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Isolation and Characterization of Lipase producing bacteria from Gangotri glacier, Western Himalaya, India.

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ABSTRCT: A Psychrotrophic bacterium producing cold active lipase was isolated from soil of Gangotri glacier designated as GGPRTL-5. Out of the eight selected lipase producing isolates the best one GGPRTL- 5 was further characterized. The bacterial strain GGPRTL-5 showed maximum activity at pH 8.0 and temperature 20°C. Cold-active lipases are generally characterized by high-catalytic efficiencies at lower temperature and lower thermal stability. The metal ion like Ca^{2+},Mg^{2+},Na^{+}, Fe^{2+} and Zn^{2+} are able to enhance the activity of purified lipase while other ion like Hg^{2+}, Cu^{2+}, Co^{2+} had shown negative effect on the enzyme activity. Among the different inhibitors EDTA inhibit the cold active lipase. In contrast, lipase is stable in presence of DTT, SDS and Tween 80.

KEYWORDS: Psychrotrophic, lipase, Gangotri, cold adapted bacteria.

I. INTRODUCTION

During the past decade it has been recognized that cold adapted micro-organisms provide a large biotechnological potential, offering numerous economical and ecological advantages over the use of organisms and their enzymes which operate at higher temperatures (Margesin et al 2005, Ohgiya et al. 1999, Margesin et al. 2002a). Gangotri glacier is located in Uttarkashi district of Uttaranchal state, India between 30°44´-30°56´ N latitude and 79°04´-79°15´E longitude. It is about 30 km long flowing in a NW direction and its width varies from 0.5 to 2.5 km. Numerous small sized glaciers feed and contribute into the main glacier to form the Gangotri group of glaciers. These are smaller in size but larger in numbers so their cumulative effect is very significant. Psychrophiles and psychrotrophic organisms may play a major role in degradation of organic matter in microbial ecosystem of Gangotri glacier. Thus, it would be of much interest to study nature of extracellular enzyme such as lipase secreted by cold adapted bacteria that are active not only in permanently cold areas but in habitats which experiences temperature fluctuation (Baghel et al 2005). Psychrophilic enzymes are not only of extraordinary interest at the fundamental level to investigate the thermodynamic stability of protein, but also to understand the relationship between stability, flexibility or plasticity and their catalytic efficiency. The idea of using microorganisms to reduce environmental contamination, such as in soils and waste waters, is not new but appears to be a feasible alternative to physicochemical methods. In temperate regions, large seasonal variations in temperature reduce the effectiveness of microorganisms in degrading organic pollutants such as oils and lipids (Gerday et al 2000).

Lipases (EC 3.1.1.3) are carboxylesterases that catalyze the hydrolysis and synthesis of long-chain triacylglycerol. Lipase is a versatile enzyme that is the one of the most important biocatalysts in the laundry, food, chemical, and pharmaceutical industries (Gupta et al 2004. Jaeger and Reetz. 1998, Jaeger and Eggert. 2002). Recent advances in the study of lipase point to the discovery of novel lipases from extremophilic microorganisms. Cold-adapted lipases from psychrophilic bacteria have received increased attention for the fact that they raise considerable interest in both basic and applied research. (Y. Xiuxia et al 2008). Cold-active lipases have true enzyme potentialities for industrial applications (A.L.Giudice et al 2004) in fields of pharmaceutical preparations, cosmetics, food production, waste management, biosensors (Joshi et al 2006), organic synthesis of unstable compounds, fine chemicals, additives in laundry detergents for cold washing, bioremediation (K.N. Timmin and D.H. Pieper 1999) etc. Therefore, an attempt has been made to isolate and characterize cold active bacteria capable of producing cold active lipase from Gangotri glacier Western Himalya, India

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II. MATERIALS AND METHODS

A. *Collection of Sample*- Soil and ice sample were collected randomly from different sites of Gangotri glaciers, Western Himalaya, India for the isolation of cold adapted lipase producing microorganism.

B. *Isolation of Bacterial Flora From Soil* - For enumeration of the bacterial count in soil sample, one gram of respective sample were homogenized in 9 ml of cold sterile double sterilized double distilled water and the suspensions was serially diluted upto 10^{-6}.The diluted suspension (0.1ml) of various dilution were inoculated in triplicate for each dilution of tributyrin agar plates (TBA) and incubated for 48 hours at 4-20^0c.Bacterial growing at a temperature range between 4-20^0 C were picked and further growth on tributyrin media.

C. *Screening of Cold Active Alkaline lipase*- For the preliminary screening of lipase producing bacteria tributyrin agar media was used. Appearance of clear zone on the colonies was the principal indicator for the production of lipolytic enzymes. One hundred bacterial colony were isolated on tributyrin agar plates from eight samples of Gangotri glaciers. Eight bacteria were selected as potent lipase producer out of them one potent lipase producing strain were selected and designated as GGPRTL-5.

