigerated centrifuge. Cell free supernatant was saturated with (0–70%) ammonium sulfate with continuous stirring at 4 °C followed by centrifugation at 14,000 rpm for 20 min. Ammonium sulfate fraction was dialyzed against phosphate buffer (pH 7.0) for 6 h at 4 °C in a dialysis bag. The concentrated enzyme after dialysis was loaded onto Sephadex G-100 column. The lipase was eluted from the column at a flow rate of 3 ml/min. Enzyme fractions (5 ml each) were collected and the protein content was measured spectrophotometrically at 280 nm. Lipase assay was performed using fractions containing highest protein content.

2.4. Characterization of lipase enzyme

2.4.1. Effect of pH on the activity and stability

The effect of pH on enzyme activity was determined by incubating the reaction mixture at various pHs ranging from 4.0 to 11.0 at 50 ± 2 °C for 30 min. The buffers used were citrate phosphate buffer (pH 4.0 to 7.0), Tris HCl buffer (pH 8.0) and glycine-NaOH buffer (pH 9.0 to 11.0).

2.4.2. Temperature optimum and thermal stability

To evaluate the optimal temperature for the enzyme activity, the assay was conducted at varying temperatures ranging from 35 to 121 °C. The lipase was pre-incubated at different temperatures ranging from 30, 40 ……121 °C for 0–180 min.

2.4.3. Effect of metal ions

0.5 ml of purified lipase in 2.5 ml 20 mM Tris HCl buffer (pH 8.0) was incubated for 30 min with various metal ions (1 mM) Ca^{2+}, Mg^{2+}, Cu^{2+}, Fe^{2+}, Co^{2+}, and Zn^{2+}.

2.4.4. Shelf stability of lipase

Shelf stability of lipase was determined by pre-incubating the enzyme at 4 °C in 20 mM Tris HCl buffer (pH 8.0). Enzyme activity was determined every 3 days till 9 days.

2.4.5. Effect of media additives

To determine the influence of different additives viz. SDS, EDTA, CTAB, Tween 20, Tween 80, Triton X 100 and Glycerol etc., purified lipase in 1 M phosphate buffer (pH 7.0) was pre-incubated for 30 min at 50 ± 2 °C.

2.5. Application of purified lipase as a laundry additive lipase from B. Methylotrophicus PS3

Application of lipase as a detergent additive had been evaluated in terms of its washing performance on white cotton cloth pieces (5 × 5 cm) that were stained separately with different oils i.e. olive oil, black grease, butter, vegetable oil, and white grease. The stained cloth pieces were taken in separate petriplates and the following washing performances sets were

prepared: Petriplates with distilled water (100 ml) + stained cloth (each cloth stained separately with olive oil black grease, butter, vegetable oil, and white grease), petriplates with distilled water (100 ml) + 1 ml detergent (7 mg/ml) + stained cloth; petriplates with distilled water (100 ml) + 1 ml detergent (7 mg/ml) + 2 ml enzyme solution + stained cloth to analyze the efficiency of lipase in removing stains from the clothes and to observe the efficiency of lipase in removing strains.

3. Results and discussion

3.1. Isolation of lipolytic bacterial isolate

The least explored and highly probable sources of lipolytic enzyme producing microorganisms is the soil contaminated with oil at service stations, which has been selected in the present study for isolation of lipolytic microorganisms. Soil samples collected from different sites of Himachal Pradesh were processed for isolation of lipase producing microorganisms by directly plating the serially diluted samples onto the tributyrin agar plates. All the bacterial isolates were preliminarily characterized based on their morphological and cultural characteristics with round, smooth, viscous, convex, opaque and yellow white of morphological characteristics on nutrient agar medium (Plate 1).

3.2. Qualitative screening of lipolytic microorganisms

Among the variable isolates PS3 showed the maximum zone formation of 13.61 mm on tributyrin medium at 37 °C of incubation after 24 h (Plate 2).

