Research Article

# Screening and Characterization of Thermolabile Protease and Alkaliphilic Lipase Producing Psychrotrophic *Stenotrophomonas* sp. and *Pseudomonas* sp.

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**Abstract** | The study was performed to isolate cold active alkaline protease and alkaline active lipase producing psychrotrophic bacteria from water and soil samples collected from different glaciers of Karakorum Range of mountains, Pakistan. Serial dilution and plating approaches were exploited for bacterial isolation and the isolates were qualitatively screened for proteolytic and lipolytic activity with skim milk and Tributyrin agar (TBA plate) assay method. From the collected samples, a total of 20 bacterial isolates (PAK-01 to PAK-20) were exhibiting psychrotrophic physiology. About 8 isolates were capable of proteolysis in alkaline range of pH; and 6 bacteria exhibited lipolysis. Based upon primary and secondary qualitative screening, One isolate (PAK-01) exhibited maximum proteolysis on 1 % skim milk while another isolate (PAK-03) demonstrated maximum lipolysis on Tributyrin Plate Assay. On the basis of morpho-physiology, biochemistry and 16s rRNA sequence, the PAK-01 was identified as *Stenotrophomonas sp*. MG662181 and PAK-03 was identified and tagged as *Pseudomonas* sp. MG687270. Based upon genetic signatures and physiological parameters, our strain presented less than 79% of homology to *Stenotrophomonas* sp. and *Pseudomonas* sp. which makes it a novel species. Both of the strains were non-pathogenic and potentially industrial strains for the production of thermolabile protease and alkaliphilic lipase.

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**Keywords** | Cold active alkaline protease, Lipase, *Stenotrophomonas* sp., *Pseudomonas peli*, Morpho-physiological characterization, Biochemical characterization, 16S rRNA

## Introduction

Psychrotrophic microbes provide a wide-ranging biotechnological potential and offering abundant financial and eco-friendly advantages as compared to thermophilic as well as mesophilic bacteria and their biocatalysts (Margesin et al., 2002; Soror et al., 2007). Thermolabile biocatalyst at low temperatures exhibited extraordinary catalytic efficiency and have been exploited in biotechnological applications recently as compared to their counterparts such as mesophilic and thermophilic bacteria (Margesin et al., 2005).

Such biocatalyst application enables less processing time and reduction of temperature deprived of a loss of catalytic efficiency, that leads to save energy and time consumption. Enzymes act as biocatalysts

to enhance metabolic rate of chemical reactions. An enormous number of biocatalysts could be produced *in vivo* because of their great significance in industries. Protease enzyme is the most imperative biocatalysts produced industrially to speed up the chemical reactions. Naturally protease enzymes exist in all creatures and correspond to 1-5% of the entire protein content (Singh et al., 2012). Protease enzyme is found as the third principal group of industrial biocatalysts and has 60% of the worldwide sale (Akcan and Uyar, 2011). Proteases perform the amino acid hydrolysis in proteins and they are also termed proteolytic enzymes, peptidase or proteinase (Sharma et al., 2015). Protease enzymes are able to generate eco-friendly products and also performance a fundamental role in modern-day biotechnology industrial applications (Abebe et al., 2014).

Lipases belong to the family of serine hydrolases that catalyze equally hydrolysis and synthesis of ester bonds of long-chain triacylglycerides. Lipases are significant amongst biocatalysts due to their potential to speed up a wide diversity of chemical reactions. They are also an essential group of biotechnologically important biocatalyst and have considerable applications (Mohammed, 2013). Lipases from bacterial origin are more beneficial as compared to the lipases from plant and animal origin. Since, microbes have numerous variability's in their hydrolytic activities and they are easily to manipulate genetically and can be grown easily with rapid growth in cheap media (Mongkolthanaruk and Boonmahome, 2013; Veerapagu et al., 2014; Jinyong, 2014). Bacterial lipases have gained significance from industrial point of view because of their stability, selectivity and comprehensive substrate specificity. Enzymes from microbial origin are also more stable as compared to plant and animal biocatalysts and their production is safer and more convenient (Veerapagu et al., 2013). Existing detergents in the market consist ingredients including dyes, salts and soda which damage the shine and quality of fiber and hence fabric as a whole. They also cause a significant addition to the sanitary pollution and are considered to be allergens. Besides these issues, the existing detergents are expensive. To the best of our knowledge, there is no available formulation of bio detergents in the market so far. To tackle the issues, cost effective and environment friendly bio detergents should be fabricated and launched in the market. The sustainable cost and quality of the fabric can be maintained by using the detergents designed through bio-based material of low economic value. The bio detergents consisting hydrolase enzymes particularly the lipase and protease could be helpful to sort out the issue. Washing at low temperature in optimized settings and higher pH can be sorted by extracting the lipase and protease from low temperature active bacteria and proliferating at higher pH.

Thermolabile alkaliphilic lipases and protease covering a broad-ranging spectrum of biotechnological uses for example applications in bioremediations technology, detergents formulation, food industries additives, biotransformation and also achieve significant functions in molecular biology and the heterologous genes expression in psychrophilic microbes to prevent the growth of inclusion elementary bodies (Joseph et al., 2008). Thermolabile alkaliphilic lipases and protease enzyme usage has a vast potential in bacteriological contamination in diverse industrialized practices and likewise in terms of lower energy costs (Alquati et al., 2002).

Considering the significance of cold active alkaliphilic protease and lipase enzyme, there is a great urge to search for novel lipases and proteases of industrial uses. This study was aimed at screening, characterization and maintaining the potential psychrotrophs from soil and water samples of Karakorum Range Glaciers of Pakistan, which were capable of producing low temperature active alkaliphilic protease and lipase of commercial interest.

## Materials and Methods

### *Sample collection*

Water and soil samples were collected during field visits during July 2017 from three diverse localities of polar glaciers of Gilgit-Baltistan Pakistan including Juglot (latitude 35°41′06″, longitude 74°37′26″), Jutial (latitude 35°54'276″, longitude 74°19'841″) and Rakaposhi (latitude 36°14'368″, longitude 74°26'576″) (Table 3). Microbiological prospects like the sterility of instruments, personnel's and handling of samples were performed according to standard microbiological techniques.

