

# An Approach to the Identification and Characterisation of a Psychrotrophic Lipase Producing *Pseudomonas* sp ADT3 from Arctic Region

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## Abstract

The aim of this research work was to explore psychrotrophic microbes from soil sample of Ny-Alesund, Svalbard, arctic region and to investigate their potential use as an effective tool for industrial application. A novel psychrotrophic bacterial strain showed good growth on minimal medium containing lipid as the only carbon source. Microbiological characterisation of the isolate showed that it was a gram negative rod. The strain was tested for the production of extracellular lipase enzyme. The enzymes were partially purified by 90% saturated ammonium sulfate and dialysis for desalting. The bacterium was identified as *Pseudomonas* sp ADT3 by 16S rRNA amplification and sequencing which had been deposited in the NCBI GenBank with accession number JX914667. Phylogenetic tree was also constructed with MEGA5 software and showed the highest level of sequence similarity with *Pseudomonas* sp. HC3-13 strain. The microorganism had a growth optimum at pH 8.0 and temperature 22°C. Optimization of different parameters e.g. temperature, pH, incubation time, cofactors etc. was performed for the extracellular lipase activity. The hydrolytic activity of the enzyme was enhanced 5 times by Pb<sup>2+</sup> but strongly inhibited by heavy metals Hg<sup>2+</sup> as well as EDTA and β-mercaptoethanol. For the molecular weight estimation of enzyme SDS-PAGE was done which showed an inducible band of approximately 13.9 KDa. Activity staining and mass spectrometry techniques were also performed.

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## Keywords

**Psychrotrophic; Arctic; Extracellular Lipase; 16S rRNA; Mass Spectrometry**

## 1. Introduction

Microbial enzymes have a great number of usages in food, pharmaceutical, textile, paper, leather and other industries. Their applications have been increasing rapidly. Among industrially important enzymes, hydrolases come in the first place and include enzymes with wide substrate specificity. Carbohydrases, proteases, pectinases and lipases are classified into hydrolases. They catalyze the hydrolysis of natural organic compounds. We will focus on lipase from a cold-adapted bacterium in this particular study. Lipases belong to the class hydrolase (EC 3.1.1.1). It hydrolyzes acylglycerides into free fatty acids and glycerol [1] and also catalyzes a variety of organic transformations which are chemo-, regio-, enantio selective besides esterification under reduced non-aqueous conditions [2] [3]. Due to such multifaceted properties, it is one of the important industrial enzymes that have found usage in a wide array of industrial applications, such as food, detergent, and chemical industry and also in biomedical sciences. Lipases are widely distributed in the earth's flora and fauna but are found more abundantly in microbial flora comprising bacteria, fungi and yeast [4]. For commercial bulk production, microbial sources are used, because of the great variety of catalytic activities available, the high yields possible, ease of genetic manipulation, regular supply due to absence of seasonal fluctuations and rapid growth of micro organisms or inexpensive media [5]-[10]. Microbial lipases are also more stable than their corresponding plant and animal enzymes and their production is more convenient, safer and can be obtained in bulk at low cost [11] [12]. There is growing interest in large scale purification of lipases. The chemo-, regio- and enantio-specific behaviour of these enzymes has caused tremendous interest among scientists and industrialists. Lipases from a large number of bacterial, fungal and a few plant and animal sources have been purified to homogeneity. Lipase producing bacterial sources is enormous but only few wild or recombinant strains have been used for commercial production of lipases.

Large area of the earth's surface is occupied by cold environments, such as the Arctic, Antarctic, alpine region, and abysses. Arctic soils can be considered an extreme environment colonized by special microorganisms, plants and animals which are adapted to these ecological niches. Many microorganisms are known as potential producers of extracellular lipases, including, yeast, and fungi [13] but there are few reports on extracellular lipase producing bacteria under cold condition [14]. The number of available lipases has increased since the 1980s. This is mainly a result of the achievements made in the cloning and expression of enzymes from microorganisms, as well as of an increasing demand for these biocatalysts with novel properties such as specificity, stability, pH, and temperature [15] [16]. The present piece of work has been designed by taking the futuristic view and will provide a base for commercial exploitation of enzymes as an alternative to chemicals.

In the present communication isolation and identification of a psychrotrophic arctic *Pseudomonas* producing lipase, purification of the extracellular lipases and characterization were done to find out its usage in appropriate industry.

## 2. Materials and Methods

### 2.1. Chemicals

All chemicals used were of analytical grade, obtained from Himedia (India) and Sigma-Aldrich Company.