3.3. Quantitative screening of lipolytic microorganisms

Qualitatively screened lipolytic bacteria PS3 based on formation of zone of hydrolysis on tributyrin agar plates, were subjected to quantitative screening by assaying their enzyme activity under submerged fermentation. Maximum activity of 127.31 IU possessed by PS3 was finally selected for further studies. Literature survey revealed similar reports for lipolytic microorganisms. Aliyu et al. [12] collected the palm oil mill effluent from west oil mill of sime darby Sdn. Bhd. Carey Island, Malaysia.

Sequences of these isolate obtained were submitted to NCBI database and matched with already existing sequences.

Plate 1 Morphological characteristics of PS3.

Plate 2 Zone of hydrolysis formed by the lipolytic strains PS3.

Partial 16S rRNA gene sequence was quire to NCBI BLAST (http:// www.ncbi.nlm.nih.gov/Blast) and the nearest neighbor of the isolate was determined. Sequence similarity search (mBLAST, NCBI) for PS3 showed 99% homology with nucleotide sequence of *Bacillus methylotrophicus* | LN999829.1| 16S ribosomal RNA gene, partial sequence. Unrooted phylogenetic tree based on comparison of 16S rRNA sequence data of *Bacillus methylotrophicus* PS3| KX758632 | with its closest phylogenetic relatives in the NCBI, GenBank, USA, was constructed by Neighbor joining (NJ) tree algorithm using bootstrap value of the MEGA 7.0 (see Plate 3) (http://www.megasoftware.net/) [13]. Numbers on the tree indicate percentage of bootstrap sampling derived from 1000 random samples (Fig. 1).

3.4. Production of lipase under submerged fermentation

The growth and enzyme production of the organisms are strongly influenced by different process parameters viz. temperature, pH, incubation period, inoculum size, divalent ions, surfactants, media types etc. An enhanced lipase titers of 320.0 IU have been obtained at 40 °C temperature in nutrient

Lane 1: Marker

Lane 2: DNA

Plate 3 Molecular identification of screened isolates by 16S rRNA gene technique.

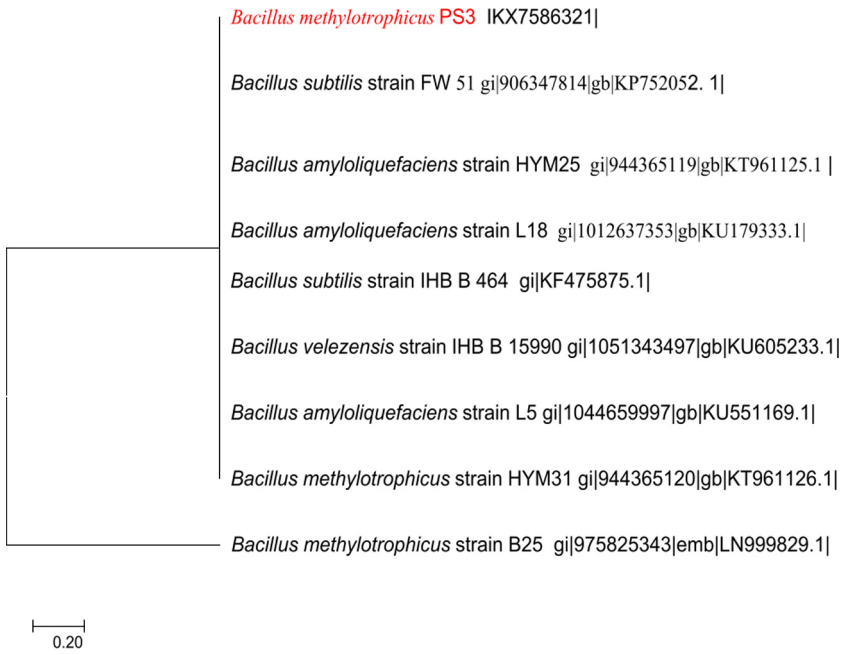

Fig. 1 Neighbor-joining tree with 1000 bootstrap values in MEGA 7.0 showing phylogenetic relationship of *Bacillus methylotrophicus* PS3 based on a distance matrix analysis of 16S rRNA sequences.

broth medium with 1% tributyrin and pH 7.0, 10% of inoculum size and 60 h of incubation.

