

## Isolation, Partial Purification and Characterization of a Cold Active Lipase from *Pseudomonas* sp., Isolated from Satopanth Glacier of Western Himalaya, India

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**Abstract:** A Psychrotrophic bacterium producing cold active lipase enzyme at low temperature was isolated from soil sample of Satopanth glacier. It was identified as *Pseudomonas fluorescens* based on colony morphology, biochemical characteristics. Out of the seven selected lipase producing isolates the best one SGPR-4 was showing activity maximum at 20° C temperature. The cold active lipase was purified to 6.44 fold by DEAE cellulose column chromatography. The bacterial strain SGPR-4 showed maximum activity at pH 8.0 and temperature 20°C and it showed thermostability up to 30°C. Therefore these properties of enzyme could be explored for industrial application and commercialization.

**Keywords:** Lipase, Satopanth Glacier, Tributyrin agar, Psychrotrophic

### 1. Introduction

Extremophiles are microorganisms, which belong to the domains Archaea and Bacteria. They thrive in environmental conditions considered by human standards to be extreme and the enzymes they produce are called extremozymes, which show utmost stability under extreme conditions [1], [2]. The earth's biosphere is largely adapted to extreme conditions below the zero temperatures in the polar ice area, to boiling temperature in the hydrothermal valves [3], [4]. More than three-quarters of our planet is comprised of environments which experience extremely low temperatures ( $\leq 15^{\circ}\text{C}$ ) therefore, that the majority of Earth's biomass is generated at temperatures below 5°C [5]. These kinds of environment sustain a wide diversity of microorganisms, which naturally thrive in cold environments, known as psychrophilic microorganisms. Microbial Adaptation of the catalytic, structural and regulatory functions of the protein to extreme conditions are of particular interest both theoretically and practically opinions. The survival at extreme environmental conditions involves the optimization of enzymatic tools allowing metabolic rates that are able to exist at low or high temperatures, of lipid-rich habitats and enzymes from psychrophilic organisms have high catalytic activity at low temperatures and low thermal stability [6]. Consequently, much attention has been given to the microorganisms that are able to thrive in cold environments such as glacier regions. Thus, biocatalysis using psychrotrophs as well as cold adapted enzyme is rapidly being transformed from an academic science to an industrially viable technology.

Lipases were first discovered in 1856 by Claude Bernard when he studied the role of the pancreas in fat digestion [7]. Lipase (Triacylglycerol hydrolases EC 3.1.1.3) is an enzyme able of hydrolyzing lipids into fatty acids and glycerol [8]. Several reports have been shown that lipase have emerged as an significant enzyme in the fast-growing biotechnologies for numerous properties that may find use in a wide range of industrial applications, such as food technology, pharmaceuticals, chemical industry and biomedical sciences agrochemicals, bio surfactants and bioremediation etc [9-12]. It has emerged as one of the leading biocatalysts with proven potential to contribute billions of dollars to be used in the biotechnology industry [13]. Precious finding of Tripathi [14] Lipase producing bacteria was isolated from the pulp and paper mill and identified as *Microbacterium* sp., can be a good source for the production of lipase enzyme to be used in transesterification reaction for biodiesel production.

Cold-adapted bacteria produce lipases which work effectively at low temperatures, with high rates of the catalysis compared to mesophilic or thermophilic lipases show little or no activity at low temperatures. In addition, the maximum level of activity of the lipase is shifted to lower temperatures and reduced thermal stability. In past a number of cold active lipase from various sources

have been studied in recent years such as *Candida Antarctica*, *Bacillus subtilis* and *Pseudomonas fluorescens* [15-16]. Microbial lipases are more useful than enzymes derived from plants and animals, since they have great variety of catalytic activities and microorganisms are easy to manipulate genetically and capable of rapid growth on inexpensive media.

Satopanth glacier is the source of the Alaknanda River, a major tributary of Ganga. It lies in the North West side of Nilkantha a major peak of the Garwal division of the Himalayan. The glacier spreads over a large area of 13 km long with an average width of 750 m, covering an area of 21.17 km [17]. Cold active enzymes from cold adapted bacteria play a major role in biodegradation of organic matter in Gangotri glacier and which are active in cold active region [18]. In view of the above characteristic and their applications in numerous commercial processes, the present study was carried out with an objective to isolate and characterize lipase producing microorganisms from soil samples of Satopanth glacier.