A total of 20 samples were collected from 10 different sites and coded as AMBL-1-AMBL-20. Samples of water and soil were collected carefully and transferred to their respective portable ice boxes. The materials

included a manual drill, sampler, sterile gloves and sample bags, pH strips, thermometer, GPS, Ice cabins, ethanol, methylated spirit, spray bottles, tissues and cotton, organized Petri plates with nutrient agar medium.

Geographic coordinates, height and atmospheric pressure were recorded using a GPS device. Dissolved oxygen (DO) was measured with Portable Dissolved Oxygen Meter. The pH was recorded using pH indicator strips and ice was cut into pieces and collected in sterile sample bags. The water samples were obtained in sterile bottles and soil samples were collected in sterile bags.

The soil and water samples were collected in sterile bags, labelled, and transported to the basecamp in their intact physical form. The samples were immediately transported back to the laboratory in Islamabad in same physical condition using ice containers as described by Sagar et al. (2013). The soil and water samples were preserved at 4°C in the lab and isolation of bacteria was done within 24 hours after preservation.

*Media, culture conditions and isolation of bacteria*

Soil and water samples were subjugated for isolation of psychrotrophic bacteria as mentioned in Table 1. Soil samples from Glaciers were used for bacterial isolation by formulating dilutions of 2 g of soil to 8 mL of distilled water by serial dilution method. Around 100 µL of every dilution and water samples were spread on nutrient agar medium (peptone 0.5%, NaCl 0.5%, yeast extract 0.5%, agar 2% and 9 pH of Tris-HCl buffer). Aerobically the duplicate spread dishes were incubated for about 1 week at 4, 10 and 20°C.

**Table 1:** *Isolation of psychrophilic bacteria from various samples collected from glaciers.*

| Source | | Sample Codes | Isolates |
|---|---|---|---|
| Glacial samples and isolates | Rakaposhi Glacier: water | 1.7 | PAK-01 |
| | Rakaposhi Glacier: soil | 2.3 | PAK-03 |

The microorganisms were isolated from soil (Abd Rahman et al., 2007) and water samples in the Applied Microbiology Research Laboratory, Department of Biological sciences, International Islamic University Islamabad, Pakistan and identified. From the collected samples, a total of 20 bacterial isolates (PAK-01 to PAK-20) were screened and 8 protease producing and 6 lipase producing isolates were finally selected based on their proteolytic activity. Among them, one isolate (PAK-01) was identified as *Stenotrophomonas* sp. and another isolate identified was *Pseudomonas peli* (PAK03) were selected for further study due to their higher proteolytic and lipolytic activity.

*Primary and secondary screening of cold active protease producing psychrotrophic bacteria*

Primary screening was done by spot inoculation of all the protease producing isolates on skim milk agar plates at 1 % exploiting sterilized toothpick and were incubated at 25°C. The psychrotrophic isolates producing zones of clearance above 10 mm were preferred and exposed to secondary screening. This screening was performed with the culture filtrates of the 8 preferred isolates by means of well diffusion technique.

Entirely, the 8 isolates were cultivated in nutrient broth after that 1 mL of all tested inoculums with 0.6 OD has been inoculated to 200 mL of growth medium and incubated at 180 rpm in a rotatory shaker at 25°C. The bacterial cultures were harvested subsequently after 48 hours of incubation and centrifuged at 15000 rpm for 20 min at 4°C and the supernatant was collected. Wells in skim milk agar dishes were prepared using sterile cork borer. Filtrate of bacterial culture of all the isolates in each well were placed at 200 µL and incubated at 25°C for two days. Tannic acid solution (10%) in all wells was flooded and incubated for 24 h at 25°C, 9 pH and Subsequently, after the incubation phase, isolates efficient of producing cold active protease were investigated on the basis of zone of clearance and colony size ratio on plates. Consequently, the potential colonies were selected and streaked for culturing pure psychrotophic bacterial colonies.

*TBA plate assay for screening of cold active lipase producing psychrotrophic bacteria*

Lipase producing bacteria isolates created a zone of hydrolysis after their suitable dilutions were inoculated on the TBA medium comprising of peptone, 5g/l; tributyrin, 10 mL/l; beef extract, 3g/l and agar-agar, 20g/l. The size of cleared zone was experimented after 48 h of incubation at 25°C.

*Maintenance of cultures*

Selected Glacial bacterial isolates were consistently maintained at 2-5°C on nutrient agar slants comprised

of 20g Agar, 3g yeast extract, 5g peptone, 1000 mL of Double-distilled water (dd$H_2O$) with pH 9.

*Identification of bacterial isolates*

Potential pyschrotrophic bacterial isolates were identified on the basis of morphological characteristics, biochemical characterization and 16S rRNA sequencing technique.

*Morphological and biochemical classification*

The potential psychrotrophic bacterial isolates were Gram's stained and were observed with a high potency magnifying glass under the light microscope. Morpho-physiological physiognomies and motility characteristics of the cells were evaluated on the basis of endospore staining, elevation; margins, capsule staining, odor, color, motility test, optimum temperature, respiration and pH.

Selected bacterial isolates were categorized on the basis of biochemical tests such as indole test, nitrate reduction test, voges proskauer test, methyl red test, catalase test, oxidase test, simmons citrate test, urease test, acid formation from sugars, hydrogen sulfide and starch hydrolysis test (Reiner, 2010; Shields and Cathcart, 2010; Urszula et al., 2009; Rajeswari et al., 2013; Kumara et al., 2015)

*PCR Amplification for identification*

Psychrotrophic strains were identified on the basis of PCR Amplification and partial sequencing of the 16S ribosomal RNA with universal bacterial primers. The sets of universal primers used were 27F- 5'LAGAGTTTGATCCTGGCTCAG3' and 1492R5' LTACGGTTACCTTGTTACGACTT3' as shown in Figure 1.

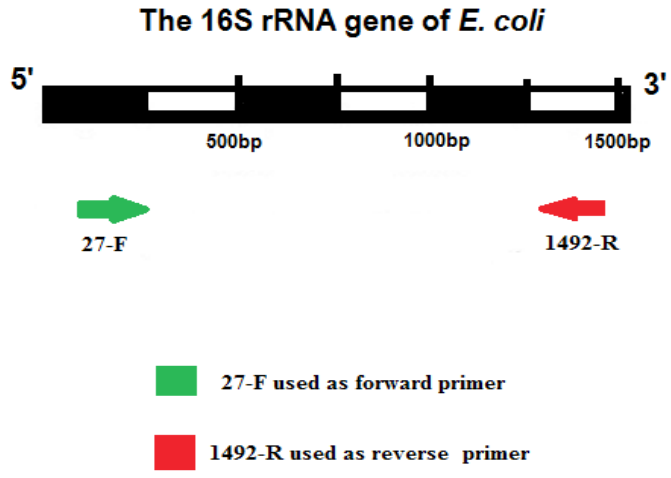


**Figure 1:** *E. coli 16S rRNA gene structure.*

*PCR reaction*

Reaction mixture was exploited to amplify 16S ribosomal RNA of selected bacterial isolates. The optimized PCR total reaction mixture volume used was 50 µL for each reaction as showed in Table 2.