3.5. Lipase purification

Lipase purification was done to get a protein of interest and to remove unnecessary one. Purification process of lipase occurs in sequential manner. The enzyme produced over 48 h of culture was purified by ammonium sulfate precipitation for salting out the proteins for increased enzymatic activity; desalting was performed for removing the traces of salt. An extracellular lipase from *B. methylotrophicus* PS3 was purified by ammonium sulfate precipitation and Sephadex G-100 column chromatography with a total yield of 24.10% and 2.90-fold purification (Table 1 and Fig 2).

Enzyme activity was determined which was concentrated and subjected to SDS-PAGE with molecular mass of the purified lipase from *B. methylotrophicus* PS3 was 31.40 kDa on SDS–polyacrylamide gel electrophoresis (Plate 4).

Bae et al. [14] purified lipase from *Pichia lynferdii* Y-7723 with 33 purification fold using chromatographic techniques and the purified lipase represented maximum lipolytic activity. Tripathi et al. [15] purified lipase from *Microbacterium sp.* by sequential methods of ammonium sulfate precipitation and Sephadex G-75 gel column chromatography. This purification procedure resulted in 2.1-fold purification of lipase with a 20.8% final yield. The purified lipase exhibited maximal hydrolytic activity at a temperature of 50 °C and a pH of 7.0.

3.6. Characterization of purified lipase

3.6.1. Effect of temperature on lipase

Lipase activity was examined at a temperature range of 35–100 °C, with maximum activity at 55 °C as observed in Fig 3 with 98.44% of relative activity and 752.97 IU/mg of specific activity. The lipase was also active from 55 to 80 °C retained small amount of activity with relative activity in the range of 52.58–10.01%, the enzyme activity decreases almost half at 100 °C about 7.62% of relative activity. At elevated temperature the enzymatic protein denaturation occurs and decreases the lipase activity. Thus an elevated temperature decreases the lipase activity. The relative activity remains almost stable from 35 °C to 55 °C i.e. 93.82% to 98.44%. The thermostability of the lipase at high temperature indicates its industrial applications, because of unique nature of protein and its thermostable nature. Similarly Gokbulut and Arslanoglu [16] purified a lipase from capable of tolerating a temperature range of 45 °C. A lipase from *P. aeruginosa* was found stable at 55 °C with 46% of the relative activity [17].

3.6.2. Effect of pH

Enzyme being protein is sensitive to changes in the environment in which they work, affecting the activity of enzyme. pH optimum for lipase was explored at constant temperature of 50 °C. Purified lipase from *B. methylotrophicus* PS3 was most stable at pH 7.0, and activity remains stable from pH 7 to 9, the activity start losing at above the 9 and at lower acidic range of pH, indicating the neutral behavior of the purified lipase. Change in pH has a varied effect on the enzyme activity, as altering the structure of enzyme and substrate and inhibiting the catalysis of reaction (Fig. 4).

Similarly, Akshatha et al. [18] performed the characterization of purified lipase from LBCK having pH stability in the range of 7–9. Lipase from *B. licheniformis* MTCC 6824 was optimally active at pH ranging from 6.0 to 10 [19]. The *Bacillus subtilis* PCSIR NL-39 lipase are active in pH range of 3.5–9.0. The previous reports have shown that lipases are mostly produced by bacteria especially *Bacillus* species whose pH is 7.0 [20].

Table 1 Purification profile of lipase from *B. methylotrophicus* PS3.