## **2. Materials and Method**

### **2.1 Sample collection and isolation of cold active lipase producing bacteria**

Soil samples were collected from different niches of Satopanth glacier (latitude 30°42'-30°50'N and longitude 79°13'-79°29'E, and altitude 4,600 m) located in western region of Himalaya, India. The samples were collected aseptically from different sites and depths and immediately transported intact at ambient temperature in sealed polyethylene bags for further processing at laboratory. For screening of lipolytic bacteria, serially diluted samples were spread on tributyrin agar plate (TBA). The colonies showing a clear zone formed by the hydrolysis of tributyrin after 48 h of incubation at 20°C was selected for further studies [19]. Bacterial colony, showing largest zone, was isolated by repeated pure culture technique and selected for further studies. Stock cultures were maintained as 50% glycerol stocks at 20°C.

### **2.2 Identification of isolates**

The selected isolates was completely identified and characterized based on morphological and biochemical characteristics. It was followed by enzyme production and partial purification [20]. Evolutionary analyses were conducted in MEGA 6 [21].

### **2.3 Lipase activity assay**

Lipase activity was determined using p-nitrophenylpalmitate (p-NPP) substrate as described in 1979 by Winkler and Stuckmann [22]. In brief, the substrate was prepared in phosphate buffer (90 ml) containing gum arabic (100 mg) and sodium deoxycholate (207 mg). The substrate p-NPP (30 mg) was dissolved in 10 ml of isopropanol and mixed with buffer solution. Freshly prepared p-NPP substrate solution (2.4 ml) was mixed with 0.1 ml of sample, pre-incubated at 15°C for 15 min along with a control (without enzyme). The release of p-nitrophenol (p-NP) was measured spectrophotometrically at 405 nm. One unit of lipase activity is defined as the amount of enzyme releasing 1 mol p-NP min<sup>-1</sup> under assay condition. Protein concentration was measured by using bovine serum albumin as a standard [23].

### **2.4 Enzyme production and partial purification**

The microorganism was grown at 20°C in twelve, 500 ml Erlenmeyer flasks containing 100 ml tributyrin broths (pH 8.0) for 24 h at incubator shaker of 150 rpm. The culture was centrifuged at 10,000 rpm for 10 min at 4°C to obtain cell free supernatant. Supernatant was subjected to ammonium sulphate fractionation of 0–40%, 40–60% and 60–80%. Ammonium sulphate fraction (60–80%) having maximum activity was dialyzed (Dialysis membrane Himedia) against glycine–NaOH buffer (pH 9.0–11.0) for 24 h at 4°C. Lipase activity was determined spectrophotometrically by measuring the amount p-nitro phenol palmitate as a substrate by following methodology of [24] with some modification.

### **2.5 Effect of pH and temperature on lipase activity and stability**

The crude enzyme used for assay was the culture broth after separation of cells and particles. The enzyme was normally stored at 4°C until used. To study the effect of temperature on the activity of the partially purified enzyme, assay reaction mixture was incubated at different temperatures ranging from 10 to 60°C for 15 min and activity was determined. To study the enzyme stability at a different temperature, purified enzyme was dissolved in 50 mM phosphate buffer (pH 8.0), pre-incubated at different temperatures ranging from 10 to 60°C for 1 h, rapidly residual activities were measured by the standard assay procedure. The

activity of the purified lipase at different pH values was measured adjusting pH of the reaction mixture using (0.1 M) of following buffers: citrate buffer (pH 5.0–6.5), Tris-chloride buffer (pH 7.0–9.0), glycine–NaOH buffer (pH 9.0–11.0). The enzyme activity was assayed by the method described before. To study the stability at different pH, purified lipase was dissolved in above-mentioned buffers. These enzyme solutions were preincubated at 15°C for 1 h and residual activity was measured at pH 8.0 enzymatic activity was measured according to a standard protocol with *p*NPP as the substrate.

