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Lipase from *Bacillus pumilus* RK31: Production, Purification and Some Properties

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Abstract: A new lipolytic strain of *Bacillus* sp. was isolated from oil contaminated soil sample. On measurement of biochemical parameters and 16S rDNA sequence information the strain was identified as *Bacillus pumilus* RK31 (NCBI accession no GQ463238). The lipase purification steps involved, 60% ammonium sulphate saturation, gel filtration chromatography using Sephadex G200 and ion exchange chromatography with DEAE cellulose. A purification fold of 186 was achieved following only three step purification process with specific activity of 3525.6 U/mg to apparent homogeneity as evident by a single band of 62.2kDa on sodium dodecyl sulphate polyacrylamide gel electrophoresis and reverse phase HPLC chromatogram. The purified enzyme exhibited optimum activity at temperature 60°C, pH 6.0, Km of 1.83 mM l^{-1} and the Vmax of 10.0 mM l^{-1} min^{-1}. EDTA-K almost completely inhibited the lipase activity. The enzyme had property to tolerate a wide range of organic solvents which make it attractive towards industrial applications.

Key words: Purification fold · Characterization · Lipase · Column chromatography

INTRODUCTION

Among many ubiquitous enzymes, lipases (triacylglycerol acylhydrolases, E.C. 3.1.1.3) are of considerable physiological significance with wide industrial applications. The unique features of lipases include the functionality at aqueous and nonaqueous phase, the wide spectrum of substrate, high stability towards extremes of temperature, pH and organic solvents and chemo, regio and enantioselectivity [1, 2]. Among lipases of plant, animal and microbial origin, it is the microbial lipases that find immense applications. This is because microbes can be easily cultivated and their lipases can catalyse a wide variety of hydrolytic and synthetic reactions. Commercially useful lipases are usually obtained from microorganisms that produce a wide variety of extracellular lipases [3]. Lipase producing microorganisms have been found in diverse habitats such as industrial wastes, vegetable oil processing factories, dairies, soil contaminated with oil, oilseeds, decaying food, compost heaps, coal tips and hot springs [4]. Microbial lipases are of special interest because of their stability in organic solvents, lack of requirement for cofactors, broad substrate specification and high enantioselectivity [5]. Lipases have been used for development of flavors in cheese ripening, bakery products and beverages. Also, lipases are used to aid removal of fat from meat and fish products [6]. Lipases can be further exploited in many newer areas where they can serve as potential biocatalysts. The present study reports on the production, partial purification and properties of a novel lipase from newly isolated bacterium strain.

MATERIALS AND METHODS

Enrichment and Screening of Lipase Producing Bacterial Strain: A number of soil isolate have been screened for lipase production. Enrichment cultivation was done from oil contaminated soil samples. Enrichment

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medium contains (g l^{-1}): peptone, 5.5; yeast extract, 2.5; agar, 20 and tributyrin, 1% (v/v) and initial pH was adjusted to 7.0. After 15 days of enrichment isolates were selected by appearance of halo zone around growing colonies [7]. On further subculturing for 6 days, few isolates were selected as potent lipase producers.

Identification of Isolated Bacterium: Identification was done by morphological, biochemical and 16S rDNA sequence analysis. For rDNA sequence analysis prokaryotic 16S rRNA specific primers were used to amplify 16S rDNA from isolated bacterium using PCR. The sequence of forward and reverse primer was 5'-AGAGTRTGATCMTYGCTWAC-3', 5'-CGYTAMCTTWTTACGRCT-3' respectively. The PCR mixture contains 400 ng each primer, 1µl DNA, 4 µl dNTPs (2.5mM each), 10 µl 10X Taq DNA Polymerase assay buffer, 1 µl Taq DNA Polymerase enzyme (3U μl^{-1}) and total reaction volume was leveled to 100 µl with water. Sequencing of 16S rDNA was performed by Chromous Biotech Pvt. Ltd., Bangalore, India.

Enzyme Assay- Spectrophotometric Method: Lipase activity was assayed using p-nitrophenylpalmitate (pNPP) as substrate [8]. Enzyme solution (50 µl) was added to 950 µl of the substrate solution consisting of one part solution A (5.0 mM pNPP in 2-propanol) and nine parts solution B (100 mM potassium phosphate buffer pH 7.0, 0.4 % Triton X-100 and 0.1 % gum arabic), which was freshly prepared before use. The reaction mixture was incubated at 37°C for 20 min and absorbance was read at 410 nm. Enzyme activity and specific activities were calculated as per the following formula:

$$\text{Lipase activity (U/ml)} = \frac{A \times B}{C \times D \times E}$$

Where, A- µmol of p-Nitrophenol released, B- Total volume, C- Vol. used in spectrophotometric determination, D- vol. of enzyme used in assay and E- Time of incubation

$$\text{Specific activity (U/mg)} = \frac{\text{Enzyme activity (U/ml)}}{\text{Protein content (mg/ml)}}$$

Other Methods: Protein concentration was estimated according to the method of Lowry *et al.* [9]. Protein content in each fraction was determined by reading absorbance at 280 nm. SDS-PAGE was performed on a 12% polyacrylamide slab gel by the method described by Laemmli [10] and also used to determine the molecular mass of the enzyme.