Purification step	Total Volume (ml)	Enzyme activity (IU/ml)	*Total activity	**Total protein	***Specific activity	****Purification fold	*****% Recovery/ Yield
Crude enzyme	300	360	108000	450	240	1	100
Ammonium sulfate fractionation (30–90%)	100	1050	105000	200	525	2.20	97.20
Dialysis	25	1100	27500	45	611	2.54	25.4
Gel exclusion chromatography (Sephadex G-75)	20	1300	26000	37.50	693	2.90	24.10

* Total activity: Enzyme activity in given volume (IU).
** Total protein: mg/ml.
*** Specific activity: Enzyme activity per unit protein concentration (IU/mg).
**** Purification fold: increase in specific activity.
***** Percent recovery is remaining protein concentration as % of the initial protein concentration.

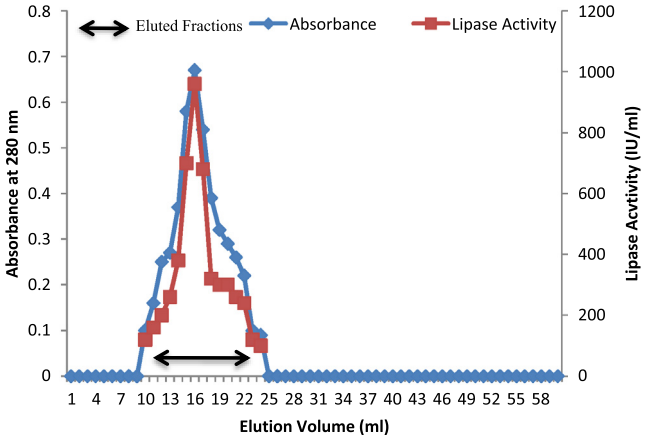

Fig. 2 Protein and enzyme activity profile of fractions of Sephadex G-75 column chromatography of the dialyzed lipase of *B. methylotrophicus* PS3.

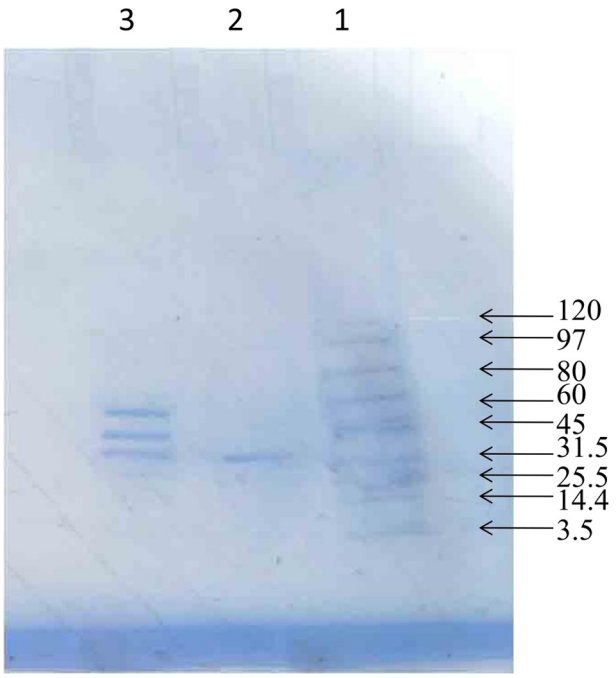

Plate 4 SDS-PAGE of *B. methylotrophicus* PS3 lipase at various stages of purification. Lane 1: Marker; Lane 2: Purified Lipase; Lane 3: Crude enzyme.

3.6.3. Effect of metal ions on activity of purified lipase of B. methylotrophicus PS3

The activity of various enzymes is influenced by the presence of metal ions either by directly involving in their catalysis or by structural modifications. Thus, the effect of variable metal ions i.e. Ca^{2+}, Mg^{2+}, Cu^{2+}, Fe^{2+}, Co^{2+}, Zn^{2+} on lipase activity was investigated and the results were given in Table 2. Mg^{2+} and Ca^{2+} stimulate the lipase effect with 100 and 98.40% of relative activity respectively indicating the requirement of metalloprotein, Ca^{2+} increases the thermal stability of enzyme due to the presence of more binding sites. However a small decrease in relative activity of 52% was observed with Cu^{2+} as metal ion, an enhanced decrease in lipase activity was observed using Fe^{2+}, Co^{2+}, Zn^{2+} with about 35.36%, 24.60% and 16.91%. The result indicates the stability of lipase in the metalloprotein. Moreover, the activity of KE38 lipase in the presence of Ni^{2+} was a distinguishing characteristic compared to other *Pseudomonas* sp. lipases, since they were generally activated only by Ca^{2+}, but not by Ni^{2+} [21].