### 3. Results and Discussion

#### 3.1 Screening and identification of lipolytic bacteria

Total Twenty-one bacterial colonies were isolated on nutrient agar media from seven soil samples from Satopanth Glacier, Western Himalaya. After that, seven isolates were screened for extracellular lipase production on tributyrin agar plate. A loopful of isolate was streaked into the tributyrin medium and incubated at two different temp 4°C and 20°C at pH 8.0. After incubation, four isolates (SGPR-2, SGPR-4, SGPR-9 and SGPR-15) were found to be capable of producing lipase at slight alkaline pH and at different temperature ranging from 4 to 20°C. Among them on the basis of zone diameter of hydrolysis at low temperature, i.e. 20°C one isolate was selected as potent lipase producing strain, designated as SGPR-4 Selected for further studies.

Colony morphology was circular, pulvinate, opaque and smooth in appearance while pigment is whitish in colour. The isolate studied was gram negative, aerobic, rod shaped, motile bacteria and was able to grow at 4–50°C and at a wide range of pH 5.0–10.0. It was positive for catalase, starch hydrolysis, citrate utilization, casein hydrolysis, activities. Positive carbon sources were galactose, dextrose, sucrose while showing variable activities for glucose. The bacterial isolate was negative for methyl red, VP test and hydrogen sulfide production and assimilation for arabinose, maltose (Table. 1) Thus, on the basis of its characteristics the isolate SGPR-4 was identified as *Pseudomonas*. The almost complete 16SrRNA gene was sequenced and the analysis clearly demonstrated that strain SGPR-4 was a member of the genus *Pseudomonas* and it was found to have 95- 99% identity with different strains of *Pseudomonas*. Among them, it showed maximum similarity (98.98%) with *Pseudomonas fluorescens* strain CIP 105469. After 16SrRNA sequencing it has been reported that large number of cold adapted bacteria by different researcher such as *Pseudomonas Vancouverensis*, *Pseudomonas ficuserectae*, *Cryobacterium psychrophilum*, *Pseudoalteromonas* sp. from Antarctic marine, glaciers, arctic and polar region that producing lipase enzymes [25-27].

The objective of purification was to get rid of unwanted protein, while retaining the enzyme activity. Most purification schemes for lipases are based on multi step strategies. Cold active lipase (1200 ml) was partially purified by precipitating with ammonium sulfate (60-80%) and using a single step ion-exchange chromatography on a DEAE-cellulose (Table 2). Partially purified lipase was eluted out as fractions (with 0.1–1M NaCl gradient) from DEAE-cellulose column with 6.44 fold purification and specific activity of 120.47 U/mg. In the present study, the yield of cold active lipase from *P. fluorescence* is 9.10%, low after partial purification. Lipase production by psychrotrophs varies with species, as does the optimum temperature, optimum pH and enzyme specificity [28]. In similar study of [29] reported an extracellular lipase from *Pseudomonas aeruginosa* KKA-5 was purified using ammonium sulphate precipitation and successive hydroxyl aptite chromatography and the enzyme was purified 518 fold. In reference to mesophilic enzyme lipase from psychrophiles was partially purified with 17.74-fold purification and specific activity of 3,244.44 U mg<sup>-1</sup> [30]. The presence of lipases has been noticed earlier for *B. pyocyaneus* and *B. fluorescens* which are now called *Pseudomonas aeruginosa* and *Pseudomonas fluorescens*, respectively [31-32].

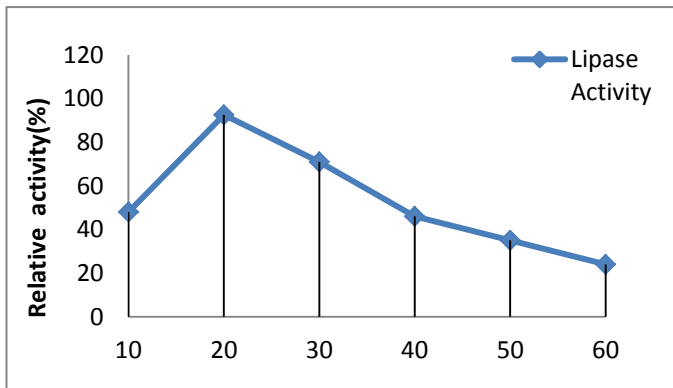
**Table 1: Morphological, physiological and biochemical characteristics of the isolate SGPR-4**