### 2.2. Sample Collection

The soil samples were collected from 6 different sites of Ny-Alesund (78°56"N, 11°54") Svalbard, Arctic region during the Indian Arctic Expedition 2009, organized by National Centre for Antarctic and Ocean Research, Goa under the Ministry of Earth Science. During sampling and processing, careful attention was put to maintain sterile conditions. All soil samples were stored at -20°C.

### 2.3. Isolation of Microorganisms

1 g of soil was used for enrichment in a medium that consisted of 0.5% (w/v) glucose, 0.4% (w/v) NH<sub>4</sub>NO<sub>3</sub>, 0.1%

(w/v) yeast extract, 0.025% (w/v)  $\text{KH}_2\text{PO}_4$ , 0.025% (w/v)  $\text{Na}_2\text{HPO}_4$ , 0.1% (w/v)  $\text{MgSO}_4$ , and 0.5% (v/v) Tween 20 [17] at 22°C for 10 - 15 days at uniform shaking. Enriched soil samples were then serially diluted and plated on minimal medium containing 0.3% (w/v)  $\text{KH}_2\text{PO}_4$ , 0.6% (w/v)  $\text{Na}_2\text{HPO}_4$ , 0.05% (w/v)  $\text{NaCl}$ , 0.1% (w/v)  $\text{NH}_4\text{Cl}$ , 2 mM  $\text{MgSO}_4$ , 0.1 mM  $\text{CaCl}_2$  and 2% (w/v) agar containing 1% (v/v) olive oil at pH 8.0 by spread plate method. Plates were incubated at 22°C for 48 hrs.

## 2.4. Screening of the Isolates for Lipase Activity

Lipolytic organisms were screened by qualitative plate assay. Isolates were grown on phenol red agar base plates and incubated at 22°C for 2 days. A change in colour was observed from red to yellow due to hydrolysis of olive oil to free fatty acid. Three isolates designated: ADT3, ADT4 and ADT5 showed positive result. The ADT3 isolate showing maximum zone of clearance was chosen for further investigation. The isolate was grown and sub-cultured in minimal medium slants with 1.0% olive oil.

## 2.5. Morphological and Biochemical Characterisation

The pure colony isolated was characterised by various biochemical tests using the Bergey's manual of Determinative Bacteriology, as summarised in **Table 1**. The bacterial culture was streaked on respective plates containing tryptone agar, EMB (Eosin Methylene Blue) agar and blood agar. The plates were incubated at 22°C for 24 hrs. The different colony characters were recorded.

For antibiotic sensitivity test the bacterial culture was spreaded on Muller Hilton agar and the antibiotic octadisc combination (Himedia) placed on it. Plates were incubated at 22°C for 48 hrs.

## 2.6. Scanning Electron Microscopy

The morphology of the bacterium was studied using Hitachi, S530 SEM at University Science Instrumentation Centre; Burdwan University. The bacterium was fixed with glutaraldehyde (4% v/v stock) at 4°C for 4 hrs. After centrifugation, the cells were dehydrated by gradually lowering the concentration of alcohol: 95%, 70%, and 50%. Cells were incubated 30 minutes in each of the alcohol concentrations followed by washing. Finally, the cells were diluted with 50% alcohol. One drop of this cell suspension was placed on a cover slip and studied with Hitachi, S530 SEM.

**Table 1.** Morphological and biochemical characterization.

Shape	Rods (Bacillus)
Lactose Fermentation	Non-fermentative
Gram Character	Negative
Indole	Negative
Oxidase	Negative
H <sub>2</sub> S	Positive
Catalase	Negative
Methyl red	Positive
Voges proskauer	Negative
Amylase	Negative
Gelatin	Positive
Endospore formation	Positive
Growth	Aerobic
pH range	3 - 9
Optimum pH	8.5
Temperature range	10°C - 25°C
Optimum temperature	22°C
NaCl range	0.01 M - 2 M
Optimum salt concentration	0.8 M
Growth in presence of oil	0.5% - 1.5%
Optimum olive oil concentration	1%

## 2.7. Physiological Characterisation

### 2.7.1. Temperature

ADT3 cells were grown in minimal medium with 1.0% peptone and 1.0% olive oil and incubated at temperatures ranging from 10°C to 70°C. Growth was determined by measuring O.D. at 620 nm after 48 hours.

