JOURNAL OF PURE AND APPLIED MICROBIOLOGY, June 2016. Vol. **10**(2), p. 1435-1442

Purification and Characterization of Extracellular Lipase from *Pseudomonas aeruginosa* KF 853103

Baljot Kaur[1], Monica Sachdeva Taggar[2]*, Poonam Sharma[3] and Manjeet Kaur Sangha[1]

[1]Department of Biochemistry, [2]School of Energy Studies for Agriculture, [3]Department of Plant Breeding and Genetics, Punjab Agricultural University, Ludhiana – 141004, India

(Received: 03 February 2016; accepted: 09 March 2016)

An extracellular lipase produced by *Pseudomonas aeruginosa* KF 853103 was purified to 15-fold with a specific activity of 1671.9 pkat mg^{-1} protein after ammonium sulphate precipitation and DEAE-cellulose column chromatography. The purified enzyme showed pH optima of 8.0 and temperature 40°C and was relatively stable within the temperature range of 30-40°C. The enzyme retained 96.5, 80.3 and 93.4 of its maximum activity in the presence of methanol, ethanol and acetone, respectively. However, the relative activity of enzyme was comparatively less in the presence of hexane and propanol (64.8 and 52.8%). The K_m and V_{max} values for lipase were found to be 0.136mM and 71.4 pkat ml^{-1}, respectively. From the study of effect of temperature on K_m and V_{max}, free energy change (ΔG), enthalpy change (ΔH), entropy change (ΔS) and energy of activation (E_a) were reported to be -23.2 kJ mol^{-1}, 15.9 kJ mol^{-1}, 124.7 J K^{-1} mol^{-1} and 19.1 kJ mol^{-1}, respectively.

Keywords: Extracellular lipase, *Pseudomonas aeruginosa*, Purification.

Enzymes such as lipases (triacylglycerol acyl hydrolases, EC 3.1.1.3) are water- soluble enzymes having the ability to hydrolyse triacylglycerols to release free fatty acids and glycerol. Lipases constitute a major group of biocatalysts that have immense biotechnological applications. Lipases have been isolated and purified from fungi, yeast, bacteria, plant and animal sources[1]. Microbial lipases have gained special industrial attention due to their stability, selectivity and broad substrate specificity[2]. Microbial enzymes are also more stable than their corresponding plant and animal enzymes and their production is more convenient and safer[3]. Many lipases are limited in use because they are substrate-specific and regioselective. However, majority of the lipases are capable of converting triglycerides, diglycerides, monoglycerides and free fatty acids to fatty acid ethyl esters in addition to fat hydrolysis[1, 4]. Biochemical characterization of lipases is a preliminary requirement to identify their unique position and/or stereo-specificity, which will enable them to be used as candidate biocatalyst for concentrating a specific class of fatty acids[5]. The present study highlights the production, purification and characterization of an extracellular lipase from *Pseudomonas aeruginosa*.

* To whom all correspondence should be addressed.
E-mail: monicasachdeva@pau.edu

MATERIALS AND METHODS

The present study was carried out at Biodiesel Laboratory, School of Energy Studies for Agriculture, Punjab Agricultural University, Ludhiana (Punjab).

Procurement of microbial cultures

Pseudomonas aeruginosa KF 853103 was procured from the Pulses Microbiology Laboratory, Department of Plant Breeding and Genetics, Punjab Agricultural University, Ludhiana. The bacterial strain was repeatedly sub-cultured and maintained on nutrient agar slants.

Determination of growth and lipase activity of bacterial strain

Pseudomonas aeruginosa KF 853103 was studied for its growth and extracellular lipase activity on minimal salt media using olive oil as carbon source. Lipase production in liquid culture was carried out by the method of Gokbulut and Arslanoglu (2013)[6] with slight modifications. The minimal medium used for lipase production consisted of 200 ml of 5X minimal salt solution ($Na_2HPO_4.7H_2O$, 64 g/l; KH_2PO_4, 15 g/l; NaCl, 2.5 g/l and NH_4Cl, 5 g/l), 800 ml of water and 0.02 per cent gum arabic. The medium was adjusted to pH 7.0, autoclaved and cooled. Then 2 ml 1M $MgSO_4$ (sterile filtrated) and 0.1ml 1M $CaCl_2$ (sterile filtrated) was added to medium.