<b>Morphological Tests</b>	<b>SGPR-4</b>
<b>Colony morphology</b> Configuration, Surface, Margin, Elevation, Surface, Pigment, Opacity, Density, Size	Round, Smooth, Undulate, Pulvinate, Whitish, Opaque Translucent, Short
<b>Gram's reaction</b>	Negative
Cell shape	Rod
<b>Spore(s)</b>	-
Motility	Motile
<b>Physiological tests</b>	
<b>Growth at temp.</b>	
4°C	+
10°C	++
20°C	+++
30°C	++
40°C	+/-
50°C	-
<b>Growth at pH</b>	
pH 5.0	+
pH 6.0	+
pH 7.0	+
pH 8.0	++
pH 9.0	+
pH 10.0	-/+
Growth under anaerobic condition	-
<b>Biochemical Tests</b>	
Methyl red test	-
Voges -Praoskauer Test	-
Citrate utilization	+
Starch hydrolysis	+
H <sub>2</sub> S production	+/-
Catalase test	+
Casein hydrolysis	+
<b>Acid production from carbohydrates:</b>	
Arabinose, Maltose	-
Sucrose, Galactose, Dextrose	+
Glucose	+/-

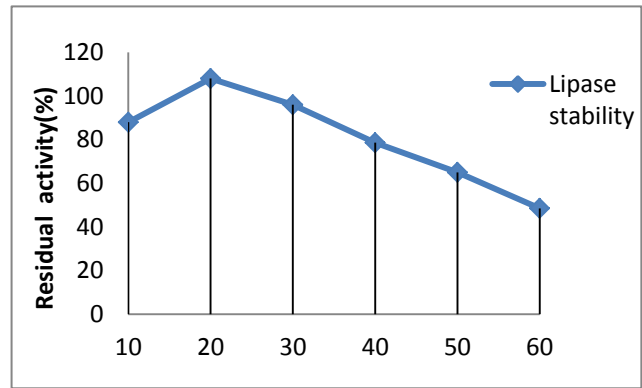
(+ Growth, ++ Moderate growth, +++ Strong growth, – negative, +/- variable)

**Table 2: Partial purification summary of lipase from *Pseudomonas* sp., SGPR-4**

<b>Purification</b>	<b>Total protein (mg)</b>	<b>Total activity (U)</b>	<b>enzyme activity (U/mg)</b>	<b>Specific activity (U/mg)</b>	<b>Purification fold</b>	<b>Yield (%)</b>
<b>Crude enzyme</b>	135.5	2532	18.68	18.68	1	100
<b>(NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> ppt. dialysis</b>	33.6	1730	51.48	51.48	2.75	24.79
<b>DEAE Cellulose</b>	2.1	253	120.47	120.47	6.44	9.10



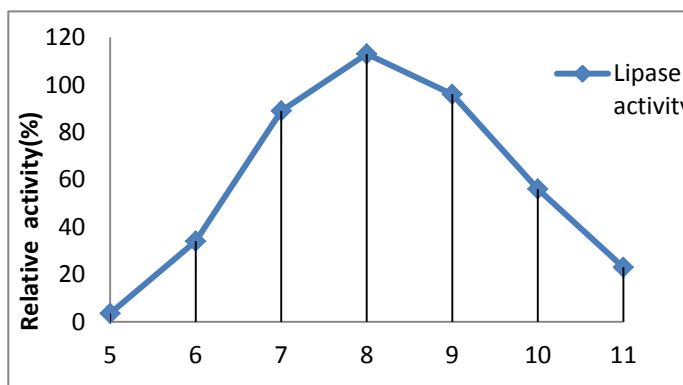
**Graph-1 Effect of temperature on enzyme activity**



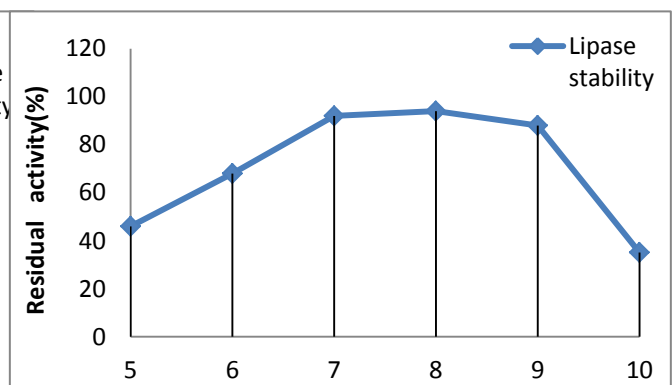
**Graph-2 Effect of temperature on enzyme stability**