**Table 2:** *Optimized PCR conditions.*

| No. | Ingredients | Concentration | Volume µL |
|---|---|---|---|
| 1 | Template (0.2 ng/ µL) | 1ng | 5 |
| 2 | primers (10 µmol) each | 1µL | 1 |
| 3 | dNTPs Mix (10 mmol) | 1µL | 1 |
| 4 | PCR Standard Buffer | 5µL | 5 |
| 5 | *Taq* DNA Polymerase | 1 Unit | 0.25 |
| 6 | dd$H_2O$ | 35.75 µL | 35.75 |
| 7 | $MgCl_2$(50 nmol) | 1 µL | 1 |

*Statistical analysis*

The statistical analysis was performed on MS Excel where required. The mean values are presented with their standard deviations (±SD).

## Results and Discussion

Isolation of the enzyme producing microorganism has gained great attention due to its application in many biotechnological processes such as textile; detergents; oil processing; dairy industries, chiral pharmaceuticals synthesis and surfactant production. Microorganisms like bacteria, fungi and yeast produce certain enzymes for growth on organic insoluble substrates. For example, the lipase enzyme is produced and favored because of having greater stereo specificity, high reaction specificity and stability and less energy consumption than the enzymes from plants and animals source (Lee et al., 2015). A lot of studies have been previously conducted for the production of lipase and protease enzymes from different bacterial species from different samples (Abd Rahman et al., 2007; Grbavčić et al., 2011; Lee et al., 2015). Glaciers of the Karakorum mountain Range in Pakistan are said to be the home and archive of psychrotrophic bacteria. Bacterial cold active alkaliphilic protease and lipase are frequently produced throughout starvation and sporulation other than temperature stress and pH etc. In this study, two facultative psychrotrophic bacteria were isolated from glacial water and soil samples of Gilgit-Baltistan Pakistan. A thermolabile alkaliphilic protease and lipase has been characterized from the glacial isolates identified as *Stenotrophomonas* sp. (MG662181) PAK-01 and *Pseudomonas peli* PAK-03 (MG687270).

**Table 3:** *Sampling data sheet.*

| Glacial Isolates | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| GPS Coordinates | | | | | | | | |
| Rakaposhi Glacier | | | | | | | | |
| Sr.# | Location | Transect name | Latitude | Longitude | Temp (°C) | Pressure | pH | Height |
| 1.1 | Water | Lake water | 36°14'36.8 | 074°26'57.6 | 6 | 795 | 6 | 2823 |
| 1.2 | Glacier | Glacier ice | 36°14'35.6 | 074°26'58.1 | -1 | 790 | 6.5 | 2868 |
| 1.3 | Ice | Lake ice | 36°14'35.6 | 074°26'57.7 | 0 | 794 | 6 | 2823 |
| 1.4 | Lake head | Stationary lake water | 36°14'34.2 | 074°26'58.3 | 6 | 796 | 6 | 2816 |
| 1.5 | Soil | Deep soil | 36°14'34.0 | 074°26'57.2 | 18 | 790 | 6.5 | 2821 |
| 1.6 | Pond | Stagnant water | 36°14'34.8 | 074°26'56.7 | 15 | 793 | 6 | 2841 |
| Juglot Glacier | | | | | | | | |
| 2.1 | Water | Lake water | 35°41'06.2 | 074°37'26.2 | 4 | 795 | 6 | 1983 |
| 2.2 | Soil | Surface soil | 35°41'06.5 | 074°37'26.2 | 15 | 790 | 6.5 | 1988 |
| Jutial Glacier | | | | | | | | |
| 3.1 | Ice | Lake ice | 35°5427.6 | 074°19'84.1 | -1 | 784 | 5.5 | 1511 |
| 3.2 | Water | Lake Water | 35°5427.3 | 074°19'84.4 | 6 | 785 | 6 | 1510 |
| 3.3 | Soil | Deep soil | 35°5426.7 | 074°19'83.7 | 0 | 784 | 6 | 1510 |

### *Sampling of psychrotrophic bacteria*

Soil, ice and water samples were obtained from glaciers (Juglot, Jutial and Rakaposhi) in Northern Areas of Pakistan (Table 3). On average, the glaciers were found at 35˚36 and 74˚27 at globe with average physical parameters as: height, 3000 m; temperature range, -3 to 18°C; atmospheric pressure, ~790 mb and pH 5.5 to 10.

### *Isolation and screening of cold active protease producing bacteria*

The twenty Glacier samples collected were treated by serial dilution and spread plate scheme for the quarantine of psychrotrophic bacteria. 20 bacterial strains were isolated on the basis of clear zone of proteolysis. All strains were screened primarily for cold active alkaline protease production on 1% skim milk agar plate technique (Abirami et al., 2011; Geethanjali and Subash, 2011; Sevinc and Demirkan, 2011; Smita et al., 2012; Sinha et al., 2013). Amongst 20 strains, eight (08) isolates were capable of exhibiting significant zone of proteolysis on 1% skim milk agar plate as given in Table 4. Among them, one strain *Stenotrophomonas* sp. MG662181 (PAK-01) demonstrated maximum zone of clearance. This cold adapted strain was identified and exploited for further findings. Willsey et al. (2015) investigated nine bacterial isolates from the potable water system and checked the impact of individual community member absence on the resulting community production of exoenzymes (extracellular enzymes) involved in lipid and protein hydrolysis.

