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Original Article

Extracellular cold active lipase from the psychrotrophic *Halomonas* sp. BRI 8 isolated from the Antarctic sea water

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Abstract

An extracellular cold active lipase-producing psychrotrophic bacterium (BRI 8) was isolated from the Antarctic sea water sample. The 16s rRNA sequence study revealed that the isolate belongs to the genus *Halomonas* (929 bp). The present paper reports optimization of fermentation conditions for production of lipase (EC 3.1.1.3) from *Halomonas* sp. BRI 8. Highest lipase production was observed in the medium containing olive oil and peptone. The optimum pH and temperature for enzyme catalysis were 7.0 and 10°C respectively. The enzyme was relatively more stable in acidic pH range and retained 50% activity when incubated at 30°C for 1 h. The enzyme was stable in various organic solvents and showed more than 100% activity in presence of isoamyl alcohol. Significant enzyme activity was also observed in the presence of metal ions and detergents. The molecular mass of partially purified lipase was found to be around 66 kD.

Keywords: *Halomonas*, Antarctic, cold active lipase, optimization, stability

1. Introduction

Lipases have become commercially valuable due to their role in various sectors including food, chemical, pharmaceuticals, cosmetic, detergent, leather processing, biodiesel etc. (Gupta *et al.*, 2004). Hence, detection of newer and stable lipases active at extreme pH and temperature have become the need of the hour. In recent years, a wide range of enzymes produced by microorganisms from extreme environments has widely contributed to industrially common processes.

Lipases have been reported from various microorganisms with stability at temperatures up to 50°C (Gupta *et al.*, 2004). However, comparatively few reports are available on cold active lipases. Psychrotrophs are essentially mesophilic microorganisms that can also grow at or below 15°C. The cold adaptation of these microorganisms may be attributed to their ability to produce cold active enzymes which exhibit higher catalytic abilities at low temperatures. Lipases from marine microbes are valuable catalysts since they i) act under mild conditions, ii) are highly stable in organic solvents (Villeneuve *et al.*, 2000; Gessesse *et al.*, 2003; Davidson, 2006)and iii) their purification, if extracellular, is comparatively easy (Rapp and Backhaus, 1992). Lipase activity has been reported from comparatively fewer species of *Halomonas* (Vargas *et al.*, 2004; Wang *et al.*, 2008; Dang *et al.*, 2009; Khunt *et al.*, 2012). As per our knowledge this is the first report on cold active lipase from *Halomonas* sp. These enzymes show relatively high specific activity in the range of 0- 20°C and are a good candidate for low temperature processes.

In the present work, we have isolated and identified pyschrotrophic *Halomonas* sp. BRI 8 from an Antarctic sea

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water sample. The culture conditions were optimized for maximum lipase production. The cold active lipase from *Halomonas* sp. BRI 8 was found to be active in the presence of a wide range of organic solvents, metal ions and detergents suggesting its potential for industrial use.

2. Materials and Methods

2.1 Organism

Halomonas sp. BRI 8 was isolated from an Antarctic seawater sample (Latitude S 59°40'24.6" and Longitude E 68°33'23.7"). Its lipolytic activity was screened using tributyrin agar base at 15°C and pH 7.0. *Halomonas* sp. BRI 8 was maintained on Marine Salt Medium (MSM) (Jadhav *et al.*, 2010).

2.2 Chemicals

All the media components and chemicals were purchased form Hi Media and Merck (Mumbai, India) and were of analytical grade.

2.3 Isolation of genomic DNA

The genomic DNA of BRI 8 was isolated as described by Ausubel *et al.*, 1987.