D. *Identification of Isolate showing lipase activity*- The morphological characterization of lipase producing isolates was done by examining the configuration, margin, elevation size, margin arrangement and Gram's staining of bacterial colonies**.** Biochemical tests like MR-VP, citrate utilization, Indole production, H_2S production and Catalase test etc. were done.All the eight isolates were identified by studying morphological and biochemical characteristic according to Bergey's Manual of Systematic Bacterialogy (Holt etal 1994).

E. *Production of enzyme and partial purification*-The extracellular lipase production was carried out in a medium composed of 3% w/v yeast extract, 1% w/v KH_2PO_4, 0.1w/v $MgCl_2.7H_20$, 0.5% w/v maltose and 0.2 v/v olive oil at pH 7.0. Medium was sterilized and incubated with 1% inoculum prepared in nutrient broth followed by incubation at 37^0c for 48 hr at 160 rpm in a shaker incubator. The cells were centrifuged at 10000 rpm for 15 min and the supernatant was subjected to Ammonium sulphate fractionation of 0-40%, 40-60% and 60-80%. Fraction was dialyzed against glycine NaOH buffer. Cold-active lipase was purified to homogeneity by precipitating with ammonium sulphate fractions and using a single step ionexchange chromatography on a DEAE-cellulose. Enzyme was eluted from the column as unbound fractions with 0.7M NaCl gradient. The purified enzyme served as a enzyme source for further characterization.

F. *Assay of Lipase*- The lipase activity in the culture supernatant was determined by p-nitrophenylpalmitate (p-NPP) substrate as described by Winkler and Stuckmann (1979) with some modifications. The reaction mixture consisted of 135 ml of 0.4% (w/v) Triton-X , 0.1% (w/v) gum Arabic in 50 mM Tris-HCl buffer, pH 7.0) and 15 ml of substrate (16.6 mM p-nitrophenyl substrate in 2-propanol). The mixture was pre-reacted at 10^oC for 10 min and 50 ml of enzyme solution was subsequently added. The color change was measured at 405 nm using multi-well plate reader after 30 min of incubation at 10^oC. Protein concentration was measured by the method of Bradford (Bradford, 1976) with bovine serum albumin as a standard. One unit of lipase activity is defined as the amount of enzyme releasing 1 µ mol p-NPP min^{-1} under assay condition.

G. *Effect of pH on Activity*-The optimum pH for lipase produced by GGPRTL-5 was measured by assaying at various pH as citrate buffer (pH 5.0–6.5), as tris-chloride buffer (pH 7.0–9.0), glycine- NaOH buffer (pH 10). Activity of the purified lipase at different pH was measured by adjusting pH of the reaction mixture using (0.1 M) of the above mentioned buffer. To determine the pH stability of the enzyme partially purified enzyme was incubated at various pH (pH 7–11) for 30 min in the presence of 0.05 mg/ml BSA at 20^0C, and the residual lipase activity was determined.

H. *Effect of temperature on lipase Activit*-The apparent optimum temperature for lipase of GGPRTL-5 was measured by assaying its hydrolytic activities toward olive oil at various temperatures (10-50°C). To examine the enzyme stability at different temperature(10-50^{o}C), purified enzyme was dissolved in 50 mM Tris–HCl buffer (pH 8.0) containing 0.05 mg/ml of bovine serum albumin (BSA) for 30^0 min. at different temperature then the residual activity was determined.

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I. Effect of metal ion/ inhibitors/detergent-The effect of organic compound and inhibitor on the stability of purified lipase was investigated by the modified procedure of Ogino et al (2000). The enzyme was incubated with various inhibitors (1mM) compounds such as EDTA, SDS, and dithiothreitol (DTT) that may inhibit the enzyme, metal ions Zn^{2+}, Mg^{2+}, Ca^{2+} , Na^{2+} and Fe^{3+} in the form of (5 mM): $ZnSO_4$, Mgcl2, $CaCl_2$, $NaSO_4$ and $FeSO_4$, detergents (5%): sodium dodecylsufate (SDS), triton X-100 and tween 80 at 20°C for 30 min. and the residual activity was measured with *p*-nitrophenyl butyrate as the substrate. Residual activities were measured and compared with a control (without metal ions/ inhibitors and detergents).