The inoculum was prepared by transferring a loopful of stock culture to minimal media broth (20 ml) in 150 ml flasks containing 1 per cent (w/v) olive oil as carbon source. The cultivation was performed at ±28°C with constant shaking at 150 rpm in an orbital shaking incubator until absorbance at wavelength of 600 nm (A_{600}) reached to 0.6. Lipase production was carried out by transferring 10 ml of prepared inoculum into 250 ml triplicate flasks containing 100 ml of minimal media broth supplemented with olive oil (1% w/v) as carbon source. The inoculated flasks were incubated at ±28°C with constant shaking at 150 rpm in an orbital shaking incubator. The aliquots of culture broth were withdrawn at an interval of 24, 36, 48, 60, 72, 96 and 120 h to determine the lipase activity. For this the bacterial cells were harvested from broth by centrifugation at 10,000 rpm for 15 min at 4°C. The cell free supernatant, thus, collected was used as crude extract to determine the activity of extracellular lipase. The relative growth of bacterial strain was measured in terms of absorbance ($A_{600\ nm}$) using visible spectrophotometer at different hours after incubation (24, 36, 48, 60, 72, 96 and 120 h).

Determination of lipase activity

Lipase activity was determined spectrophotometrically using p-nitrophenyl palmitate (p-NPP) as substrate by the method of Winkler and Stuckmann (1979)[7] with slight modifications. The substrate solution consisted of solution A and solution B. Solution A was prepared by adding 40mg of p-NPP in 12 ml of isopropanol. Solution B was prepared by adding 0.1 g of gum arabic and 0.4 ml Triton X-100 in 90 ml of distilled water. Finally, 1 ml of solution A was added to 19 ml of solution B drop wise with continuous stirring to obtain a substrate emulsion that remained stable for 2 h. The reaction mixture for lipase assay contained 1 ml of substrate emulsion, 0.5 ml of buffer (50 mM Tris HCl, pH-8.0), 0.1 ml of crude enzyme and volume was made up to 3 ml with distilled water. The reaction mixture was incubated at 40°C for 45 min in a temperature-controlled water bath. The reaction was stopped by adding 0.5 ml of isopropanol and the absorbance was measured at 410 nm against blank. The standard curve was prepared by using p-nitrophenol (0.01 to 0.2 µmoles). The amount of enzyme activity was expressed as pkat of p-nitrophenol (p-NP) produced per ml under standard assay conditions. The protein content of enzyme extracts was estimated by method of Lowry *et al* (1951)[8]. The specific activity was expressed as pkat of p-nitrophenol (p-NP) produced per mg protein.

Production of lipase enzyme

The bacterial strain was grown in 1.0 litre minimal medium broth containing 1 per cent olive oil (v/v) at ±28°C with constant shaking at 150 rpm in an orbital shaking incubator. The culture broth was collected after 72 h of incubation and centrifuged at 10,000 rpm at 4°C for 20 min. The cell-free culture supernatant was used as crude lipase and partially purified by ammonium sulfate precipitation followed by DEAE-cellulose column chromatography.

Partial purification and characterization of lipase enzyme

Ammonium sulphate precipitation

The ammonium sulfate precipitation was carried out by adding solid ammonium sulfate to crude enzyme mixture until 0-30 per cent saturation (w/v) with continuous stirring at 4°C. The mixture was then allowed to stand overnight at 4°C with gentle stirring and centrifuged at 10,000 rpm for 15 min. The precipitates were dissolved in minimum

volume of 0.1M sodium phosphate buffer, pH 7.2 and was dialyzed against the same buffer (20 times diluted) overnight at 4°C. The supernatant thus obtained, was subsequently fractionated to 30-60 and 60-80 per cent ammonium sulphate saturation. The enzyme activity of these fractions (0-30%, 30-60% and 60-80%) was determined as described earlier.

DEAE-cellulose coloumn chromatography

The ammonium sulphate fraction exhibiting maximum lipase enzyme activity was applied to DEAE-cellulose column (50 × 1.8 cm) pre-equilibrated with 0.1M sodium phosphate buffer (pH 7.2). The enzyme was eluted with the same buffer using stepwise gradient (0.1 M each) of increasing molarity of NaCl (0.1-1.0 M) at a flow rate of 0.5 ml min^{-1}. From this, 5 ml fractions were collected and assayed for lipase activity and protein content.