Lipase Production and Cell Free Extract: For inoculum development, 100 ml tributyrin agar medium was inoculated with loopful culture of isolate RK31. After 24 h of incubation, 2% inoculum was transferred to 250 ml of production medium (g l^{-1}: peptone, 5.5; yeast extract, 2.5; agar 15 and tributyrin 1% (v/v), pH 7.0). The flasks were incubated for 24 h at 37 °C in orbital shaker incubator under shaking conditions of 180 rpm.

Purification of Extracellular Lipase Enzyme: Precipitation of proteins was performed according to the chart as mentioned by Gomori [11]. The 30 ml retentate was treated with 50, 60, 70, 80 and 90% saturation of ammonium sulphate. For each saturation percentage, lipase activity and protein concentration was measured and specific activity was calculated.

Dialysis was carried out to remove the traces of ammonium sulphate. Fifteen ml sample was poured in the Dialysis apparatus (Wonder Lyzer, India) and was kept in inverted position in 500 ml beaker containing distilled water. It was then kept on magnetic stirrer for 24 h at room temperature and after every 6 h distilled water was changed.

Gel filtration chromatography was performed as described by Bayoumi *et al.* [12]. The concentrate was applied to the column packed with Sephadex G-200 (1.2 x 20cm) equilibrated with Triss-HCl buffer, pH 8 and then the enzyme was eluted with the same buffer containing 0.1N NaCl. The flow rate was adjusted to 2 ml min^{-1}, the fraction volume of 5 ml each was collected and processed for determination of specific enzyme activity.

Ion exchange chromatography was performed using DEAE cellulose as described by Kim *et al.* [13]. Two ml sample was applied to the column packed with DEAE cellulose resin (20x1.2cm column size) and equilibrated with Triss HCl buffer pH 8.0. Fractions of 5ml each were harvested by 0.1% NaCl.

The enzyme was electrophoresed on a 12% Sodium dodecyl sulfate Polyacrylamide gel (SDS-PAGE) according to the method of Laemmli [10] on a Genei-Bangalore, India, electrophoretic system. PAGE was run on 4% stacking gel and 12% resolving gel with 25 mA initial and 60mA running current. Protein bands were located by coomassie brilliant blue stain.

Analytical RP-HPLC of Purified Lipase: Analysis of purified lipase was done by RP-HPLC [14] under following conditions: Column C-8, Phenomenax (250 x 4.6 mm, 5 µm); flow rate 1 ml min^{-1}. Initial solution (sol A): 0.1%

trifluoroacetic acid in water, elution with 60% acetonitril in sol A (sol B); total volume: 50 ml. Protein was detected at 280 nm.

Properties of Purified Enzyme

Effect of Temperature and pH: For the effect of temperature, the purified enzyme was incubated at 4°C, 30°C, 40°C, 50°C, 60°C and 70°C for a period of 1h using p-nitropalmitate as substrate. After incubation the enzyme was immediately cooled in ice bath for 20 min and the residual lipase activity was determined.

To study the effect of pH, the lipase activity was measured at pH of 4, 5, 6, 7, 8 and 9. The pH of the reaction mixture was varied using acetate buffer, pH 4; Phosphate buffer, pH 6 and Triss HCl buffer, pH 8. One hundred and fifty µl purified lipase was incubated in different buffers for 24 h at 37°C and residual specific activity was determined.

Effect of Organic Solvents: The effect of organic solvents viz. diethyl ether, hexane, ethanol, petroleum ether, acetone, methanol, 1- propanol, 2- propanol, ethyl acetate, chloroform, DMSO, cyclohexane, butanol and benzene were investigated at 10% concentration. The enzyme was incubated in presence of organic solvents for 30 min and relative activity (%) was calculated.

Km and Vmax Determination: Michaelis-Menten constant for lipase substrate pNPP was determined by incubating with concentrations of substrate ranging from 10 to 80 mmol/l with purified lipase. The value of constant was calculated according to the Lineweaver-Burk graphic method.

Effect of Inhibitors: Effect of $CaCl_2$, $MgCl_2$ and EDTA-K was studied for their inhibitory effect on purified lipase at 1mM and 10mM concentrations.