### 2.7.2. pH

ADT3 cells were grown in minimal medium with only olive oil as the carbon source (1.0%) at 22°C. pH was varied from 3 to 9. Optimum pH for growth was determined by measuring O.D. at 620 nm after 48 hours. pH was rechecked after the experiment.

### 2.7.3. Carbon Source

The ADT3 cells were grown in minimal medium with 0.2%, 0.4%, 0.6%, 0.8% and 1% of each of the carbon source respectively: fructose, glucose, sucrose, maltose and galactose. Growth in presence of different carbon source was determined by measuring O.D. at 620 nm at 24 hrs, 48 hrs and 72 hrs respectively.

### 2.7.4. Substrate Concentration

The cells were grown at pH 8 in minimal medium with olive oil as the sole carbon source at 22°C for 48 hrs. The amount of olive oil added was varied from 0.1% to 1.5%.

### 2.7.5. NaCl Concentration

Cells were grown in minimal medium with olive oil (1.0%) as sole source of carbon at 22°C for 48 hrs, with salt concentration from 0.01 M to 2 M. Optimum salt concentration for growth was determined.

## 2.8. Identification of the ADT3 Isolate by 16S rDNA Gene Amplification and Sequencing

### 2.8.1. DNA Extraction

Cells obtained from 50 ml culture were resuspended in lysis buffer [4.5 ml TE buffer, 100 µl lysozyme (10 mg/ml), 450 µl of 10% SDS, 10 µl of 20 mg/ml proteinase K] and incubated for 1 hour at 37°C. DNA was purified by phenol chloroform extraction and ethanol precipitation (Sambrook *et al.* 1989).

### 2.8.2. 16S rRNA PCR Amplification

The amplification was performed on 1.5 µl DNA extract in 20 µl using High Fidelity PCR System (GeneAmp PCR System 2400). PCR was carried out by using the universal primers: 27F: 5' AGA GTT TGA TCC TGG CTC AG 3' and 1492R: 5' TAC GGY TAC CTT GTT ACG ACT T 3' (Chromous Biotech Ltd., Bengaluru, India) [18]. Amplification reactions were performed in a 25 µL volume, containing: 20 mmol/L Tris-HCl (pH = 8.4), 50 mmol/L KCl, 2.0 mmol/L MgCl<sub>2</sub>, 200 µmol/L of dNTPs, 1 µmol/L of each primer, 30 ng of genomic DNA and 1.5 U of Taq DNA polymerase. The amplification conditions were: 94° for 3 min for the first step, 30 cycles comprising of 94° for 1 min, 52° for 45 sec, 72° for 1 min and the final extension step of 72° for 3 min. The PCR product obtained was purified and sequenced by an automated DNA sequencer.

### 2.8.3. Phylogenetic Analysis

The forward and reverse sequences were assembled through DNABaser V35.0 software and submitted to GenBank through NCBI online sequence submission tool: Sequin V12.21. MEGA5 software was used to construct phylogenetic tree by aligning ADT3 sequence with reference sequences obtained from NCBI GenBank.

## 2.9. Extraction of Crude Lipase

On the day of maximum lipase activity in the lipase production medium, the culture was centrifuged at 10,000 g for 15 min at 4°C and the cell free supernatant was the source of crude extracellular lipase enzyme. The pellet so obtained was properly vortexed in Tris-HCl buffer, pH 8.0 and used as intracellular lipase enzyme.

### Partial Purification of Enzyme

The crude extracellular enzyme was subjected to different fractions of ammonium sulphate precipitation *i.e.* 0 -

10%, 10% - 30%, 30% - 50%, 50% - 70% and 70% - 90% with continuous stirring in magnetic stirrer overnight at 4°C. The resulting precipitate was centrifuged (15,000  $\times$ g) for 20 min at 4°C, and the supernatants discarded. The crude precipitate was dissolved in minimal amount of 10 mM Tris-HCl buffer of pH 8.0, and then dialysed extensively against the same buffer overnight at 4°C. The enzyme activities in the dialysate were again assayed.

### 2.10. Determination of Lipid Hydrolysis by Titrimetric Assay

Extracellular and intracellular lipase activity in bacterial culture supernatants after centrifugation (12,000 g for 20 min) was determined by titrimetric assay using olive oil as substrate. The reaction mixture contained 1 ml of 0.1 M Tris-HCl buffer pH 8.0, 50 mM KCL, 200  $\mu$ l Tween 20, 1 ml of olive oil as substrate, 1 ml of culture supernatant at 22°C for 3 hrs. The reaction was stopped and fatty acids were extracted by addition of 3 ml of ethanol. The amount of fatty acids liberated was estimated by titrating with 10 mM NaOH using phenolphthalein indicator.