**Table 4:** *Determination of zone of proteolysis by isolated strains.*

| Isolates | Zone of proteolysis (mm) |
|---|---|
| PAK-01 | 25 ± 1.25 |
| PAK-02 | 17± 1.12 |
| PAK-03 | 13± 0.82 |
| PAK-04 | 17± 1.2 |
| PAK-05 | 20± 1.21 |
| PAK-06 | 13± 0.74 |
| PAK-07 | 14±0.86 |
| PAK-08 | 19±0.72 |
| ATCC-13637 (control) | 18± 1 |

### *Screening and isolation of cold active lipase producing bacterial strain*

Different samples collected from glaciers were managed for quarantine of a maximum lipolytic bacterial strain by means of qualitative screening. Phychrotrophic bacterial strains on nutrient agar plates were exposed on TBA plates assay as qualitative assessment for maximum lipolytic bacterial strains (Kempka et al., 2008; Kim et al., 2001). The 20 psychrotrophic bacterial strains were tested for zone of hydrolysis on tributyrin agar (TBA) assay at temperature of 20 °C at pH 9. Among the 20 tested bacterial strains, five (05) strains were capable to demonstrated lipolytic

activity on tributyrin agar (TBA) plate as compared to the control strain ATCC 13637 as given in Table 5. The maximum zone of lipolysis demonstrated by the isolate *Pseudomonas* sp. PAK-03 (MG687270) on Tributyrin Plate (TBA) Assay is illustrated in Figure 2. Size of cleared zone by PAK-03 on TBA plate assay was increased from 2 to 8 mm as incubation period was increased from 24 to 48 hrs.

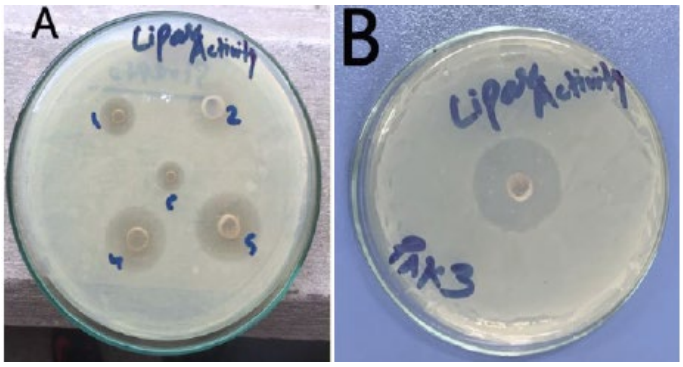


**Figure 2:** *TBA plate showing zone of hydrolysis of tested bacterial strains. A) Samples 1, 2, 4 and 5 are bacterial strains with lipolytic activity. Sample C is control one. B) Sample PAK-03 showing maximum zone of hydrolysis of Pseudomonas peli PAK-03.*

**Table 5:** *Lipolytic activity in terms of zone of hydrolysis (in mm) in tributyrin agar (TBA) plate.*

| Isolates | Zone of Lipolysis (mm) |
|---|---|
| PAK-01 | 3± 0.9 |
| PAK-02 | 2± 0.35 |
| PAK-03 | 8± 0.6 |
| PAK-04 | 6± 0.55 |
| PAK-05 | 7±0.94 |
| ATCC-27853 (control) | 2+0.46 |

Isolate PAK-03 showing cleared zone on TBA assay might be due to the esterase producer. Tributyrin agar plate enzyme assay is a common technique for quantifying lipase hydrolysis by mean of the appearance of haloes degradation on growth media supplemented with tributyrin as substrates (Selvin et al., 2012; Prasad, 2014). For lipase screening and production, Kumar et al. (2012) isolated *Bacillus* sp. strain DVL2 from common city garbage using the tributyrin as substrate in Karnal district of Haryana, India. They used three production media viz, PM1, PM2 and PM3 for lipase/esterase production. They observed maximum intracellular (112 IU/L) and extracellular (33 IU/L) lipase production in production medium 2 after 24 and 36 hours, and greater production of esterase was noticed in production medium 2 after 24 hours. Their results showed that the lipase/esterase from DVL2 strain of *Bacillus* sp. esterifies stearic acid with ethanol that causes the formation of ethyl stearate and were confirmed by thin layer chromatography. In a study, Soleymani et al. (2017) used *Bacillus sp. ZR-5,* for maximum lipase production by optimizing culture media compositions using "one variable at a time" strategy. They showed a significant increase in lipase activity with usage of low-cost sources. In other study, oil-contaminated soil samples were screened in Malaysia to assess and promote the growth of lipolytic bacteria for enzyme production with olive oil as the sole carbon source (Abd Rahman et al., 2007).

### *Identification of the isolates*

Many bacterial strains are widely exploited for the purpose of protein production due to the very high product production, immense fermentation nature, and the very low level of toxic by-product. *Stenotrophomonas* sp. and *Pseudomonas* strains also produce a large amount of the alkaliphilic proteases and lipase which have high significant proteolysis and lipolysis activity and are stable at high pH and low temperature.

In this study, maximum cold active protease and lipase secreting bacterial strains were selected depending on the highest zone of proteolytic and lipolytic hydrolysis. From the experiment, it was observed that the psychrotrophic bacterial strains PAK-01 and PAK-03 both exhibited largest zone of hydrolysis of 25± 1.25 mm and 8± 0.6 mm as compared to other strains and these strains were designated for further study.

### *Morpho-physiological and biochemical characterization*

The 16S rDNA-based sequencing is the better and efficient approach for identification of bacteria. Nevertheless, the traditional method of biochemical characterization and Gram staining does not only aid in the identification of bacteria, but also provides information on the extracellular secretions of the bacteria (Lee et al., 2015). Results from the Morpho-physiological and biochemical characterization studies such as catalase, voges-proskauer, oxidase, starch hydrolysis, gram staining, acid formation from sucrose, methyl red, Hydrogen sulfide and urease, Sugar utilization test, Nitrogen utilization, optimum pH and temperature and respiration is presented in Table 6.

On the basis of morphological and biochemical observations, the selected potential strains were identified and classified as *Stenotrophomonas* sp.

(PAK-01) and *Pseudomonas peli* (PAK-03) due to maximum protease and lipase catalytic activity as compared to other diverse isolates. These identified potent strains were utilized for future research.