Chemicals: *p*-nitropalmitate and sephadex G-200 were purchased fron Sigma, Aldrich USA; DEAE Cellulose was from Himedia Chemicals, Mumbai, India and all other chemicals were of high purity. Protein low molecular weight markers were purchased from Chromous Biotech Pvt. Ltd., Bangalore, India.

RESULTS

Selection and Identification of Lipase Producing Bacterial Strain: Sixty six bacterial isolates were grown in tributyrin agar medium, pH 7.0 at 37°C in order to isolate bacteria for potent lipase producing ability. A screening test of lipolytic productivity of all bacterial isolates resulted in the fact that only ten bacterial isolates were found to be very good lipase enzyme producer. The one most potent bacterial isolate RK31 (enzyme activity 1.98U/ml) was selected for further study.

Identification was carried out by morphological, biochemical and 16S rDNA sequence analysis and the strain was identified as *Bacillus pumilus* RK 31. The 16S rDNA sequence was submitted to NCBI Gene Bank depository under the accession no GQ463238. 16S rDNA sequence of isolated bacterium exhibited the homology with other bacterium, *Bacillus pumilus* ATCC 7061 (99%), *Bacillus pumilus* SAFR-032 (99%), *Bacillus amyloliquefaciens* FZB42 (97%), *Bacillus subtilis* JH642 (97%) and *Bacillus subtilis* NCIB3610 (97%).

Purification of Extracellular Lipase: To precipitate lipase by ammonium sulphate, experiment was conducted at 50%, 60%, 70%, 80% and 90% saturation of ammonium sulphate salt. Results revealed that 60% saturation was proved to be effective for maximum specific activity of 123.82U/mg with purification fold of 6.53 (data not shown). The 60% ammonium sulphate saturated and precipitated protein was dialyzed against distilled water for 24 h. Dialysis removed 10-20 kDa impurities from the sample and it proved very effective raising specific activity to 211.40 U/ml with 11.74 purification fold.

The purification fold of lipase increased to 127 after applying Sephadex G-200 column chromatography technique. A total 51 fractions of 5 ml each were collected and the maximum specific activity (2406.60 Uml^{-1}) was shown by fraction no 41. This fraction was loaded to ion exchange chromatography with DEAE cellulose column and a total of 51 fractions of 5 ml each were collected. The maximum specific activity (3525.60 Umg^{-1}) was shown by fraction no. 44. This fraction was stored at 4°C pending uses. The overall purification steps protocol resulted in raising the purification fold to 186.04 times. The overall purification scheme is summarized in Table 1.

SDS-PAGE analysis showed a single band of purified lipase after DEAE- Cellulose column chromatography. Comparison with low molecular weight Protein markers, the molecular weight of purified lipase was determined to be 62.2kDa (Figure 1). Figure 2 represents the RP-HPLC chromatogram of DEAE Cellulose fraction. The purified lipase showed two close peaks with retention time of 5.57 min and 5.87 min.

Table 1: Summary of Purification Scheme for extracellular lipase enzyme from *Bacillus pumilus* RK31

Technique	Volume before (ml)	Volume after (ml)	Protein Conc. (µg/ml)	Total protein (mg)	Activity (U/ml)	Total Activity (U)	Specific Activity (U/mg)	Purification fold
Speed Centrifugation	500	450.0	844.00	379.80	16.00	7200.0	18.95	1
Amm. Sulphate Saturation (60%)	200	30.0	500.00	15.00	61.91	1857.3	123.82	6.53
Gel filteration (Sphadex G-200)	0.5	5	89.00	0.445	214.18	1070.90	2406.6	127
Ion exchange (DEAE cellulose)	2	5	76.00	0.380	270.13	1350.65	3525.6	186

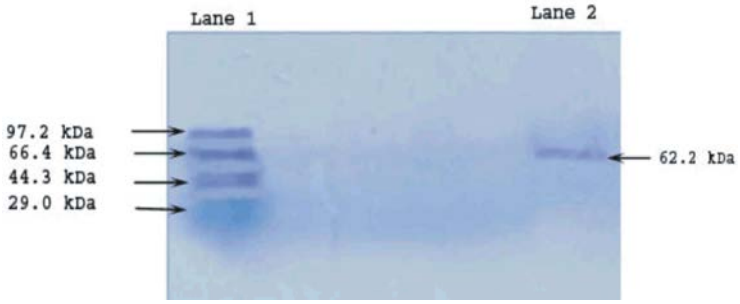

Fig. 1: Photographic representation of SDS-PAGE Gel (Lane 1, Marker Proteins; Lane 2 showing lipase single band after DEAE Cellulose column chromatography)