2.4 Amplification of 16S rDNA and sequence analysis

The PCR assay was performed using Applied Biosystems, model 9800 (Foster, California, USA) (50ng of DNA). The primers used were 8F (5' -AGAGTTTGATCCTGGCTCAG 3') and 1391R (5'- GACGGGCGGTGTGTRCA -3') The PCR product was purified by PEG-NaCl method and sequenced in a 3730 DNA analyzer (Applied Biosystems, Foster City, USA) following the manufacturer's instructions. The primers used were 704F (5'GTAGCGGTGAAATGCGTAGA3') and 907R (5' CCGTCAATTCMTTTGAGTTT 3') (Amann *et al.*, 1992; Hauben *et al.*, 1997; Pidiyar *et al.*, 2002; Ben-Dov *et al.*, 2006). The sequences of bacterial 16S rDNA were analysed using Sequence Scanner (Applied Biosystems) software. The 16S rDNA sequence contigs were generated using Chromas Pro and then analysed using online databases, viz. NCBI-BLAST, to find the closest match of the contiguous sequence. Phylogenetic analysis was carried out using MEGA software package version 5.0 (Tamura *et al.*, 2011)

2.5 Lipase assay

The isolate BRI 8 was cultivated in the medium consisting of peptone (3%), NaCl (0.5%) and tributyrin (1%) (w/v) pH 7.0, 15 °C, 120 rpm for 24 hours [h]. The extracellular culture broth was used as enzyme source. Lipase activity was measured by spectrophotometric method using paranitro phenyl palmitate (pNPP) as substrate. For optimization of temperature for enzyme catalysis, crude enzyme was assayed over different temperatures (5-60°C) at pH 7.0 in 50 mM phosphate buffer. To determine optimum pH, enzyme was incubated at optimized temperature in the pH range of 5.0-9.0. The following buffers were used: pH 3.0-5.0 citrate phosphate buffer, pH 6.0-8.0 phosphate buffer and pH 9.0-10.0 carbonate bicarbonate buffer. For enzyme assay, 0.1 ml of enzyme was added to a 0.9 ml of pNPP substrate solution (2 mg /ml in isopropanol) and 1 ml phosphate buffer (50 mM, pH 7.0). The mixture was incubated for 1 hour at optimized conditions. The amount of para- nitrophenol (pNP) liberated was measured spectrophotometrically at 410 nm (Winkler and Stuckmann, 1979). One unit (U) of lipase activity is defined as the amount of enzyme necessary to hydrolyze 1 μmol of pNPP /min under assay conditions.

2.6 Optimization of media and cultivation conditions for lipase production

Optimization of different nutrient and physical parameters was carried out by maintaining all the factors unaltered except the one being studied. The basal medium used was composed of peptone (crystalline peptone) (3%), NaCl (0.5%) and tributyrin (1%) (w/v) with pH adjusted to 7.0. No additives were used in the basal medium. The effect of various media components, viz. carbon sources, nitrogen sources and NaCl concentrations, were studied on lipase production. For determination of the best carbon source for lipase activity tributyrin in the basal medium was replaced with different compounds, viz. olive oil, coconut oil, castor oil, ghee and palm oil individually at 1% concentration. Furthermore, the medium containing optimum carbon source was used to determine the effect of various nitrogen sources on lipase production. For this, peptone in the medium was substituted by ammonium chloride, sodium nitrate, urea, yeast extract and tryptone individually at 3% concentration. All experiments were done in triplicates. For all experiments 5% inoculum was used.

Effect of temperature on lipase production was studied by growing the isolate at various temperatures (15-37°C) in the medium containing optimized carbon and nitrogen source. Effect of pH on lipase production was studied by cultivating the isolate at optimum temperature by varying the pH (2.0-10.0) of the production medium. After optimizing these parameters, enzyme production was monitored for 3 days, by removing the samples after every 8 h to determine the effect of incubation period on enzyme production.

2.7 Effect of temperature and pH on lipase stability

Thermal stability of crude enzyme was investigated by incubating the enzyme in phosphate buffer (50 mM, pH 7.0) at different temperatures ranging from 5-60°C for 1 h and aliquots were assayed for residual activity. The pH stability was tested by incubating the enzyme at 10°C in the pH range of 3.0-10.0 using different buffers at 50 mM concentration for

1 h. Buffer systems used were the same as mentioned earlier. The aliquots were assayed for residual enzyme activity. All experiments were done in triplicates.