Effect of pH on enzyme activity

The effect of pH on lipase activity was determined in the pH range of 4.0-10.0. The various pH buffer systems, *viz*. sodium acetate buffer (50 mM, pH 4.0-5.0), phosphate buffer (50 mM, pH 6.0-7.0), Tris-HCl buffer (50 mM, pH 8.0-9.0) and glycine- NaOH buffer (pH 10.0) were used. The optimum pH was determined by plotting relative activity (%) versus pH.

Effect of temperature on activity and stability of the enzyme

The effect of temperature on lipase activity was determined by measuring the enzyme activity at different temperatures (30-80°C) at optimum pH. The optimum temperature was determined by plotting relative activity (%) versus temperature. Similarly, the thermostability of partially purified enzyme was determined by measuring the lipase activity after pre-incubating the enzyme at different temperatures (30-80°C) for 2 h. The lipase activity without any prior incubation at its optimum pH and temperature was taken as 100 per cent.

Effect of organic solvents on the enzyme activity

The effect of various organic solvents, *viz*. methanol, ethanol, propanol, hexane and acetone on the enzyme activity was investigated. The enzyme was incubated in the presence of organic solvents (30% v/v) at optimum pH and temperature for 30 min. The relative activity of the enzyme was expressed by taking activity without the addition of organic solvent as 100 per cent.

Determination of K_m and V_{max}

The effect of substrate concentration on the reaction velocity of lipase was studied by using p-nitrophenyl palmitate (p-NPP) as substrate. The enzyme was incubated with different concentrations of p-NPP ranging from 0.12 to 0.37 mM. Lineweaver-Burk plots were plotted to determine K_m and V_{max} values. Similar studies were performed at different temperatures (30°, 35° and 40°C) in order to determine the effect of temperature on K_m and V_{max} of enzyme. The free energy change ΔG (kJ mol^{-1}), enthalpy change ΔH (kJ mol^{-1}), entropy change ΔS (J K^{-1} mol^{-1}) and energy of activation E_a (kJ mol^{-1}) were calculated for the enzyme catalyzed reaction at its optimum pH and temperature.

RESULTS AND DISCUSSION

Growth and lipase activity of bacterial strain in olive oil liquid culture

The growth of bacterial strain measured in terms of absorbance of bacterial cells at 600 nm (A_{600}) in olive oil liquid culture ranged from 0.21 to 0.96 at different hours after incubation and the growth was observed to be maximum at 72 h and thereafter, a decline in the growth was observed (Fig. 1). Lipase production was correlated with growth and the lipase activity of bacterial strain ranged from 88.46 to 710.05 pkat ml^{-1}. Maximum extracellular lipase activity (726.35 pkat ml^{-1}) was observed at 72 h of incubation, i.e the stationary phase of growth and thereafter, the lipase activity decreased. The decrease in lipase activity at later stages could be due to changes in pH as well as proteolytic degradation of enzyme by proteases released into the culture medium at the end of exponential phase of growth[7]. These results are in accordance with the work done by Gokbulut and Arslanoglu (2013)[6] who reported maximum lipase production during stationary phase of growth (80 to 96 h after incubation). Saeed *et al* (2005)[9] studied that the extracellular lipase enzyme production *of P. aeruginosa* increased gradually at the end of log phase and reached its maximum level (74.66 U/ml) after 48 h of inoculation.

Partial purification and characterization of lipase enzyme

The crude lipase of *P. aeruginosa* was

purified by ammonium sulphate precipitation, followed by dialysis and DEAE-cellulose coloumn chromatography. Among the three different levels of ammonium sulphate saturation, the maximum activity of enzyme was detected at 30-60 per cent ammonium sulphate saturation (Table 1). The enzyme was purified to 2.8-fold with a specific activity of 312.0 pkat mg^{-1} protein relative to crude extract. The enzyme solution obtained after DEAE-cellulose column chromatography was purified to 15-fold with a specific activity of 1671.9 pkat mg^{-1} protein. The elution profile of enzyme (Fig. 2) purified by DEAE-cellulose column chromatography revealed a single protein peak that overlapped with the lipase activity.