Metal ions may stimulate the enzyme activity by acting as a binding link between enzyme and substrate combining with both and so holding the substrate and the active site of the enzyme. It is suggested that Ca^{2+} increases the thermal stability of the enzyme due to the presence of more binding sites [22]. These results suggested that Ca^{2+} and Mg^{2+} both stimulants were required for the stability of enzyme.

3.6.4. Effect of surfactants and inhibitors

Substances that reduce the activity of an enzyme catalyzed reaction are known as inhibitors. They act by either directly or indirectly influencing the catalytic properties of the active site. Inhibitors can be foreign to the cell or natural components of it. In the latter instance, they can represent an important element of the regulation of cell metabolism. Perusal of Table 3, the maximum relative activity was observed in Triton X-100 of 106.16% for *B. methylotrophicus* PS3, while in case of other

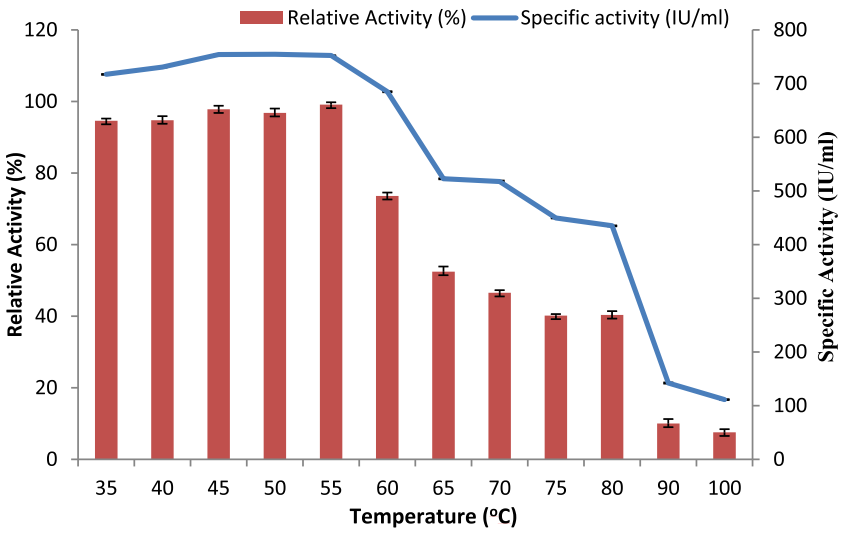

Fig. 3 Effect of temperature on purified lipase *B. methylotrophicus* PS3.

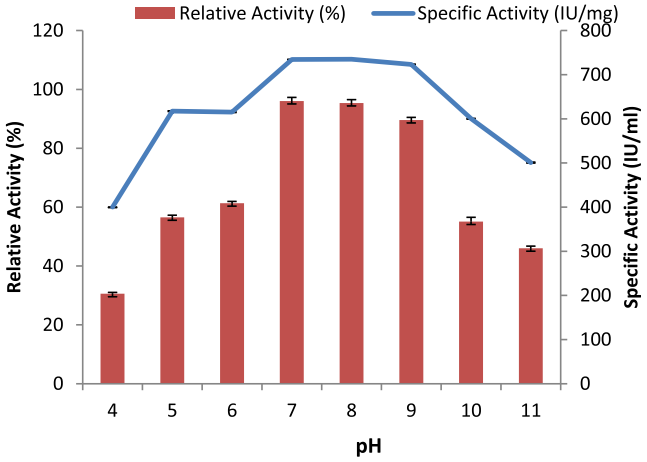

Fig. 4 Effect of pH on purified lipase *B. methylotrophicus* PS3.

Table 2 Effect of divalent ions on the activity of purified *B. methylotrophicus* PS3.