2.8 Effect of additives and metal ions on stability

The effect of various organic solvents was analyzed by incubating the enzyme at 10°C with organic solvents (1:1), viz. hexane, heptane, hexadecane, isoamyl alcohol, methanol, butanol, dimethyl sulfoxide (DMSO), ethanol and acetone for 1 h. Likewise, the effect of metal ions on enzyme activity was studied by incubating the enzyme at 10°C with 0.1 mM, 1 mM and 10 mM concentrations of each of copper (Cu^{2+}), calcium (Ca^{2+}), lead (Pb^{2+}), zinc (Zn^{+}), manganese (Mn^{2+}), magnesium (Mg^{2+}), iron (Fe^{3+}) and potassium (K^{+}) for 1 h. Stability of lipase in the presence of synthetic detergents (SDS, Tween 80 and Triton X 100) and commercial detergents (Active Wheel, Rin Matic, Henko Matic, Ariel and Tide) was studied. The enzyme sample was incubated with 1% (w/v) of each of the detergents individually at 10°C for 1 h. Aliquots were assayed for residual enzyme activity. Resulting enzyme activities were compared to that of standard enzyme reaction carried out in identical conditions without any additives.

2.9 Partial purification of lipase

BRI 8 was cultivated in the medium containing olive oil (1% w/v), NaCl (0.5% w/v) and peptone (3% w/v) at 25°C, pH 7.0 for 16 h. The culture filtrate was collected by centrifugation and used for ammonium sulphate precipitation at 90% saturation. The mixture was allowed to precipitate and then centrifuged at 5000 × g for 15 min. The precipitate was collected and dissolved in minimum amount of 50 mM phosphate buffer (pH 7.0). The solution was then dialyzed against the same buffer to remove the residual ammonium sulphate. For the determination of molecular mass of the partially purified lipase, proteins were analyzed by 12% SDS-PAGE (Laemmli *et al.*, 1970) and detected by staining with Coomasie Blue R-250.

2.10 Statistical analysis

Statistical analysis of variance (ANOVA) was performed on all values and tested for $p<0.05$ for significance.

3. Results and Discussion

3.1 Isolation and phylogenetic analysis

BRI 8 was identified and the sequence (929 bp) was deposited in EMBL+Genbank under the accession number JQ 899256. BRI 8 belonged to G*ammaproteobacteria* and is a member of the genus *Halomonas*. Phylogenetic analysis of BRI 8 is shown in Figure 1.

3.2 Lipase assay

The enzyme was active in the range of 5-60°C with optimum temperature of 10°C. Studies on effect of pH on enzyme activity showed that the enzyme was optimally active at pH 7.0. Similar pH optimum (near neutral) was observed for lipase from *Halomonas salina* (pH 6.8) (Khunt *et al.*, 2012). However, its optimum temperature was found to be 37°C. Cold active lipase from *Micrococcus roseus* had lower temperature optimum of 15°C resembling that of BRI 8 (this work). However, it was optimally active at pH 8.0 (Joseph et al., 2011).

3.3 Optimization of fermentation parameters

Maximum production of 18 U /ml activity was obtained with olive oil (1%) as a carbon source after 24 h of cultivation (Figure 2A). Similarly, peptone was found to be the best nitrogen source amongst all the sources tested (Figure 2B). A 6.2% increase in activity was observed in the medium containing olive oil as compared to initial production medium containing tributyrin. Optimum NaCl concentration was found to be 0.5% for enzyme production (Figure 2C). Thus, maximum lipase production was obtained in the medium containing olive oil (1% w/v), NaCl (0.5% w/v) and peptone (3% w/v). This medium was used for all other experiments. Mobarak-Qamsari *et al.* (2011) have also reported olive oil and peptone as the optimum carbon and nitrogen source with 0.46 and 0.17 U/ml of lipase activity respectively in *Pseudomnas aeruginosa* KM110. Similarly, Kumar and Valsa