**Table 6:** *Morpho-physiological and biochemical properties of glacial Isolates.*

| Characteristics | PAK03 | PAK01 |
|---|---|---|
| Color | white | white |
| Margin | smooth | smooth |
| Gram's staining | - | - |
| Shape | rod | rod |
| Spore | - | - |
| Motility | - | - |
| Oxygen utilization | aerobic | aerobic |
| pH range | | |
| Lower limit | 4 | 4 |
| Upper limit | 11 | 11 |
| Temperature limits | | |
| Lower limit (°C) | 2 | 4 |
| Upper limit (°C) | 30 | 30 |
| Biochemical tests | | |
| Catalase | + | + |
| Gelatinase | + | + |
| Methyl Red | - | - |
| Nitrate Reductase | + | - |
| Simmon citrate | + | - |
| Triple sugar Iron | - | + |
| Urease | - | - |
| Voges-Proskauer | - | - |
| oxidase | + | - |
| Citrate utilization | + | - |
| Hydrolysis of casein | - | + |
| Hydrolysis of Tween 80 | + | - |
| Acid formation from sucrose | - | - |
| Formation of indole | - | - |
| Acid formation from glucose | - | - |
| Hydrogen sulfide | - | - |
| OF (Oxidation/Fermentation) test | Oxidative | - |
| b-Galactosidase | + | + |
| Arginine dehydrogenase | + | - |
| Coagulase | - | - |

### *Molecular Identification of glacial isolates*

Molecular procedures exploit target DNA amplification to provide alternating methods for analysis and identification (Kurtzman and Robnett, 1997). The cold-active protease and lipase producing potential bacterial strains were also identified on the basis of sequences of their 16S ribosomal RNA gene sequence technique. The sequences were BLAST searched in NCBI GenBank database (MIDILABS Inc., USA) and the sequences based upon genetic signatures and physiological parameters, our strain exhibited less than 79% of homology for both *Stenotrophomonas* sp., (Nakagawa et al., 2003) and *Pseudomonas* sp. was recorded (Gupta et al., 2008), which renders it a novel species.

The phylogenetic tree was constructed with the help of MEGA 7 software program (Tamura et al., 2013). Conclusive sequences of PAK-01 and PAK-03 (Zhang et al., 2009) was submitted to the NCBI GenBank under the accession numbers of MG662181 for *Stenotrophomonas* sp. (PAK-01) and MG687270 for *Pseudomonas peli* (PAK-03).

### *Phylogenetic studies of glacial strains*

The 16S rDNA-based sequencing is the better and efficient approach for identification of bacterial strains (Lee et al., 2015). The details of 16S rDNA-based sequencing for the two isolated bacterial strains are given below.

### *Stenotrophomonas sp. MG662181*

On the basis of BLAST search, it was observed that *Stenotrophomonas* sp. MG662181 has very intimate sequence resemblance with *Stenotrophomonas maltophilia* strain (KP858919) with 78% sequence analysis with e-value 0.0, which renders it a novel species.

Highest parsimony exploration was accomplished with 1000 bootstrap replications, with the numbers presented at the nodes and two evolutionary steps were signified by the scale bar (Figure 3).

### *Pseudomonas sp. MG687270*

Based on BLAST search, it was observed that *Pseudomonas peli* (MG687270) has very intimate sequence resemblance with *pseudomonas anguilliseptica* (AF439803) and *Pseudomonas cuatrocienegasensis* (JN64459) with 78% and 79% sequence analysis with e- value 0.0. Highest parsimony exploration was accomplished with 1000 bootstrap replicates, with the numbers presented at the nodes and two evolutionary steps were signified by the scale bar (Figure 4).

The secretion and the function of the enzymes in soil produced by bacterial species are the matter