Divalent ions	Relative activity (%)
Ca^{2+}	**98.40**
Mg^{2+}	**100.00**
Cu^{2+}	52.30
Fe^{2+}	35.36
Co^{2+}	24.60
Zn^{2+}	16.91
Control	100.00
SE (m)	0.50
$C.D._{0.05}$	1.54

*SE Standard Error of Mean.
C.D. Critical Difference.

Table 3 Effect of surfactants on the activity of purified lipase.

Surfactant	Relative activity (%)
SDS	10.71
EDTA	16.95
CTAB	24.68
Tween 80	30.73
Triton X 100	**106.16**
Glycerol	82.37
Control	100.00
SE (m)	0.46
$C.D._{0.05}$	1.42

*SE Standard Error of mean.
C.D. Critical Difference.

surfactant i.e. Glycerol slight decrease in relative activity of 82.37% was observed, whereas other surfactants such as SDS, EDTA, CTAB, and Tween 80 drastically decrease to 10.71%, 16.95%, 24.63% and 30.73% respectively. It was confirmed that the higher lipase yields obtained with these additives were not due to increased lipase transcription, but to the enhanced secretion. Kiran and Chandra [23] also reported retention of 90% activity in the presence of Tween 20, Tween 80 and Triton X-100. Tween 20 a surfactant similarly enhances the lipase activity from *Pseudomonas aeruginosa* SRT 9 as studied by Borkar et al. [24].

3.6.5. *Effect of organic solvents*

Organic solvent stability of enzymes is a desirable feature especially in synthesis reactions such as esterification and transesterification [25]. In order to investigate the effect of organic solvents ethanol, methanol, acetone, benzene, chloroform, and xylene were used at a concentration of 1%. Among the varied organic solvent applied, methanol enhances the relative lipase activity of by 70.72%, followed by xylene with 66.14% of relative lipase activity, other organic solvent applied i.e. ethanol, benzene and chloroform applied reduces the lipase activity by 60.33%, 61.54% and 63.09% respectively (see Table 4). Therefore above results indicate that these solvent may enhance enzyme activity without causing any denaturation by modifying the oil–water interface. The lipase from *Pseudomonas fluorescens* KE38 may have potential use in