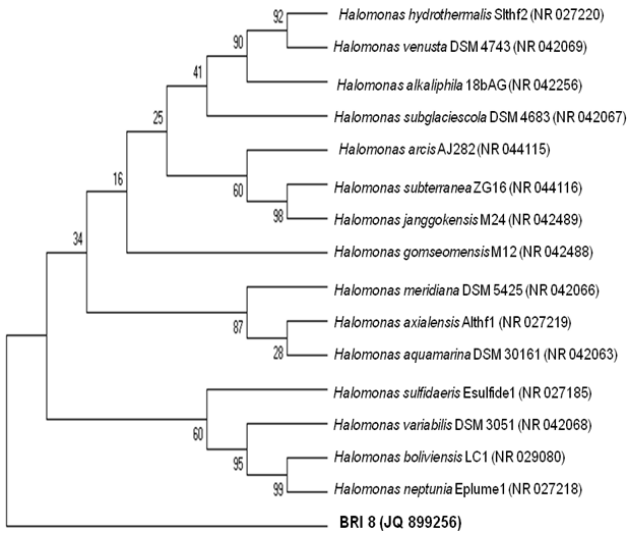

Figure 1. Phylogenetic analysis based on of 16S rRNA sequences of isolate BRI 8 and related *Halomonas* species. Gene Bank accession numbers are listed with species names. Bootstrap values were generated from 1000 replicates and are shown as percentages at nodes.

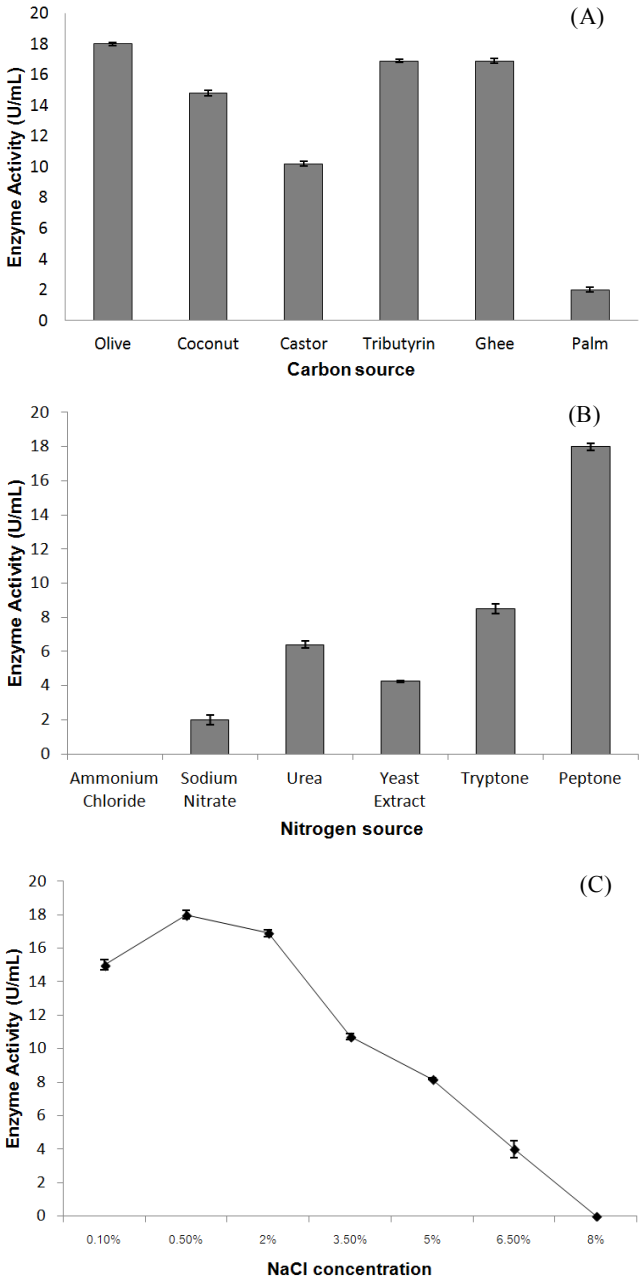

Figure 2. (A) Effect of carbon source (1%) on lipase production. (B) Effect of nitrogen source (3%) on lipase production with 1% olive oil. (C) Effect of NaCl concentration on lipase production with 1% olive oil and 3% peptone.