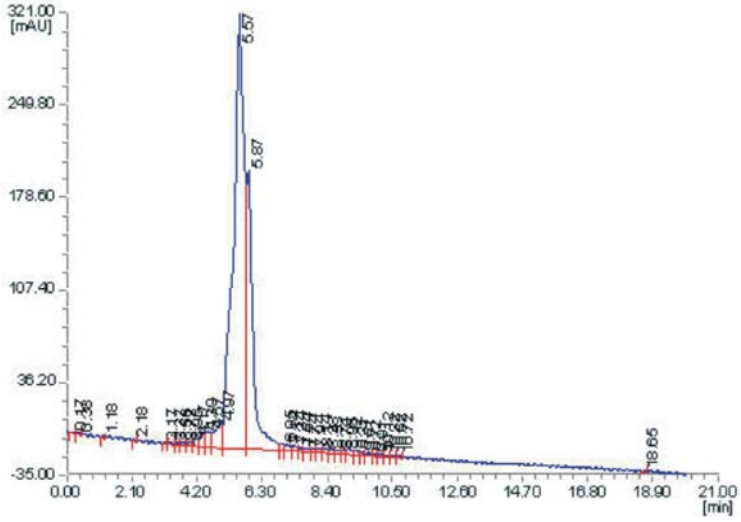

Fig. 2: RP-HPLC chromatogram of DEAE-Cellulose fraction of purified lipase

Properties of Purified Lipase Enzyme

Temperature and pH Effect: The purified lipase (specific activity 213.51 U mg^{-1}) was stable at 40°C, 50°C and 60°C retaining the 66%, 66% and 69% specific activities, respectively but stability decreased to 52% at 70°C (Figure 3). Results of pH effect showed that the enzyme is most active at pH 6, retaining about 84% of its original activity while at pH 8.0 about 76% of its original specific activity get destroyed. Below or above pH 6.0, activity of lipase get decreased (Figure 4).

Tolerance Against Organic Solvents: The enzyme exhibited the stability (>75%) against petroleum ether, hexane, cyclohexane, chloroform, acetone, ethanol, 1-propanol and 2-propanol organic solvents while the relative activity was in between 68% to 10.1% in diethyl ether, ethyl acetate, benzene butanol, DMSO and methanol (Table 2). The highest relative activity was achieved at 120.5% in petroleum ether with enzyme activity of 4.218 IU/ml. The relative activity of control was assumed as 100% with enzyme activity of 3.50 IU/ml.

Km and Vmax Determination: The Michaelis constant (Km) was determined from the Lineweaver and Burk plot (Figure 5) by dividing the slope of the line with the intercept. The purified enzyme was found to have Km of 1.83 mM l^{-1}. The Vmax was determined as the reciprocal of intercept and calculated as 10.0 mM l^{-1} min^{-1}.