III . RESULTS

The present work was focused on isolation and characterization of cold adapted bacterial strain capable of producing cold active enzyme lipase. One hundred lipase producing bacterial isolates were isolated from various soil and ice samples. However, eight isolates were identified as potent lipase producing species. The isolated bacterial strain was able to grow in the temperature range of 4-20^{0}C.The lipase producing isolates GGPRTL-5 was selected as best one on the basis of zone formation on TBA plates. These eight bacterial strains were further characterized on the basis of their morphological and biochemical characterization. For the identification of strain of interest cultural characteristics, morphological characteristics, and biochemical tests (KR Aneja 2007) were conducted and identified on the basis of characters as given in Bergey's Manual of Systematic Bacteriology (Holt et al 1994). Bacterial strain GGPRTL-5 was Gram-negative, rod shaped, motile and nonspore forming. Colonies of strain were found to be circular, smooth, convex and with the entire margin during growing on tributyrin agar plates for 48 hours. The biochemical and physiological characteristic of strain GGPRTL-5 are summarized in Table 1.

Tests	Results
Morphological characteristics	
Configuration, surface margin, elevation, density and color	Round, smooth, convex and with entire Margin, Creamish
Gram's reaction	Gram Negative,
shape	Rod shaped
Motility	Motile
Biochemical characteristics	
Growth temperature	4-20^{o}C
Indole	Negative
Voges-proskauer	Negative
Methyl red	Positive
Citrate	Negative
H_2S production	Negative
Indol production	Negative
Nitrate reduction	Negative
Tryptophane deaminase	Negative
Urea hydrolysis	Negative
Assimilation of glucose	
Mannitol	Positive
Inositol	Positive
Sorbitol	Positive
Rhamnose	Positive
Sucrose	Positive
Maltose	Positive
Citrate	Positive
Phenyl acetate	Negative

Table 1. Identification of potential lipolytic bacterial isolate by morphological and biochemical characterization

Out of eight lipase producing strain the best one GGPRTL-5 was selected for production, purification and characterization of lipase enzyme. Cold-active lipase was partially purified by precipitating with ammonium

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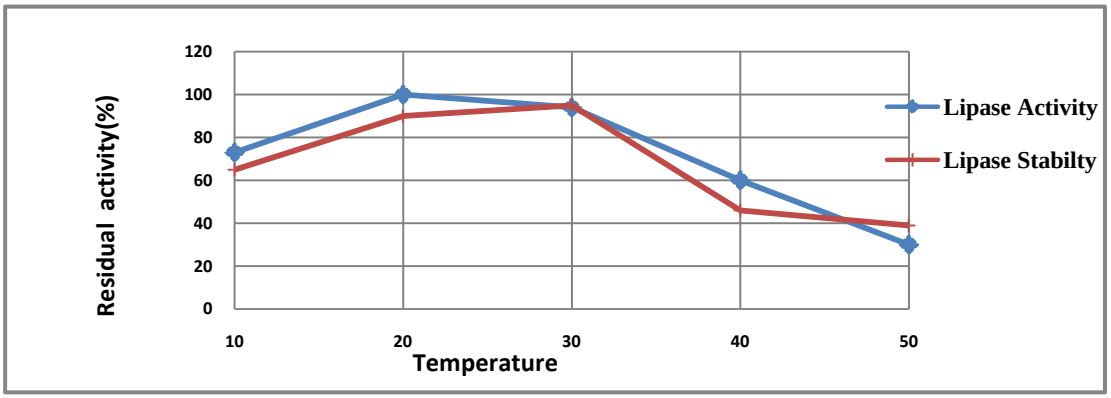
sulphate (40-60 %) and using a single step ion-exchange chromatography on DEAE cellulose (Table 2). Lipase was eluted out as fractions (with NaCl gradient) from DEAE-cellulose column with 28.80 fold purification and specific activity of 82.10 U mg^{-1}. (Table-2)

Purification step	Total activity (units)	Total protein (mg) (%)	Sp. activity (U mg−1)	Purification fold	Yield
Crude enzyme	3708	1 300	2.85	1	100
$(NH4)_2SO^4$ precipitation (dialyzed)	2300	136.57	16.84	6.2	5.90
DEAE-cellulose	156	1.9	82.10	28.80	4.2

Table 2. Summary of partial purification of cold active lipase

The purified lipase of isolate GGPRTL-5 showed maximum activity at 20°C. The enzyme was incubated at various temperatures (10, 20, 30, 40 and 50) for 30 min. and then the residual activity was measured the isolate was fairly stable up to 20-30°C, then lost almost all its activity above 50°C (Graph-1). The maximum activity of lipase of GGPRTL-5 at Ph 8 at 20°C and the enzyme was stable between pH 8 and 9 at the indicated pH range when incubated at 20°C for 24 h, but its activity decreased at pH at 10. (Graph-2).