(2007) have also found the maximum lipase activity from *Bacillus coagulans* in the presence of olive oil and peptone. Other reports on peptone as the best nitrogen source for lipase production were published by Gao *et al.* (2004) for *Serratia marcescens* and Kumar *et al.* (2012) for *Bacillus* sp. MPTK 912. However, the best carbon sources were found to be Tween 80 and glucose respectively. In psychrotrophic Antarctic bacteria, highest enzyme activity was reported in the range of 1.7-1.9 U/ ml using optimum carbon and nitrogen source (Singh and Ramana, 1998).

Maximum lipase activity was observed when the isolate was grown at 25°C (Figure 3A). As per our knowledge, this is the second report in which the optimum temperature for lipase production from *Halomonas* sp. is observed below 30°C. Previously, optimum temperature of 28°C was reported for *Halomonas* sp. (Dang *et al.*, 2009).

Optimum pH for lipase production was found to be 7.0 (Figure 3B). Similar pH optimum has been reported in

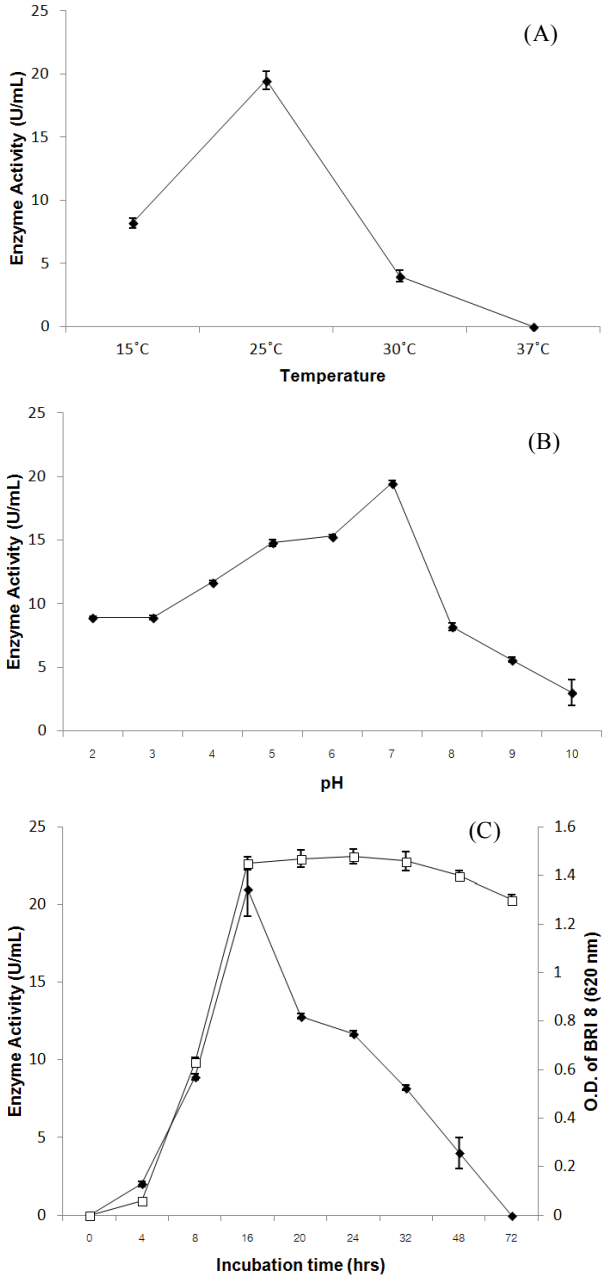

Figure 3. (A) Effect of incubation temperature on lipase production. The culture was incubated at different temperatures (15 to 37°C). (B) Effect of pH of the culture medium on lipase production. The culture was incubated in the culture medium at 25°C with pH (2.0 to 10.0). (C) Effect of fermentation time on growth and lipase production at 25°C, pH 7.0.Growth (□) lipase activity (♦)

Halomonas lutea (Wang *et al.*, 2008). In case of BRI 8 (this work) maximum production of 21 U /ml was reached after 16 h of cultivation (Figure 3C). Thus, maximum lipase production was observed at 25°C, pH 7.0 after 16 h in the optimized medium.