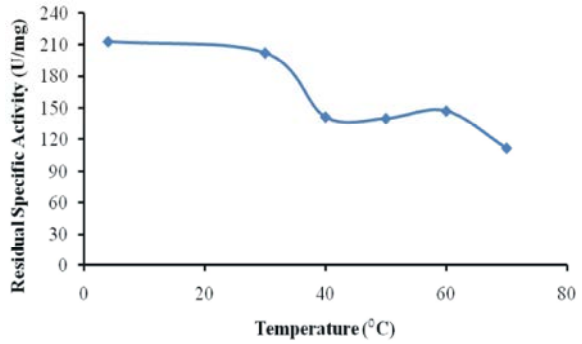

Fig. 3: Thermostability of purified lipase produced by *Bacillus pumilus* RK31

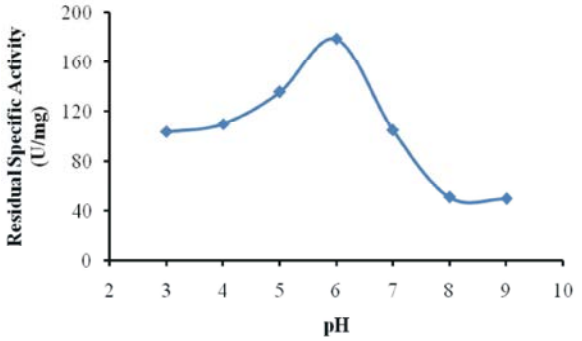

Fig. 4: pH stability of purified lipase produced by RK31 bacterial isolate

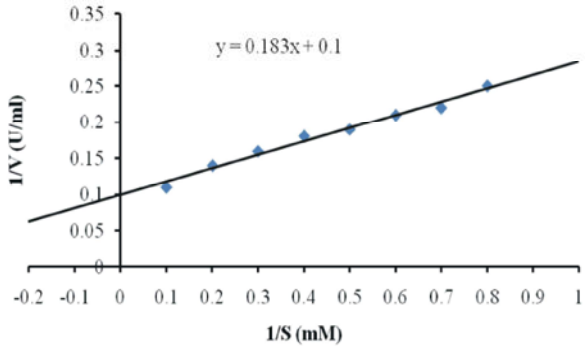

Fig. 5: Lineweaver-Burk plot for purified lipase

Stability Against Inhibitors: Enzyme stability was also checked using various inhibitors at 1mM and 10mM concentrations of $CaCl_2$, $MgCl_2$ and EDTA-K. Result revealed that $CaCl_2$ at 1 mM concentration was inhibitorier than 10 mM conc, reducing the specific activity from 213.51 U mg^{-1} to 120.55 U mg^{-1}. Unlike this, $MgCl_2$ has more inhibitory effect at 10 mM concentration than to 1 mM concentration reducing the specific activity to 105.03 U mg^{-1}. Among all tested inhibitors, maximum inhibitory effect was shown by EDTA-K at 1 mM concentration reducing the specific activity to 84.24 U mg^{-1} (Figure 6).

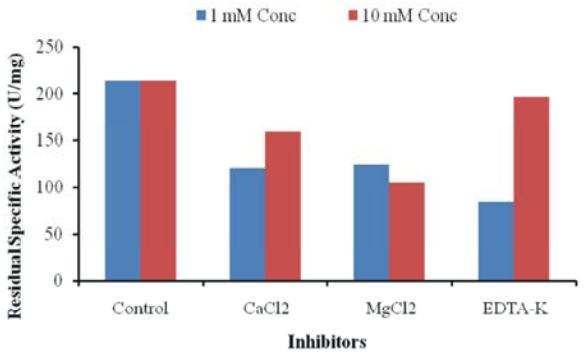

Fig. 6: Effect of inhibitors at 1mM and 10 mM concentration on purified lipase

Table 2: Relative activity (%) of *Bacillus pumilus* RK 31 lipase enzyme against different organic solvents

S.N.	Organic Solvents	Residual activity (%) at concentration of 10%
1	Control	100
2	Petroleum ether	120.5
3	Hexane	97.3
4	Cyclohexane	86.5
5	Benzene	28.8
6	Diethyl ether	10.1
7	Chloroform	84.6
8	Ethyl acetate	33.2
9	Acetone	75.4
10	Methanol	68.0
11	Ethanol	81.3
12	Butanol	63.5
13	1-Propanol	79.5
14	2-Propanol	76.8
15	DMSO	38.6

DISCUSSION

Precipitation of enzymes was carried out by ammonium sulphate since it was highly soluble in water, cheap and had no deleterious effect on structure of protein. Results revealed that 60% saturation was effective concentration with 6.53 fold specific activities, among tested different saturations. These results matches with the purification strategies followed by Moh'd and Juergen [15] from *Thermosyntropha* and Kanwar *et al.* [16] for the precipitation of lipase from *Pseudomonas* sp. G6. Further dialysis after 24h leads to denaturation of enzyme at room temperature and decrease in lipase activity.

Sephadex G-200 and DEAE- Cellulose column chromatography proved to be very effective for lipase purification increasing the fold purification to 127 and 186 times with specific activity reached to 2406.6 U mg^{-1} and 3525.6 U mg^{-1}, respectively. This would be possible

due to the removal of inhibitory and/or high molecular weight contamination during these purification stages. In view of lipase purification by many authors, the extracellular lipase by *Bacillus stearothermophilus* MC7 was purified to 19.25 fold with 10.2% recovery and a specific activity of about 12 U mg^{-1} was reported by Kambourova *et al*. [17]. In a similar study a thermostable lipase produced by a thermophilic *Bacillus* sp. J33 was purified to 175-fold by ammonium sulphate and phenyl sepharose column chromatography [18]. Lee *et al.* [19] purified a lipase from *B. thermoleovorans* ID-A (BTIDA) and *B. thermoleovoran*-ID-B (BTIDB) with a purification fold of 300 and 108 respectively, while the overall yield was 16 and 3.2%, respectively. Sharma *et al.* [20] purified a thermostable alkaline lipase from *Bacillus* sp. RSJ-1 and resulted in 201 fold purification with 19.7% final yield. Also 19 fold purification was attained using ammonium sulphate fractionation, ion exchange chromatography and gel filtration by Gopinath *et al.* [21]. Lipase from *Pseudomonas* sp. Yo103 was purified using ammonium sulphate fractionation, DEAE cellulose and Sphadex G200 to 62 fold increase and 3.7% yield by Kim *et al*. [22]. In this study the results are encouraging in the sense that only three purification steps are needed to achieve 186 fold purification and specific activity of 3525.6 U mg^{-1}. At industrial point of view the minimum steps are prerequisite to purify the enzyme at homogeneity so that the production cost can be controlled.