One unit of enzyme activity is defined as amount of alkali in  $\mu$ Eq (U) required neutralizing the fatty acids produced by hydrolysis of lipid (olive oil) per mg protein per hour.

### 2.11. Extracellular Protein Profile of the Isolate

#### 2.11.1. Extraction of Extracellular Proteins

The cells were grown in two separate flasks. One in minimal M9 media containing 1% (v/v) olive oil and in other set M9 with 1% (W/V) peptone for 48 hrs at 22°C. The cells were harvested by centrifugation at 12,000 rpm for 10 mins. The cell free supernatant was collected. The extracellular proteins were precipitated by 30% acetone overnight at 4°C. Finally the proteins were dissolved in 0.1 M Tris-HCl buffer pH 6.8.

#### 2.11.2. Estimation of Protein

Protein concentration was measured by the method of (Bradford, 1976) with bovine Serum albumin as a standard.

#### 2.11.3. SDS-PAGE of the Isolate

Proteins were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) with a 12% polyacrylamide resolving gel and a 5% polyacrylamide Stacking gel [19].

#### 2.11.4. Gel Documentation System

The protein gel was analysed in Gel documentation system (Vilbur Lourmat France) in Department of Biotechnology, Burdwan University.

## 3. Results and Discussion

10 colonies were isolated from the culture plate with lipid as sole carbon source. Most of the colonies were colourless with a few forming pigments. Among 10 isolates, one strain ADT3 showed maximal zone of clearance in phenol red plate assay which is further studied (**Figure 1(a)**).

### 3.1. Morphological, Biochemical and Physiological Characterisation

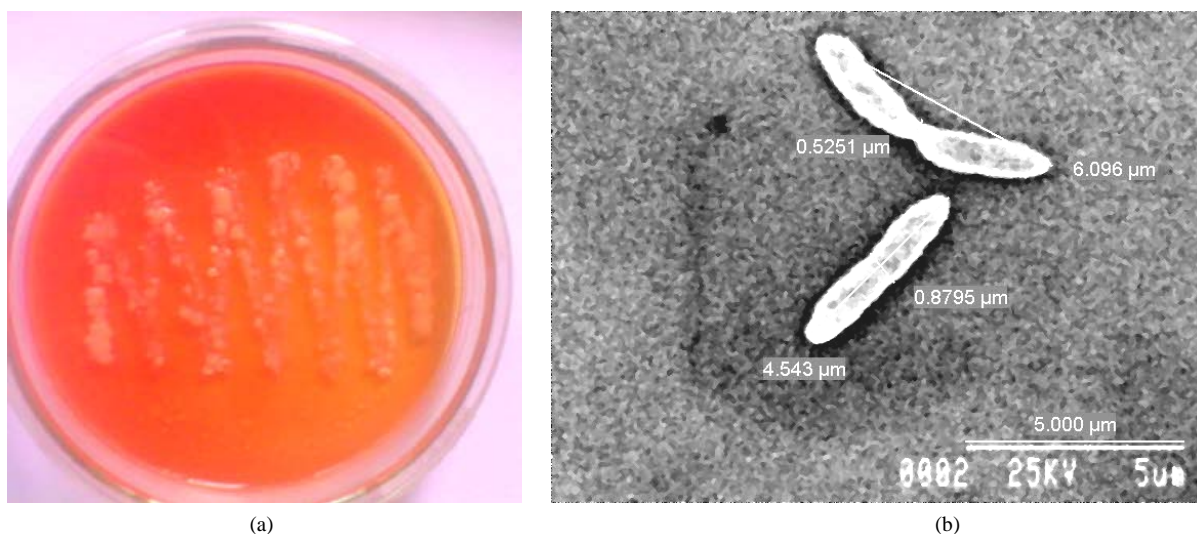
Colony formed on tryptone agar was smooth, glistening, and opaque with entire margins and deep cream in colour. Growth on eosin methylene blue (EMB) agar plate was of a pale tan colour indicating ADT3 to be a lactose non-fermenter.

ADT3 was gram negative in nature. Gram nature of the isolate was confirmed by treating a smear of the culture on a slide with 3% NaOH. Gram negative ADT3 instantly lysed and became viscous whereas a control gram positive bacillus was not affected by the alkali treatment [20]. SEM micrograph reveals that the cells are rod shaped of length 4.5  $\mu$ m and diameter 0.9  $\mu$ m (**Figures 1 (b)**). The results of experiments on Antibiotic sensitivity assay shows that ADT3 strain is resistant to Oxycillin and ampicillin (**Figure 2**).