3.4 Effect of pH and temperature on lipase stability

The enzyme showed 100% stability at 10°C (Figure 4A). In contrast, lower relative enzyme activities (40-75%) have been reported for cold active lipases from various microorganisms at 10°C (Alquati *et al.*, 2002; Hak-Ryul *et al.*, 2010). pH stability studies showed that enzyme was 100% stable at pH 7.0 (Figure 4B). Our results indicate that enzyme is relatively more stable at acidic pH. On the contrary, most of the lipases reported earlier are more active at alkaline pH range (Gupta *et al.*, 2004).

3.5 Effect of additives on stability

The activity of crude enzyme under identical conditions without addition of additives was considered as 100%. The effect of various metal ions on stability of lipase was studied (Figure 5A). Marginal increase in enzyme activity was observed in the presence of calcium ions. Enhanced enzyme activity in the presence of calcium ions was also reported for lipases from other microorganisms (Hong-wei *et al.*, 2009; Yuan-Yuan *et al.*, 2011). This increase in enzyme activity may be due to the binding of calcium ions to the enzyme and the consequent increase in the stability of the enzyme catalytic site (Barbosa *et al.*, 2012). In the presence of almost all the metals more than 90% activity was detected at 0.1 and 1.0 mM concentrations. However, a slight decrease (10- 20 %) in enzyme activity was observed in the presence of lead, manganese and magnesium at 10 mM concentration. Similar results were reported for lipase from *Bacillus subtilis* (Ma *et al.*, 2006). These observations demonstrated remarkable stability of BRI 8 lipase in the presence of various metal ions.

Lipase under study exhibited significant stability in organic solvents like hexadecane and isoamyl alcohol with residual activity of 90 and 105% respectively. The enzyme retained 60 % activity in the presence of hexane and methanol (Figure 5B). Uttatree *et al.* 2010 have reported lipase from *Acinetobacter* which also exhibited good stability in the presence of isoamyl alcohol. Organic solvents may help in increasing the solubility of substrate due to which an increase in enzyme activity is observed (Rahman *et al.*, 2006). This characteristic explains the potential of the enzyme to be used as a biocatalyst for reactions such as transesterification or chiral resolution in organic reactions. Enzyme activity could not be detected after incubation with acetone, ethanol or butanol.

Lipase from BRI 8 showed 71% residual activity in the presence of Tide, Tween 80, Triton X 100 and 54% residual activity in presence of SDS (Figure 5C). Analogous trend is

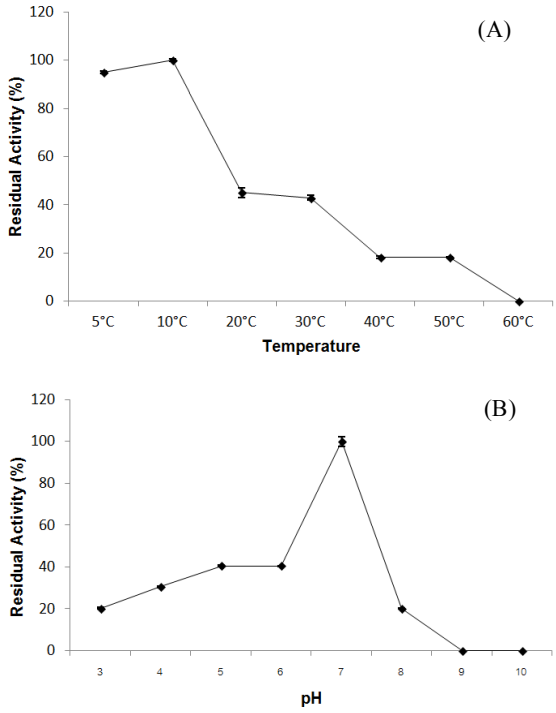

Figure 4. (A) Enzyme stability at various temperatures (5 to 60°C). (B) Enzyme stability at various pH (3.0 to 10.0).