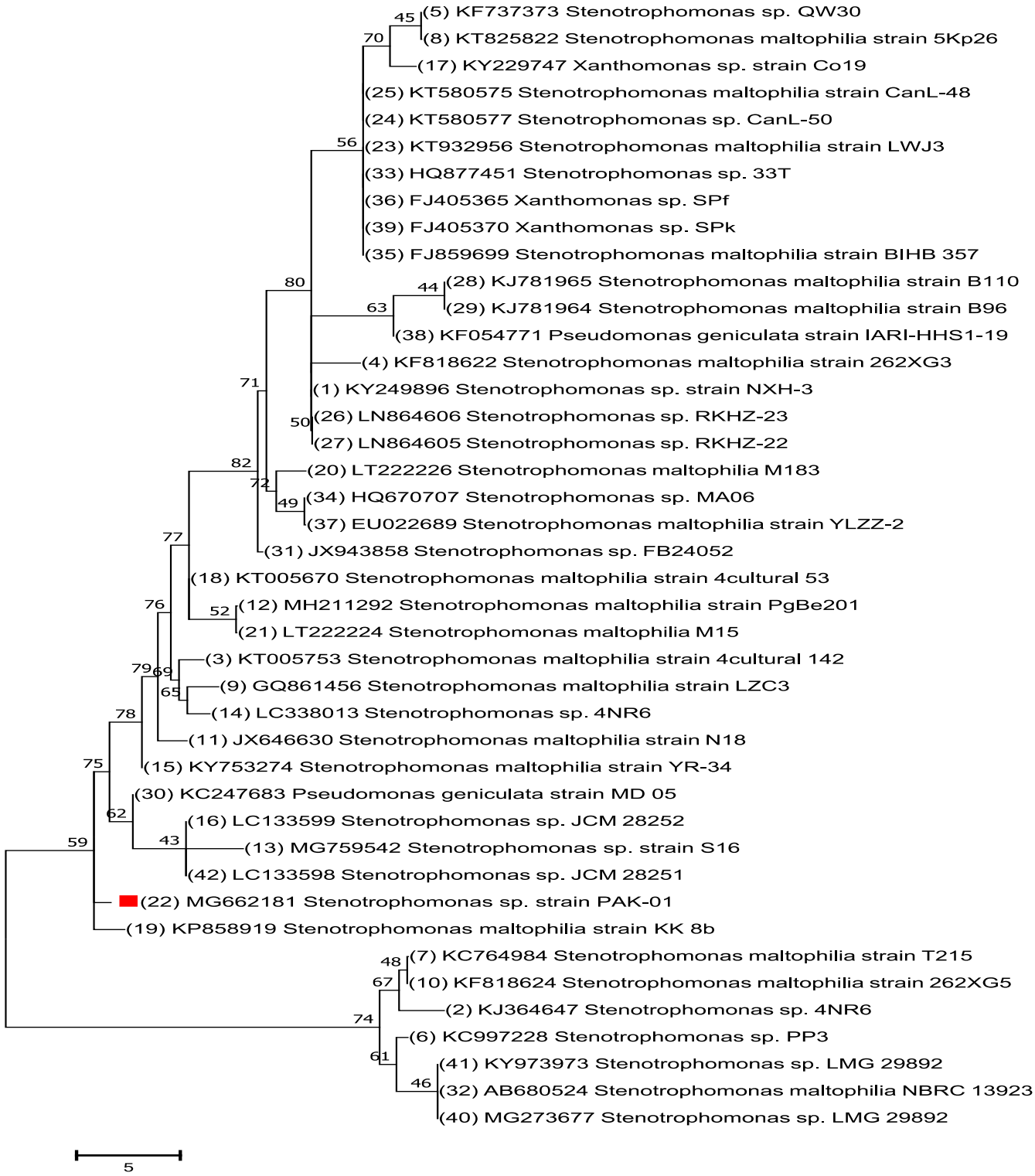


**Figure 3:** *Phylogenetic tree of Stenotrophomonas sp., PAK-01 based on 16S rRNA sequence. The red tag shows position of Pseudomonas peli PAK-03 with other bacterial species.*

of great interest for researchers (Wallenstein and Weintraub, 2008) which has got a rapid attention with the advancements in analytical and molecular techniques. As the environment is very harsh for enzymes stability, the potentially produced enzymes must be able to show stability against the denaturation effect. Henceforth, it encouraged more interest to have better understanding of the enzymes function and activity. A deeper look over the properties and function of enzymes also got practical applications (Lee et al., 2015). Our study shows that the soil and water from Glaciers are better sources to isolate lipase and protease producing microorganisms. With the tremendous potential of lipases and proteases in industrial applications, persistent and extensive screening for new sources of lipases with different

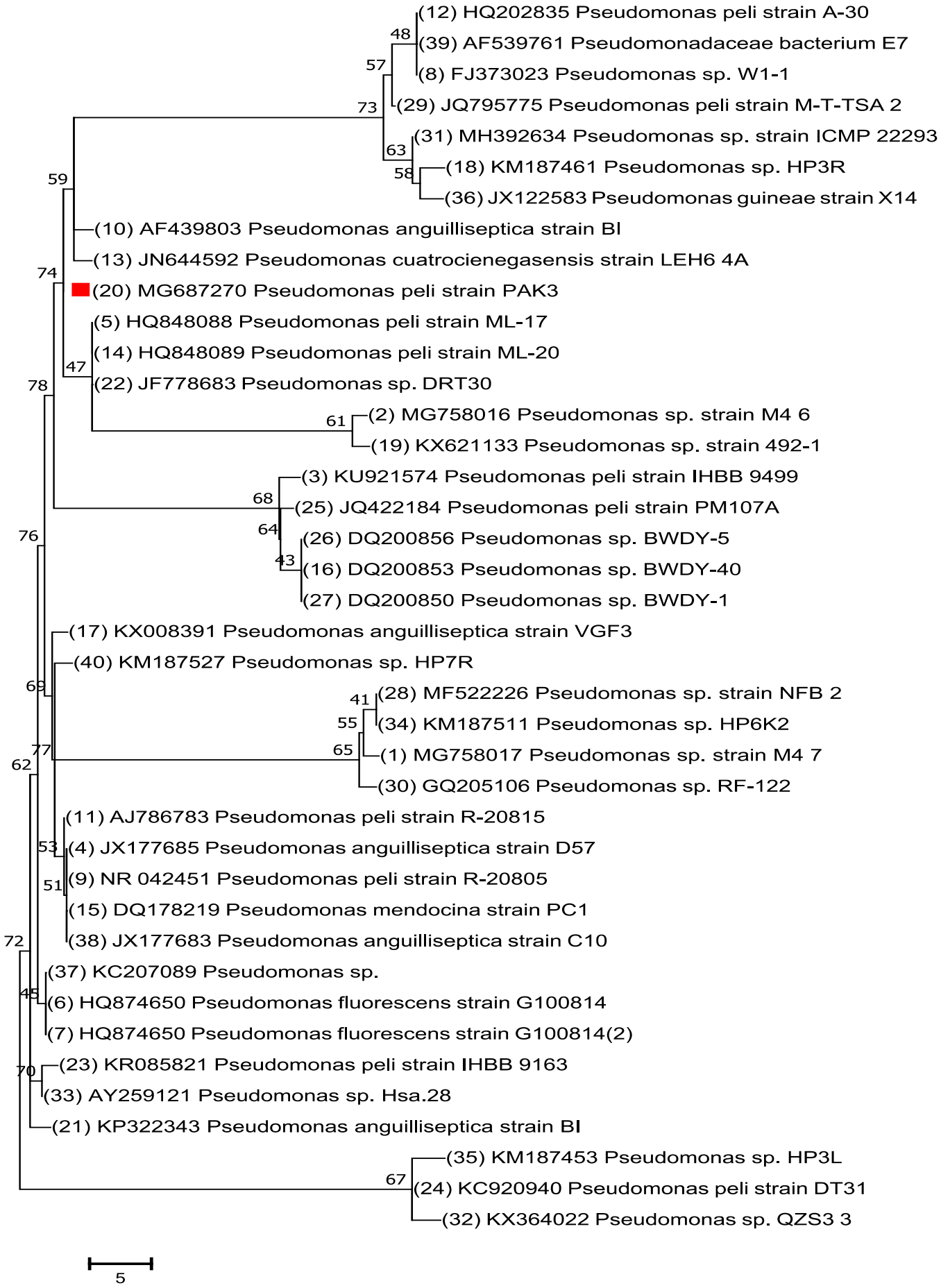


**Figure 4:** *Phylogenetic tree of Pseudomonas peli PAK-03 based on 16S rRNA sequence. The red tag shows position of Pseudomonas peli PAK-03 with other bacterial species.*

catalytic characteristics could be a matter of the highest importance.

## Conclusions and Recommendations

Protease and lipase naturally occurring in almost every organism and they are the fundamental constituent for all of the live forms. Microbes such as bacteria are the principal source of protease and lipase biocatalysts. The *Stenotrophomonas* sp and *Pseudomonas peli* identified and isolated from Glacier soil and water samples could be exploited for cold adapted alkaline protease and lipase production as these biocatalysts has an enormous application as an additive in bio-detergent industries. Under certain environmental conditions, the lipase and protease is higher in microbes. This is because of the encoding genes higher expression, that increases the level of transcripts and gives higher production of protease or lipase enzymes. Further research with the focus on identification and characterization of small RNAs or transcriptional factors or with great expression of the lipase-/protease-encoding genes from different bacterial species is necessary.