### 3.2. Analysis of 16S rRNA PCR

The PCR products were analyzed by electrophoresis on 1.5% agarose gels and stained with ethidium bromide. 100 bp ladder was used for evaluating the size of amplicons. Bands were obtained at 1.4 kb (**Figure 3**).





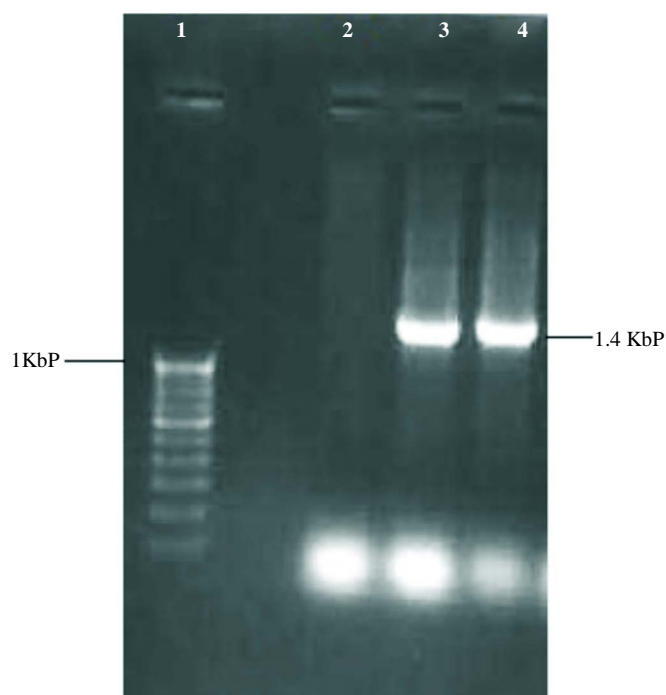
**Figure 1.** (a) Phenol red agar plate: ADT3 strain hydrolyzing the oil containing media with phenol red indicates the change in colour from red to yellow due to presence of lipolytic activity; (b) Scanning electron micrograph (Magnification 8000 $\times$ ) Isolates were grown in rich medium at 22°C for 48 hrs and fixed in 4% glutaraldehyde.



**Figure 2.** Antibiotic sensitivity assay: Antibiotic octadisc placed on ADT3 were incubated at 22°C for 48 hrs. Figure shows that ADT3 strain is resistant to Oxytetracycline and ampicillin.

### 3.3. Sequencing and Computer Analysis 16S rRNA Genes

For the validation of species identification, chosen strain was identified by 16S rRNA PCR pattern analysis on 1.5% agarose gel electrophoresis and those were selected for sequencing. The PCR amplified products were sequenced from Xcelris Labs, Ahmedabad, India. Species identification and similarity calculations were performed, comparing sequences of approximately 1500 bases of the 16S rRNA gene sequence of ADT3 with 16S rRNA gene sequences retrieved from NCBI GenBank using nucleotide BLAST network services [21]. It showed 99% similarity with *Pseudomonas* strain which belongs to the class Proteobacteria and phylum Pseudomonadaceae. The 16S rRNA sequences in this study were submitted to the GenBank database by using Sequin software under nucleotide accession number of ADT3 as JX914667 (Table 2).



**Figure 3.** 1.2% Agarose gel: The 16S rRNA PCR product of ADT3. Lanes 3 and 4 contain PCR product of 1.4 Kb. Lane 2 had water as control. Lane 1 contains the 100 b.p. ladder.

**Table 2.** 16S rRNA gene sequence accession no. from NCBI GenBank.

Sl. No.	Sample ID	BLAST details				
		Organism	Gene Bank accession no.	Max Score	Query coverage	Max Identity
01	ADT3	<i>Pseudomonas</i> sp. ADT3 16S ribosomal RNA gene	JX914667	2532	100%	99%