Graph-1 effect of temperature on enzyme activity and stability

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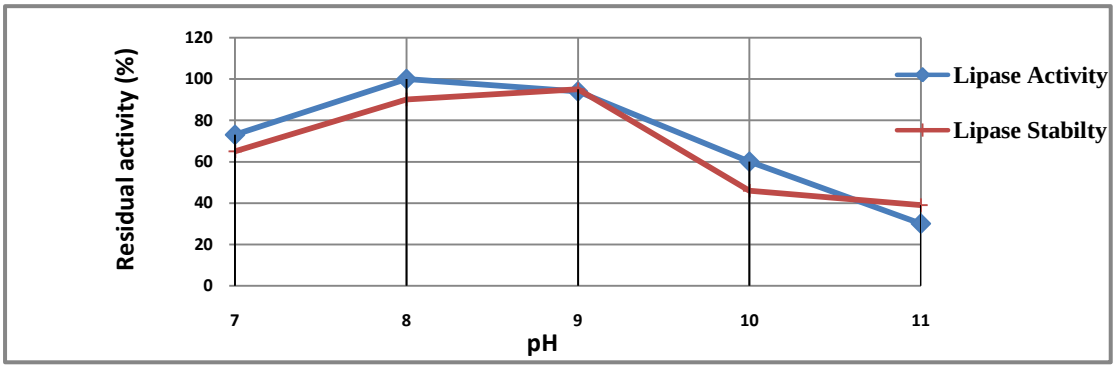

Graph-2 effect of pH on enzyme activity and stability

The enzyme was incubated with various compounds that may inhibit the enzyme, and the remaining activity was measured with *p*-nitrophenyl butyrate as the substrate at 25°C. The metal ion like Ca^{2+}, Mg^{2+}, Na^{+}, Fe^{2+} and Zn^{2+} at a concentration of 5.0 mM were able to enhance the activity of purified lipase while other ion like Hg^{2+},Cu^{2+},Co^{2+} had shown negative effect on the enzyme activity. Among the different inhibitors EDTA inhibit the cold active lipase. In contrast, lipase is stable in presence of DTT, SDS and Tween 80 **(**Table3).

IV. DISCUSSION

Cold-active enzymes from microbial sources have potential applications in biotechnology, agriculture and medicine (Feller, 2007, Moreno et al., 2013). In the present study one hundred bacteria were screened for lipase production from Gangotri glacier. Eight potent lipase producing bacteria were isolated at temperature range 4-20^{0}C. The isolated strain showed growth temperature range 4-20^{0}C indicated that isolates were belonged to psychrotrophic family (Feller and Gerday 2003).

The enzyme produced by produced the strain GGPRTL-5 maintains its highest activity at 20^{o}C, which is one of the typical characteristics found in cold-active enzymes (Wang et al., 2005, Zhang and Zeng 2008). The isolated strain was stable up to 20-30°C, then lost almost all its activity above 50°Cshowed that the enzyme was thermally unstable. The poor thermal stability of psychrophilic and psychrotrophic enzymes, which facilitates their rapid inactivation by a moderate rise in temperature is also advantageous in some technologies.

	Residual activity (%)
Inhibitors (5%)	
None	100
EDTA	32
SDS	92
Dithiothreitol	83
Tween 80	89
Metal ion(5mM)	
$FeSO_4$	91
$Cocl_2$	32
$NaSO_4$	87
$Cuco_4$	25
$HgCl_2$	19
$Cacl_2$	58
$MgCl_2$	76
$ZnSO_4$	58

Table-3 Effect of different concentration of metal ions and inhibitors on partially purified cold active lipase activity

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Enzymes from psychrophilic organisms have high catalytic activity at low temperatures and low thermal stability (Margesin and Miteva, 2011). The maximum activity of lipase of GGPRTL-5 at pH 8 at 20^{o}C and the enzyme was stable between pH 8 and 9 at the indicated pH range when incubated at 0°C for 24 h, but its activity decreased at pH at 10. Lipases showing high catalytic activity over a wide range of pH and under non-conventional conditions are of great importance to use in laundry and household detergents (Joseph et al. 2008).

Nearly one third of all known enzymes require metal ion for its catalytic activity (Voet et al 1999).The metal ion like Ca^{2+}, Mg^{2+},Na^{+},Fe^{2+} and Zn^{2+} were able to enhance the activity of purified lipase. Enhanced activity of enzyme was due to ion shown that the lipase was metal dependent enzyme. Enzyme inhibition studies primarily give an insight in to the nature of the enzyme, its cofactor requirements and the nature of the active centre (Sigma and Mooser, 1975). Other ion like Hg^{2+}, Cu^{2+}, Co^{2+} had shown negative effect on the enzyme activity. Among the different inhibitors EDTA inhibit the activity of the cold active lipase. In contrast, lipase is stable in presence of DTT, SDS and Tween 80.

V. CONCLUSION

It was found that the enzymes showed maximum activity at pH 9.0 and temperature 20°C and thus we can say that the enzymes produced from isolate GGPRTL-5 is cold active and may have various applications in industry.

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