Purified fraction from ion exchange chromatography (DEAE cellulose) showed molecular weight of lipase to be 62.2 kDa which is in accordance with the previous results [4] on *Bacillus* strain A30-1 as purified lipase was found to have molecular weight of 65 kDa. Kim *et al.* [23] isolated a lipase from *Bacillus* sp. strain 398 showing molecular weight of 50 kDa. Brune and Gotz [24] isolated a 55 kDa lipase from *Pseudomonas fluorescence* MC50 which was stable over pH 6.0-9.0. Gaur *et al.* [25] have isolated and purified a lipase which was of about 60 kDa and it was stable at 6.0-8.5 pH range.

In Contrast to SDS-PAGE where only one protein band was resolved on gel, two peaks of purified fraction were appeared on HPLC chromatogram. The two peaks probably indicate the isoforms of lipase and little difference between their retention times might indicate a strong similarity of both lipases. The presence of multiple lipases was reported for *Rhizopus* sp. [14].

Lipases appear to obey Michaelis-Menten kinetics [26]. The characteristics of enzyme were Km and Vmax which were found to be 1.83 mM l^{-1} and 10.0 mmol l min^{-1}, respectively. The *Trichoderma viride* lipase was found to have Km of 1.14 mM l^{-1} and Vmax of 0.056 mM l^{-1} min^{-1} [27].

In order to assess the utility and compatibility of enzymes with the commonly used detergents, its properties such as pH and temperature stability *etc.* should be determined. The *B. pumilus* RK31 lipase was stable over a wide temperature of 30°C to 60°C. Similar to this the lipase from *Burkholderia cepacia* ATCC 25416 appears to be more tolerant to temperature as it has been reported to be stable in the range of 30°C to 60°C [28] *Pseudomonas luteola* lipase had shown thermostability at 65°C [29].

The *B. pumilus* RK31 lipase was active over a wide range of pH from 4 to 7 with optimum activity at pH 6.0. *Serratia marcescens* Sr41 8000 lipase was stable between pH 6 to 9 [30]. Other lipases showed stability within the pH range of 5.5 to 9 [31, 32]. The wide range of pH and temperature tolerance decides the usefulness of a particular enzyme for a specific industrial application [33].

The effect of organic solvents was studied by water miscible (acetone, methanol, ethanol, butanol, 1-propanol, 2-propanol, DMSO) and water immiscible organic solvents (petroleum ether, hexane, cyclohexane, benzene, diethyl ether, chloroform, ethyl acetate) as described in Table 2. The maximum relative activity was represented in petroleum ether (120%) and in hexane (97%) which are relatively non polar solvents. Some study suggested that the enzyme exhibited more stability and activity in solutions containing hydrophilic or hydrophobic organic solutions than in organic solvent-free aqueous solution. Sometimes, the replacement of some water molecule by organic solvent molecules may stabilize the structure of the enzyme [34]. Thus the *B. pumilus* RK31 lipase bearing good stability against nonpolar organic solvents could be exploited for industrial purposes. The enzyme showed moderate stability in methanol (68%), ethanol (81.3%), butanol (63.5%), 1-propanol (79.5%) and 2-propanol (76.8%). These might be because the short-chain alcohols like methanol and ethanol have low solubility in oils; therefore, a new liquid phase appears in the system at moderate concentrations leading to an inactivation of the enzyme. The results are more or less similar with the results of Uttatree *et al*. [35] on lipase production by *Acinetobacter baylyi*. The relative activity in DMSO was comparatively less (38.6%). DMSO being water miscible has the property to dissolve the enzyme which invariably resulted in the inactivation of protein [36]. The tolerance of this enzyme against

different solvents suggested potential for its use in organic synthesis, enantioselectivity and related applications.

Lipase inhibitors have been used in the study for structural and mechanistic properties and were tested at 1mM and 10mM concentrations. It was found that EDTA-K almost inhibited the lipase activity at 1mM concentration. The $CaCl_2$ and $MgCl_2$ were found to reduce the enzyme activity to a limited extent. Similar to this, EDTA treatment strongly inhibited the *B. thermooleovorans* ID-1 lipase enzymes [19] and *Bacillus* sp. THLO27; a metallo lipase [37]. Lipase obtained from *Acenatobacter* sp. RAG-1 was found to be strongly inhibited by EDTA and stabilized by Ca^{+2} as described by Snellman *et al.* [38]. Ranjitha *et al.* [39] have reported that EDTA inhibits lipase activity almost completely. Stability of lipase activity is obviously related to its configuration and the melting point. Further, the search for lipase inhibitors is also of pharmacological interest because lipase inhibitors are used for designing drugs for the treatment of obesity and the problem of acne.