### 3.4. Phylogenetic Analysis of *Pseudomonas* spp. Based on 16S rRNA Sequence

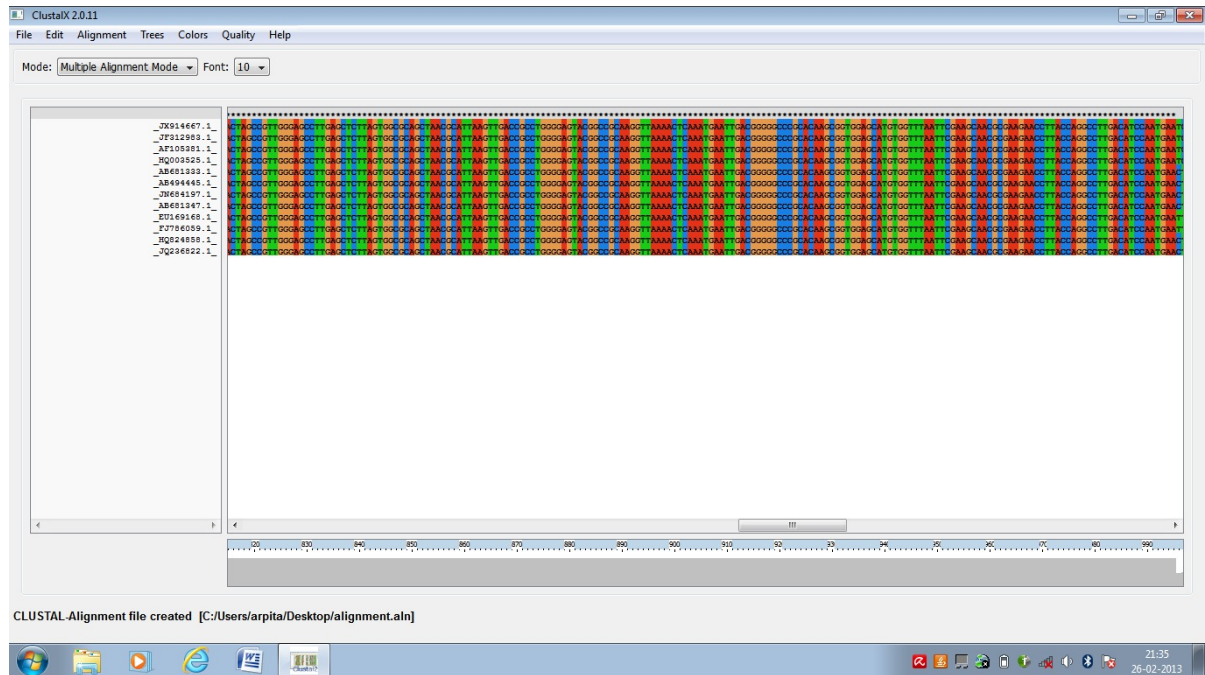
Multiple sequence alignment was done by CLUSTALW software (Figure 4). Phylogenetic relationship was outlined by MEGA5 software and found similarity index, distance using the neighbour joining method. ADT3 showed the highest level of sequence similarity with *Pseudomonas* sp. HC3-13 strain (Acc. No. JF312983.1) (Figure 5).

### 3.5. Determination of Lipase Activity by Titrimetric Assay

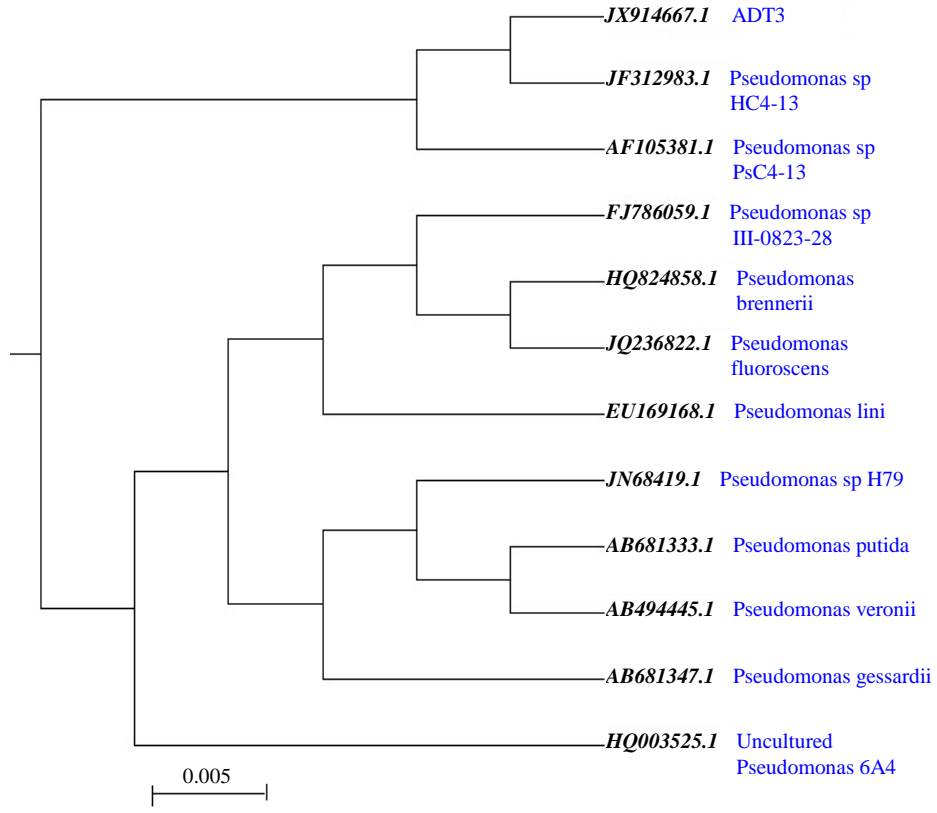
Specific activity of the lipase isolated from intracellular and extracellular fractions of ADT3 cells showed that 87% of the activity is extracellular and only 13% is intracellular. Hence, the cell supernatant was used as the source of enzyme in all studies. The lipase activity was measured titrimetrically and found 113.33 U/mg protein. Optimization of different parameters e.g. temperature, pH, incubation time, cofactors etc. was performed for the extracellular lipase activity. The lipase activity was found to increase 5 times *i.e.* 527.77 U/mg protein in presence of 1.2 mM lead when compared to control (result not shown).