There is a significant effect of temperature on cold active lipase production. The temperature of 20°C was found to be optimum to produce cold active lipase. Beyond the optimum temperature a sharp fall in the lipase production was observed. The potential application due to relatively high activity at low temperature could be used in detergent additives or for processing of volatile substances thereby making it possible to reduce temperature and thus bring down the energy costs. The activity of cold active lipase was determined at a wide range of temperature 10-60°C (Graph-1). The optimum temperature for lipolytic activity was determined to be 20°C. This marked liability of lipase together with its high catalytic efficiency near 20°C clearly denotes that it is a cold active enzyme. The enzyme activity was almost constant within 20°C -30°C and gradually declined at a temperature beyond 35°C. Similarly, cold active lipase from *Psychrobacter okhotskensis* completely lost its activity above 36°C [33].

Assessment of the thermostability of lipase was performed by measuring the residual activity at various times, following incubation at different temperatures. Thermal stability of cold active lipase was tested at different temperature ranging from 10°C to 60°C (Graph-2). The enzyme retained 88% and 96% of its maximum activity at 10°C and 30°C, respectively. The production of cold active lipase is considered temperature dependent and thermolabile [34]. Moreover in similar study the optimum temperature of the lipase from *P. fragi* and *P. mendoncina* were found to be optimally active at 35–45°C [35].



**Graph-3 Effect of pH on enzyme activity**



**Graph-4 Effect of pH on enzyme stability**

An optimum pH of 8.0 was determined for cold active lipase production from isolated strain. The data obtained indicated that there was strong influence of pH on enzyme production. The pH of medium strongly affects many enzymatic processes and transport of compounds across the cell membrane [36]. For instance, at pH 5.0 the lipase retained only 3.5% of its maximum activity and also retained 23% at pH 11.0. No activity was observed at pH 3.0 and pH 4.0. The highest lipase activity (113%) was found to be at pH 8.0 using phosphate buffer (Graph-3). Almost negligible activity was obtained in acidic range and even at

neutral pH. An increase in the enzyme activity in the alkaline range suggests that the enzyme is slightly alkaline in nature. The pH optima for the reactions catalyzed by *Pseudomonas cepacia*, *Pseudomonas pseudoalcaligenes*, and *Pseudomonas mendocina* enzymes were reported to be between 8 and 9 [37].

The stability of alkaline lipase was determined by preincubating the partially purified cold active enzyme in various buffers of different pH for 1 h. The optimum pH for the activity of the enzyme is 8.0 and it is stable over a broad range of pH 7.0–9.0 (Graph-4). Lipase obtained in our study was stable from pH 7.0 to 9.0. However, it was not stable at acidic pH range. In the previous study of Joseph et al., (2012) a bacterial strain *Microbacterium luteolum*, produced maximum lipase at 15°C, at a pH of 8.0. Therefore lipases performing high stability and activity over a wide range of pH and activity under non-traditional conditions are of great interest. The major commercial application for alkaline stable lipases is the use in laundry and household detergents. The current study demonstrated that *Pseudomonas* sp., SGPR-4 produced a new bacterial lipase was found to be cold-adapted, allowing potential application in cold washing, since psychrophilic enzymes have certain advantages under low temperature conditions.

#### 4. Conclusion

The present work has been an attempt to unravel the intact microbial biodiversity of cold active environment of Satopanth glacier in terms of biotechnological implications. Cold-adapted enzymes from microorganism living in deep-sea environments and cold regions have been found to exhibit both high activity and high stability a remarkable property that goes against the trend of substitution between activity and stability that has been observed for many enzymes. The production of cold active lipase by *Pseudomonas* owing to its low temperature activity and high stability attract special attention. Therefore these properties of enzyme could be explored in leather and detergent industry and one most promising aspect in biodegradation of waste. Further studies on these entire characteristic can improve commercial yield and also help in exploration of microorganism in extreme environment like Himalaya region.

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