CONCLUSION

The *Bacillus pumilus* RK31 strain was isolated and characterized through 16S rDNA sequence analysis with an aim to produce lipase enzyme. The enzyme was purified by chromatographic techniques by using only three main steps and further the purified enzyme was characterize so as to explore the potential of produced lipase enzyme at industrial level. The enzyme was purified 186 times with good specific activity which suggests its future role at industrial level. Further study will focus on the application of purified enzyme at therapeutic level.

REFERENCES

1. Saxena, R.K., A. Sheoran, B. Giri and W.S. Davidson, 2003. Purification strategies for microbial lipases. J. Microbiol. Methods, 52: 1-18.
2. Ghosh, P.K., R.K. Saxena, R. Gupta, R.P. Yadav and S. Davidson, 1996. Microbial lipases: production and applications. Sci. Prog., 79: 119-157.
3. Akimoto, M., Y. Nagashima and D. Sato, 1999. A kinetic study on lipase-catalyzed interesterificaiton of soybean oil with oleic acid in a continuous packed bed reactor. Appl. Biochem. Biotechnol., 81: 131-142.
4. Wang, Y., K.C. Srivastava, G.J. Shen and H.Y. Wang, 1995. Thermostable alkaline lipase from a newly isolated thermophilic Bacillus strain A30-1 (ATCC 53841). J. Ferment. Bioeng., 79: 433-438.
5. Godfrey, T. and S. West, 1996. Introduction to Industrial Enzymology. In: T. Godfrey and S. West, (ed.), Industrial Enzymology 2nd edn, New York: West Macmillan Publishers Inc., pp: 1-8.
6. Kazlauskas, R.J. and U.T. Bornscheuer, 1998. Biotransformations with lipases. In: H.J. Rehm, G. Pihler, A. Stadler and P.J.W. Kelly, (eds.), Biotechnology. New York: VCH, 8: 37-192.
7. Akatsuka, H., E. Kawai, N. Sakurai and K. Omori, 2003. The *Serratia marcescens* bioH gene encodes an esterase. Gene, 302: 185-192.
8. Iftikhar, T., M. Niaz, M. Afzal, Ikram-Ul-Haq and M.I. Rajoka, 2008. Maximization of intracellular lipase production in a lipase-overproducing mutant derivative of *Rhizopus oligosporus* DGM 31: A Kinetic Study. Food Technol. Biotechnol., 46: 402-412.
9. Lowry, O.H., N.J. Rosebrough, A.L. Farr and R.J. Randall, 1951. Protein measurement with the Folin- phenol reagent. J. Bio. Chem., 193: 265-275.
10. Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of head of bacteriophage T4. Nature, 227: 680-685.
11. Gomori, G., 1955. Preparation of buffers for use in enzyme active studies. In: S.P. Colwick and N.O. Kaplan, (Eds.), Methods in Enzymology, New York: Academic Press Inc. Pub., 1: 138-146.
12. Bayoumi, R.A., S.S. EL-Louboudey, N.M. Sidkey and M.A. Abd-El-Rahman, 2007. Production, purification and characterization of thermoalkalophilic lipase for application in bio-detergent industry. J. Appl. Sci. Res., 3: 1752-1765.
13. Kim, H.K., S.Y. Park and T.K. Oh, 1997. Purification and partial characterization of thermostable carboxyl esterase from *Bacillus stearothermophilus* L1. J. Microbiol. Biotechnol., 7: 32-36.
14. Koblitz, M., B. Gabriela and G.M. Pastore, 2006. Purification and biochemical characterization of an extracellular lipase produced by a new strain of *Rhizopus sp*. Cienc. Agrotec. Lavras, 30: 494-502.
15. Moh'd, A.S. and W. Juergen, 2007. Purification and characterization of two highly thermophilic alkaline lipases from *Thermosyntropha*. Appl. Environ. Microbiol., 73: 7725-7731.