### 3.6. Extracellular Protein Profile of the Strain

The extracellular protein profile of lipid grown cells shows one low molecular weight of 13.9 KDa inducible band (Figure 6) which may be lipase which is faint in peptone grown cell. The band seems to be lipase which is confirmed by Mass-spectrometer and activity staining.

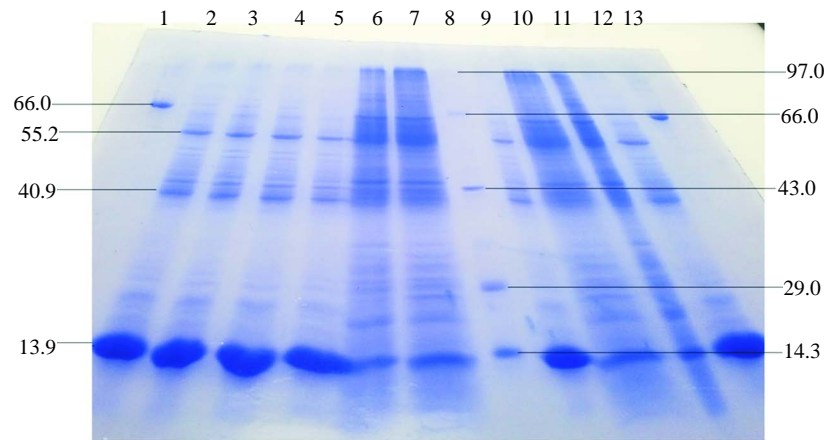


**Figure 4.** Multiple sequence alignment: Alignment of closely related 16S rRNA gene sequences of ADT3 (JX914667.1) re-retrieved from the NCBI GenBank with the software CLUSTAL W.