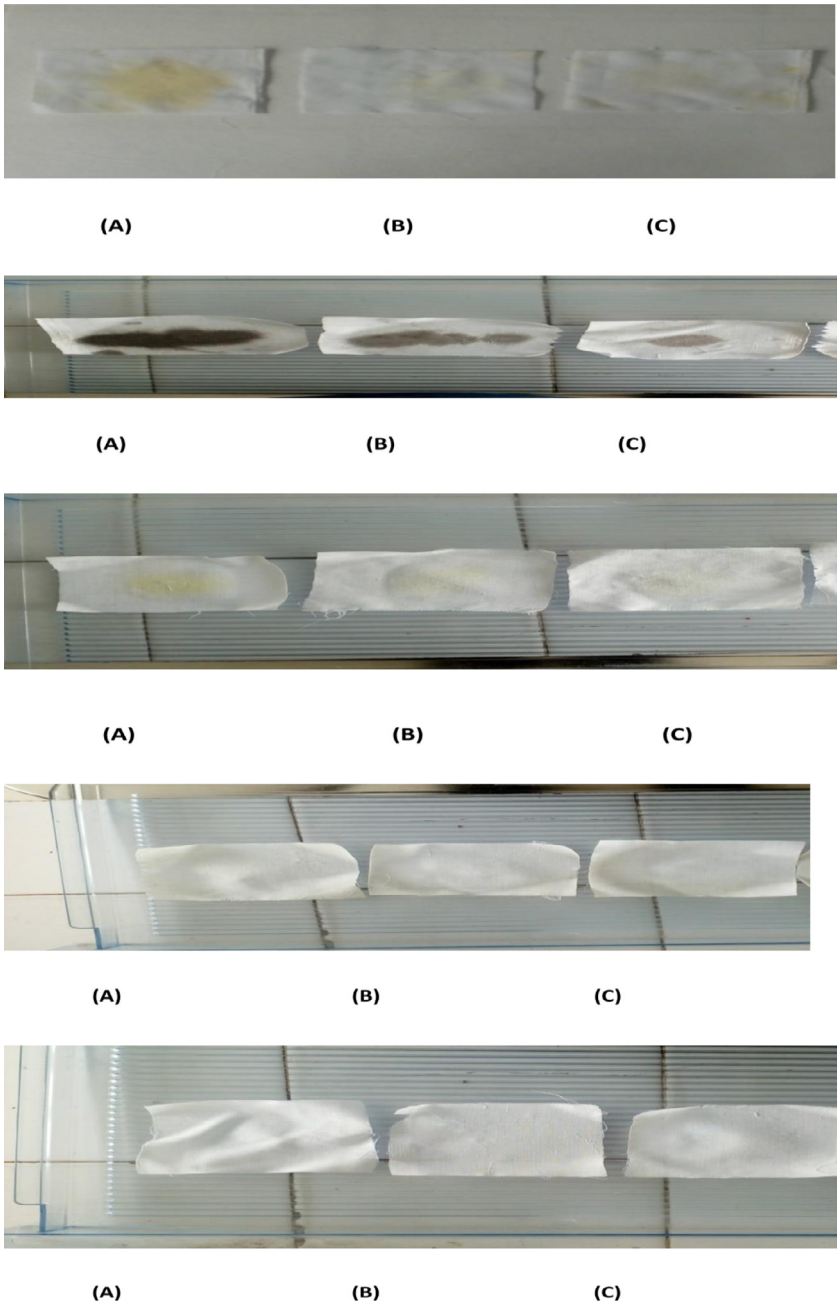

Plate 5 Washing performance of lipase from *B halotolerans* PS3 (A) Cloth stained with butter and white greece washed with distilled water (B)- washed with distilled water and detergent:(C)- washed with distilled water detergent and purified lipase from *B halotolerans* PS3.

organic synthesis and chiral resolution reactions that involve the use of DMSO and dimethylformamide as solvents [16].

3.7. Application of lipase

Lipase purified in present study from *Bacillus methylotrophicus* PS3 has shown thermostability as well as broader pH range resistance, therefore these can be considered potential candidates for laundry detergent industry, purified lipase utilized for improving the cleansing of grease, butter, vegetable oil, olive oil and grease (white) within 30 min as compared to the control (Plate 5). There was an incomplete removal of stains when washing was done only with the water and water mixed with soup, whereas an complete removal of stains occurred with addition of purified lipase into the cloth for stain removal. The enzyme containing detergents also improves the fabric quality and keeping color bright [3]. There are many different types of lipids, fatty acids, cholesterol, vitamins present in the grease and recommending its application in industrial industry.

Table 4 Effect of organic solvents on the activity of purified lipase.

Organic solvent	Relative activity (%)
Ethanol	60.03
Methanol	70.72
Acetone	66.16
Benzene	61.54
Chloroform	63.09
Xylene	66.14
Control	100.08
SE (m)	7.55
C.D. 0.05	23.12

*SE Standard Error of Mean
C.D. Critical Difference

4. Conclusion

An extracellular lipase producing bacteria *B. methylotrophicus* PS2 was purified by ammonium sulfate precipitation and gel chromatography with molecular weight of 31.40 kDa, purified lipase found maximum activity at 55 °C with optimum pH at 7.0 and metal ion Mg^{2+} has 100% relative activity with Triton X-100 surfactant providing stability to the lipase, found maximum in most of the organic solvent. The result of the present study indicates that the *B. methylotrophicus* PS3 is a potential lipase producing strain that can be efficiently utilized in detergent industry.

Compliance with ethics requirements

Authors declare that they respect the journal's ethics requirements. Authors declare that they have no conflict of interest and all procedures involving human and/or animal subjects (if exists) respect the specific regulations and standards.

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