16. Kanwar, L., B.K. Gogoi and P. Goswami, 2002. Production of a *Pseudomonas* lipase in n-alkane substrate and its isolation using an improved ammonium sulfate precipitation technique. Bioresour. Technol., 84: 207-211.
17. Kambourova, M., N. Kirilova, R. Mandeva and A. Derekova, 2003. Properties of thermostable lipase from a thermophilic *Bacillus stearothermophilus* MC7. J. Molecular Catalysis B. Enzymatic, 22: 307-313.
18. Nawani, N. and J. Kaur, 2000. Purification, characterization and thermostability of a lipase from a thermophilic Bacillus sp. J33. Mol. Cell Biochem., 206: 91-96.
19. Lee, D.W., Y.S. Koh, K.J. Kim, B.C. Kim, H.J. Choi, D.S. Kim, M.T. Suhartono and Y.R. Pyun, 1999. Isolation and characterization of a thermophilic lipase from Bacillus thermoleovorans ID-1. FEMS Microbiol. Lett., 179: 393-400.
20. Sharma, R., S.K. Soni, R.M. Vohra, R.S. Jolly, L.K. Gupta, J.K. Gupta, 2002. Production of extracellular alkaline lipase from a Bacillus sp. RSJ1 and its application in ester hydrolysis. Ind. J. Microbiol., 42: 49-54.
21. Gopinath, S.C.B., A. Hildal, T.L. Priya and G. Annadurai, 2002. Purification of lipase from Cunninghamela verticillata and optimization of enzyme activity using response surface methodology. World J. Microbiol. Biotechnol., 18: 449-458.
22. Kim, M.H., H.K. Kim, J.K. Lee, S.Y. Park and T.K. Oh, 2000. Thermostable lipase of Bacillus stearothermophilus: high level production, purification and calcium-dependent thermostability. Biosci. Biotechnol. Biochem., 64: 280-286.
23. Kim, H.K., M.H. Sung, H.M. Kim and T.K. Oh, 1994. Occurance of thermostable lipase in thermophilic Bacillus sp strain 398. Biosci. Biotechnol. Biochem., 58: 961-962.
24. Brune, A.K. and F. Gotz, 1992. Degradation of Lipids by Bacterial Lipases. In: G. Winkelmann, (ed.), Microbial Degradation of Natural Products, Weinheim, New York: Wiley-VCH Verlag Gmb, pp: 243-263, ISBN 3527283544.
25. Gaur, R., A. Gupta and S.K. Khare, 2008. Purification and characterization of lipase from solvent tolerant Pseudomonas aeruginosa PseA. Process Biochemistry, 43: 1040-1046.
26. Guit, R.P.M., G.W. Meindersma, M.J.J. Mayer, E.M. Meijer, J. Kamphuis and M. Kloosterman, 1991. Lipase kinetics: hydrolysis of triacetin by lipase from Candida cylindracea in a hollow fiber membrane reactor. Biotechnol. Bioeng., 38: 727-732.
27. Kashmiri, M.A., A. Adnan and B.W. Butt, 2006. Production, purification and partial characterization of lipase from Trichoderma Viride. African J. Biotechnol., 5: 878-882.
28. Wang, X., X. Yu and Y. Xu, 2009. Homologous expression, purification and characterization of a novel high-alkaline and thermal stable lipase from Burkholderia cepacia ATCC 25416. Enzyme and Microbial Technol., 45: 94-102.
29. Litthauer, D., A. Ginster and E.V.E. Skein, 2002. Pseudomonas luteola lipase: a new member of the 320-residue Pseudomonas lipase family. Enzyme Microb. Technol., 30: 209-215.
30. Matsumae, H. and T. Shibatani, 1994. Purification and characterization of the lipase from Serratia marcescens Sr41 8000 responsible for asymmetric hydrolysis of 3-phenylglycidic acid esters. J. Ferment. Bioeng., 77: 152-158.
31. Fox, P.F. and L. Stepaniak, 1983. Isolation and some properties of extracellular heat-stable lipases from Pseudomonas fluorescens strain AFT 36. J. Dairy Res., 50: 77-89.
32. Kumura, H., K. Mikawa and Z. Saito, 1993. Purification and characterization of lipase from Pseudomonas fluorescens No. 33. Milchwissenschaft, 48: 31-434.
33. Ramarethinam, S., K. Latha and N. Rajalakshmi, 2002. Use of a fungal lipase for enhancement of aroma in black tea. Food Sci. Technol. Res., 8: 328-332.
34. Ogino, H. and H. Ishikawa, 2001. Enzymes which are stable in the presence of organic solvents (review), J. Biosci. Bioeng., 91: 109-116.
35. Uttatree S., P. Winayanuwattikun and J. Charoenpanich, 2010. Isolation and characterization of a novel thermophilic organic solvent stable lipase from Acinetobacter baylyi. Appl. Biochem. Biotechnol., 162: 1362-1375.
36. Hun, C.J., R.N.Z. Abd Rahman, A.B. Salleh and M. Basri, 2003. A newly isolated organic solvent tolerant Bacillus sphaericus 205y producing organic solvent-stable lipase. Biochemical Engineering J., 15: 147-151.

37. Dharmsthiti, S. and S. Luchai, 1999. Production, purification and characterization of thermophilic lipase from Bacillus sp. THL027. FEMS Microbiol. Lett., 179: 241-246.
38. Snellman, E.A., E.R. Sullivan and R.R. Colwell, 2002. Purification and properties of the extracellular lipase, Lip A of Acinetobacter sp. RAG-1. Eur. J. Biochem., 269: 5771-5779.
39. Ranjitha, P., E.S. Karthy and A. Mohankumar, 2009. Purification and characterization of lipase from marine Vibrio fischeri. International J. Biol., 1: 48-56.