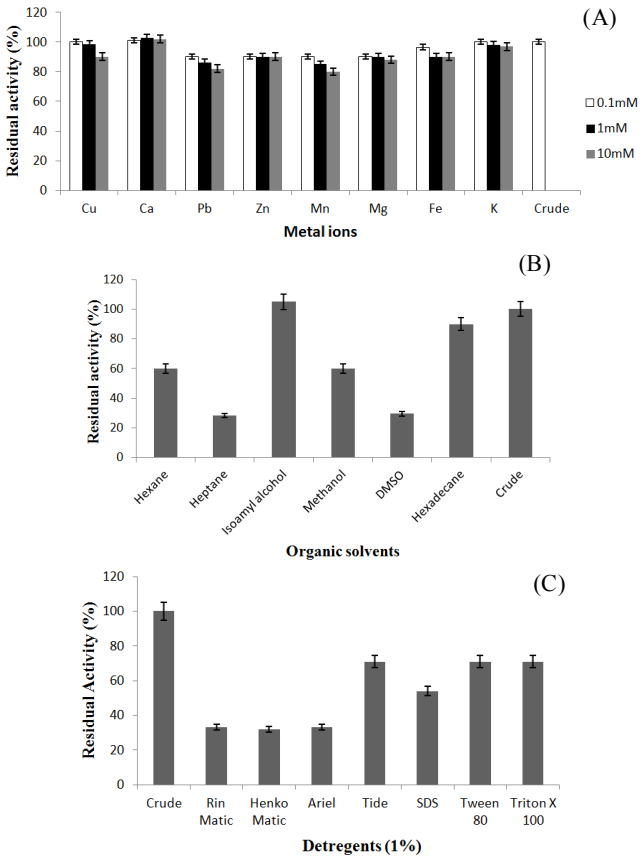

Figure 5. (A) Effect of metal ions on stability of enzyme. (B) Stability of enzyme in organic solvents. (C) Enzyme stability in presence of detergents.

Table 1. Purification of cold active lipase from *Halomonas* sp. BRI 8

Purification steps	Protein (mg/ml)	Enzyme activity (U/ml)	Specific activity (U/mg)	Yield (%)	Fold Purification
Crude enzyme	13.0	21.0	1.62	100	1
Ammonium sulphate precipitate	5.2	14.8	2.85	70.48	1.76
Dialyzate	1.0	10.0	10	47.62	10

reported for lipase from *Pseudomonas aeruginosa* KM110 (Mobarak-Qamsari *et al.*, 2011). However, lipases from *Acinetobacter* baylyi (Uttatree *et al.*, 2010) and *Pseudomonas aeruginosa* BN-1 (Sayed *et al.*, 2010) were found to drastically lose their activity in the presence of SDS and Tween 80. The cold active lipase from BRI 8 retained about 33% activity in the presence of other commercial detergents like Rin Matic, Henko Matic and Ariel at 1% concentration. However, the enzyme lost its activity when incubated with Active Wheel.

3.6 Partial purification of lipase

The crude enzyme was subjected to ammonium sulphate precipitation at 90% saturation. The precipitate thus obtained was dissolved in a minimum amount of 50 mM phosphate buffer, pH 7.0. The residual salts were removed by dialyzing the solution against the same buffer (Table 1). The partially purified lipase exhibited a single band on SDS-PAGE with an apparent molecular weight of 66 kDa (Figure 6). Jin-wei and Run-ying (2008) have reported cold adapted lipase of 64 kD from *Pseudomonas* sp. 7323, which is also a psychrotroph isolated from Antarctic sea. Lipases of molecular weight in the range of 62- 65 kD have also been reported from other microorganisms (Matsumae *et al.*, 1994; Arpigny and Jaeger, 1999).

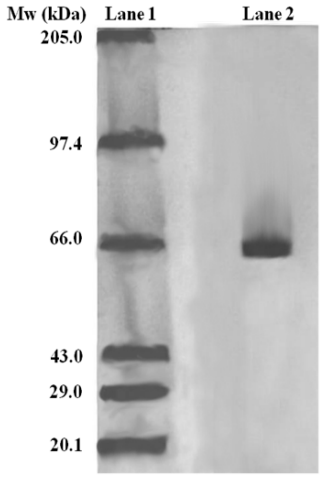

Figure 6. SDS- PAGE analysis of the partially purified lipase from *Halomonas* sp. BRI 8
Lane 1: - Protein molecular mass marker (kDa)
Lane 2: - Partially purified lipase from *Halomonas* sp. BRI 8

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