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## Novelty Statement

Two bacterial strains (PAK01 and PAK03) have been reported here as novel species. Protease and lipase enzymes produced from these species has shown as competitive candidates in industrial applications.

## Author's Contribution

**Yasir Ali:** Prepared the first draft of the manuscript. Performed field survey to collect samples. Conducted experimental work. Analyzed data and interpreted the results.

**Bashir Ahmad:** Designed and supervised the research. Provided scholarly guidance. Also wrote the manuscript.

**Naqeebullah Jogezai:** Helped in experimental work. Wrote the parts of manuscript and settled the references style and provided technical assistance to keep the manuscript on track.

**Adil Hussain:** Helped in field surveys to collect samples. Provided assistance in lab experimentation. Helped in writing manuscript and interpretation of the results, literature review, citations and manuscript editing.

## References

Abd Rahman, R.N., T.C. Leow, A.B. Salleh and M. Basri. 2007. *Geobacillus zalihae* sp. nov., a thermophilic lipolytic bacterium isolated from palm oil mill effluent in Malaysia. BMC Microbiol. 7(1): 77-86. https://doi.org/10.1186/1471-2180-7-77

Alquati, C., L.D. Gioia, G. Santarossa, L. Alberghina, P. Fantucci and M. Lotti. 2002. The cold-active lipase of *Pseudomonas fragi*. Eur. J. Biochem. 269: 3321–3328. https://doi.org/10.1046/j.1432-1033.2002.03012.x

Abebe, B., A. sago, G. Admasu, H. Getachew, P. Kassa and M. Amsaya. 2014. Isolation, Optimization and characterization of protease producing bacteria from soil and water in Gondar town, Northwest Ethiopia. Int. J. Bacteriol. Virol. Immunol. 1(3): 020-024.

Abirami, V., S.A. Meenakshi, K. Kanthymathy, R. Bharathidasan, R. Mahalingam, A. Panneerselvam. 2011. Partial purification and Characterization of an Extracellular protease from *Penicillium janthinellum* and *Neurospora crassa.* Eur. J. Exp. Biol. 1(3): 114-123.

Akcan, N. and F. Uyar. 2011. Production of Extracellular alkaline protease from *Bacillus subtilis* RSKK96 with solid state fermentation. Asian J. Biosci. 5: 64-72. https://doi.org/10.5053/ejobios.2011.5.0.8

Geethanjali, S. and A. Subash. 2011. Optimization of Protease production by *Bacillus subtilis* isolated from mid gut of fresh water fish *Labeo rohita*. World J. Fish Mar. Sci. 3(1): 88-95.

Grbavcic, S., D. Bezbradica, L. Zivkovic, N. Avramovic, N. Milosavic, I. Karadzic and Z.K. Jugovic. 2011. Production of lipase and protease from an indigenous Pseudomonas aeruginosa strain and their evaluation as detergent additives: Compatibility study with detergent ingredients and washing performance. Biores Technol. 102: (24): 11226-11233. https://doi.org/10.1016/j.

biortech.2011.09.076

Gupta, S.K., R. Kumari, O. Prakash and R. Lal. 2008. *Pseudomonas panipatensis* sp.nov., isolated from an oil-contaminated site. Int. J. Syst. Evol. Microbiol. 58(6): 1339–1345. https://doi.org/10.1099/ijs.0.65401-0

Jinyong, Y., X. Zheng, L. Du and S. Li. 2014. Integrated lipase production and in situ biodiesel synthesis in a recombinant *Pichia pastoris* yeast: An efficient dual biocatalytic system composed of cell free enzymes and whole cell catalysts. Biotechnol. Biofuels. 7(1): 55.

Joseph, B., P.W. Ramteke and G. Thomas. 2008. Cold active microbial lipases: some hot issues and recent developments. Biotechnol Adv. *26*: 457-470. https://doi.org/10.1016/j.biotechadv.2008.05.003

Kempka, A.P., N.L. Lipke, T.D.L.F. Pinheiro, S. Menoncin, H. Treichel, D.M. Freire, M. Di Luccio and D. de Oliveira. 2008. Response surface method to optimize the production and characterization of lipase from *Penicillium verrucosum* in solid-state fermentation. Bioprocess Biosyst. Eng. 31(2): 119-125. https://doi.org/10.1007/s00449-007-0154-8

Kim, E.K., W.H. Jang, J.H. Ko, J.S. Kang, M.J. Noh and O.J. Yoo. 2001. Lipase and Its modulator from *Pseudomonas* sp. Strain KFCC 10818: Proline-to-glutamine substitution at position 112 induces formation of enzymatically active lipase in the absence of the modulator. J. Bacteriol. 183(20): 5937-5941. https://doi.org/10.1128/JB.183.20.5937-5941.2001

Kumara, H., Y.S. Priyab, M. Kumarc and V. Elangovand. 2015. SEM, FTIR and biochemical characterization of bacterial pathogens causing leaf blight in mulberry leaves. Int. J. Curr. Sci. 15: 15-21.

Kumar, D., L. Kumar. S. Nagar. C. Raina. R. Parshad and V.K. Gupta. 2012. Screening, isolation and production of lipase/esterase producing *Bacillus* sp. strain DVL2 and its potential evaluation in esterification and resolution reactions. Arch. Appl. Sci Res. 4 (4): 1763-1770.

Kurtzman, C.P. and C.J. Robnett. 1997. Identification of clinically important Ascomycetous yeasts based on nucleotide divergence in the 5' end of the large-subunit (26S) ribosomal DNA gene. J. Clin. Microbiol. 35(5): 1216-1223.

Lee, L.P., H.M. Karbul, M. Citartan, S.C. Gopinath, T. Lakshmipriya and T.H. Tang. 2015. Lipase-secreting Bacillus species in an oil-contaminated habitat: promising strains to alleviate oil pollution. Bio. Med. Res. Int. 1: 1-9. https://doi.org/10.1155/2015/820575

Margesin, R., G. Feller, C. Gerday and N. Russell. 2002. Cold-adapted microorganisms: Adaptation strategies and biotechnological potential. In: Bitton, G. (ed.), The encyclopedia of environmental microbiology. Wiley, New York, pp. 871–885.