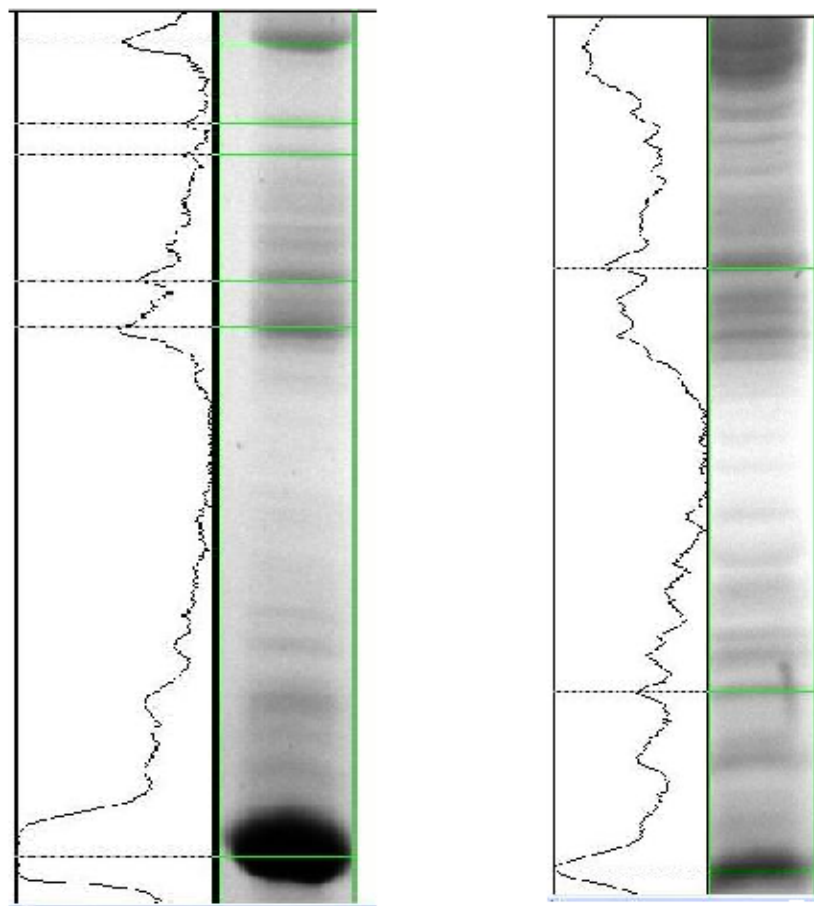


**Figure 5.** Neighbour joining tree based on ADT3 16S rRNA gene sequence comparison with 16S rRNA gene sequences re-retrieved from the NCBI GenBank, constructed using MEGA 5 software. The numbers in left are GenBank accession numbers.





(a)



Lane 2 oil grown

Lane 7 peptone grown

(b)

**Figure 6.** (a) 12% SDS PAGE of ADT3 grown in peptone and only olive oil. Lanes 6, 7, 10 and 11 show proteins extracted from ADT3 grown in 1.0% peptone. Lanes 2, 3, 4, 5, 9 and 12 contains proteins obtained from ADT3 grown in 1.0% olive oil. Lanes 1 and 13 contains BSA while lane 8 has molecular weight marker of medium and low range. All molecular weight markers are in kda; (b) Densitometric analysis of the 12% gel by Quantum Capt software showing the differential expression of proteins under varying culture conditions 1.0% olive oil and 1.0% peptone.

## 4. Conclusions

The main purpose of this study was identification, characterization and partial purification of lipase enzymes isolated from arctic soil. Through polyphasic identification strategy this lipase producer was found to be a gram negative, non lactose fermenting Proteobacteria, highly similar (99%) to *Pseudomonas* sp. The isolated bacterium was found to give its optimum temperature at 22°C. The results obtained in this study show that olive oil is the most suitable substrate for maximum lipase production by *Pseudomonas ADT3* strain. The enzyme exhibited maximum activity and stability at pH 3.5 and pH 8.5. The enzyme has two isozymes which were hence confirmed by activity staining. The remarkable stability of *Pseudomonas ADT3* lipase in this range has proved it to be a potential alkaline lipase similar to others, and a candidate for industrial applications such as detergent, leather and fine chemical industries. The lipase activity was found to increase 5 times *i.e.* 527.77 U/mg protein in presence of 1.2 mM lead when compared to control *i.e.* 113.33 U/mg protein. The enzymatic activity of the isolated lipase producing strain *Pseudomonas* sp. is stimulated highly only by lead ions but not by other elements and inhibited by Hg<sup>2+</sup> and EDTA. The mechanism of activation of this lipase by lead ions is not clear. But it is possible that lead ion binds to the core region of lipase in such a way that it leads to a change in conformation of active site. The lipase was relatively stable towards many metal ions and detergents tested. This characteristic made the enzyme suitable for lipase reactions under various conditions without special precautions.

For the protein determination of our enzyme, SDS-PAGE was done which showed approximately 13.9 KDa band. The induced band is confirmed to be lipase by activity staining and mass-spectrometry.

The characteristic of this presumably new enzyme of acting hydrolytically on fatty substrates without having to go through complicated, expensive and time consuming purification steps indicate that this enzyme would be suitable for low-scale production and utilization in industrial level. It would be recommended that the research involved in this enzyme's characterization should be continued in order to get a full image of its potential and its use in application. Further study must be performed in order to fully characterize the new lipase and design its production to serve environmental friendly purposes.

We are currently in the process of cloning this lipase gene and placing it under a strong promoter to be able to determine molecular properties of the lipase as well as increasing enzyme expression and yields for future industrial applications.

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## Authors' Contribution

The research work is really handled and designed by Arpita Dey. Pranab Roy contributes his intellectual input for the study.

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