Margesin, R., H. Dieplinger, J. Hofmann, B. Sarg and H. Lindner. 2005. A coldactive extracellular metalloprotease from *Pedobacter cryoconitis* - production and properties. Res. Microbiol. 156: 499-505.

Mohammed, H.J. 2013. Lipase activity of Acinetobacter baumannii 82 physicochemical factors affected the partial purified lipase activity of acinetobacterbaumannii "local isolates" Iraqi. J. Pharm. Sci. 22(1): 2-9.

Mongkolthanaruk, W. and P. Boonmahome. 2013. Lipase producing bacterium and its enzyme characterization. J. Life Sci. Technol. 1(4): 196-200.

Nakagawa, T., Y. Fujimoto, M. Uchino, T. Miyaji, K. Takano and N. Tomizuka. 2003. Isolation and characterization of psychrophiles producing cold-active b-galactosidase. Lett. Appl. Microbiol. 37(2): 154– 157. https://doi.org/10.1046/j.1472-765X.2003.01369.x

Prasad, M.P. 2014. Production of lipase enzyme from pseudomonas aeruginosa isolated from lipid rich soil. Int. J. Pure App. Biosci. 2: 77-81.

Rajeswari, K., R. Subashkumar and K. Vijayaraman. 2013. Decolorization and degradation of textile dyes by Stenotrophomonas maltophilia RSV-2. Int. J. Environ. Bioremed. Biodegrad. 1: 60-65.

Reiner, K. 2010. Catalase test protocol. American society for microbiology. Available at: http://www.microbelibrary.org/library/laboratory-test/3226-catalase-test protocol.htm

Sagar, K., Y. Bashir, M.M. Phukan and B.K. Konwar. 2013. Isolation of lipolytic bacteria from waste contaminated soil: a study with regard to process optimization for lipase. Int. J. Sci. Technol. 2(10): 214-218.

Selvin, J., J. Kennedy, D.P.H. Lejon, G.S. Kiran, D.W. Alan and A.D.W. Dobson. 2012. Isolation, identification and biochemical characterization of a novel halo-tolerant lipase from the metagenome of the marine sponge *Haliclona simulans*. Microb. Cell Fact. 11(1): 72. https://doi.org/10.1186/1475-2859-11-72

Sevinc, N. and E. Demirkan. 2011. Production of protease by *Bacillus* sp. N-40 isolated from soil and its enzymatic properties. J. Biol. Environ. Sci.

5(14): 95-103.

Shah, M.P. 2014. Exploring the strength of pseudomonas putida ETL-7 in Microbial Degradation and Decolorization of Remazol Black-B. Int. J. Environ. Bioremediat. Biodegrad. 2(1): 12-17.

Sharma, A.K., V. Sharma, J. Saxena, B. Yadav, A. Alam and A. Prakash. 2015. Optimization of protease production from bacteria from soil. App. Res. J. 1: 388-394.

Shields, P. 2010. Cathcart L. Oxidase test protocol. American Society for Microbiology. Available at: http://www.microbelibrary.org/library/laboratory-test/3229-oxidase-test-protocol.htm

Singh, K., H. Bose, K. Richa, L. Karthik, K. Gaurav and K.V.R. Bhaskara. 2012. Isolation and characterization of protease producing marine eubacteria. J. Agric. Technol. 8(5): 1633-1649.

Sinha, P., R.K. Singh, R. Srivastva, R. Sharma, P. Tiwari. 2013. Characterization and optimization of alkaline protease enzyme produced by soil borne bacteria. Trends Life Sci. 2(2): 2319–4731.

Smita, G.S., P. Ray and S. Mohapatra. 2012. Quantification and pptimisation of bacterial isolates for production of alkaline protease. Asian J. Exp. Biol. Sci. 3(1): 180-186.

Soleymani, S., H. Alizadeh, H. Mohammadian, E. Rabbani, F. Moazen, H.M. Sadeghi, Z.S. Shariat, Z. Etemadifar and M. Rabbani. 2017. Efficient media for high lipase production: One variable at a time approach. Avicenna J. Med. Biotechnol. 9(2): 82–86.

Soror, S.H., V. Verma, R. Rao, S. Rasool, S. Koul, G.N. Qazi and J. Cullum. 2007. A cold-active esterase of Streptomyces coelicolor A3(2): from genome sequence to enzyme activity. J. Ind. Microbiol. Biotechnol. 34: 525-531.

Tamura, K., G. Stecher, D. Peterson, A. Filipski and S. Kumar. 2013. MEGA6: Molecular evolutionary genetics analysis version 6.0. Mol. Biol. Evol. 30(12): 2725–2729. https://doi.org/10.1093/molbev/mst197

Veerapagu, M., A.S. Narayanan, K. Ponmurugan and K.R. Jeya. 2013. Screening selection identification production and optimization of bacterial lipase from oil spilled soil. Asian J. Pharm. Clin. Res. 6(3): 62-67.

Veerapagu, M., A. Sankara, K. Jeya and S. Alagendran. 2014. Isolation and identification of a novel lipase producing bacteria from oil spilled soil. Int. J. Innov. Res. Sci. Eng. Technol. 3(5): 111-119.

Urszula, G., G. Izabela, W. Danuta and L. Sylwia. 2009. Isolation and characterization of a novel strain of Stenotrophomonas maltophilia possessing various dioxygenases for monocyclic hydrocarbon degradation. Braz. J. Microbiol. 40(2): 285-291. https://doi.org/10.1590/S1517-83822009000200014

Wallenstein, M.D. and M.N. Weintraub. 2008. Emerging tools for measuring and modeling the in-situ activity of soil extracellular enzymes. Soil Bio. Biochem. 4: (9) 2098–2106. https://doi.org/10.1016/j.soilbio.2008.01.024

Willsey, G.G. and M.J. Wargo. 2015. Extracellular lipase and protease production from a model drinking water bacterial community is functionally robust to absence of individual members. PLoS One. 10(11): e0143617. PLoS One. 10(11): e0143617. https://doi.org/10.1371/journal.pone.0143617

Zhang, A., R. Gao, N. Diao, G. Xie, G. Gao and S. Cao. 2009. Cloning, expression and characterization of an organic solvent tolerant lipase from *Pseudomonas fluorescens* JCM5963. J. Mol. Catal. B. Enzym. 56(2-3): 78-84. https://doi.org/10.1016/j.molcatb.2008.06.021