The present studies are in concordance with Zouaoui and Bouziane (2011)[10] who purified the extracellular lipase produced by *P. aeruginosa* using ammonium sulphate precipitation and DEAE cellulose column chromatography. The enzyme in the broth was purified 11-fold with an overall yield of 65.51 per cent after single step purification by ion-exchange column chromatography. However, Sharon *et al* (1998)[11] reported that the extracellular lipase produced by *P. aeruginosa* KKA-5 was purified by 518-fold with a specific activity of 2238.16 U mg^{-1} protein using ammonium sulphate precipitation followed by hydroxylapatite column chromatography. Gaur *et al* (2008)[12] purified a lipase from solvent tolerant strain of *P. aeruginosa* PseA by gel exclusion chromatography. The enzyme was purified to 8.6-fold with a specific activity of 143.3 IU mg^{-1} protein.

Effect of different pH on the activity of purified lipase

Structure of proteins is greatly influenced by pH and is therefore, an important parameter affecting enzyme activity. Lipase activity from *P. aeruginosa* was examined in the pH range of 4.0-10.0 at 40°C and the optimal activity was observed at pH 8.0 (Fig. 3). The lipase retained 90.9 and 85.2 per cent of its maximum activity at pH 9.0 and 10.0, respectively. In the acidic range, significant reduction in the enzyme activity was observed. The enzyme retained only 50.0 and 50.8 per cent of maximum activity at pH 4.0 and 5.0, respectively.

The present findings are in accordance with Chakraborty and Paulraj (2009)[5] who reported that the purified lipase produced by *P. fluorescens*

Table 1. Purification of extracellular lipase from *P. aeruginosa*

Purification steps	Total activity (pkat p-nitrophenol)	Total protein (mg)	Specific activity (pkat p-nitrophenol mg^{-1} protein)	Purification fold
Crude	114420.0	1028.4	111.3	1.0
$(NH_4)_2SO_4$ saturation				
0-30%	4000.0	17.1	233.9	2.1
30-60%	6426.6	20.6	312.0	2.8
60-80%	2273.3	10.4	218.6	2.0
DEAE-Cellulose Column Chromatography	535.0	0.32	1671.9	15.0

Table 2. Effect of temperature on K_m and V_{max} of purified lipase from *P. aeruginosa*

Temperature (°C)	K_m (mM)	pK_m ($-\log K_m$)	V_{max} (pkat p-nitrophenol ml^{-1})	$\log V_{max}$
30	0.166	0.78	55.5	1.74
35	0.153	0.82	66.6	1.82
40	0.136	0.87	71.4	1.85

MTCC 2421 was active in the pH range of 7.0 to 9.0 and the optimum activity was exhibited at pH 8.0. The enzyme retained only 27.44 and 18.89 per cent of maximum activity at pH 6.0 and 7.0, respectively. Gaur *et al* (2008)[12] reported that *P. aeruginosa* PseA lipase showed pH optima at 8.0. The lipase showed good pH stability retaining 100 per cent of the activity in pH range of 6.5-8.5.

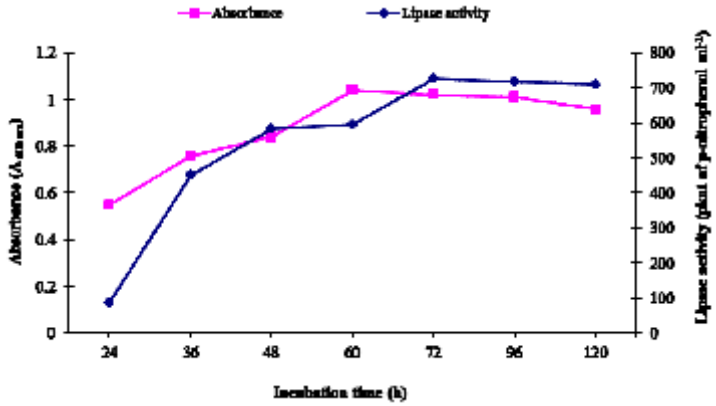

Fig. 1. Time course dependent lipase activity and bacterial growth curve of *P. aeruginosa*

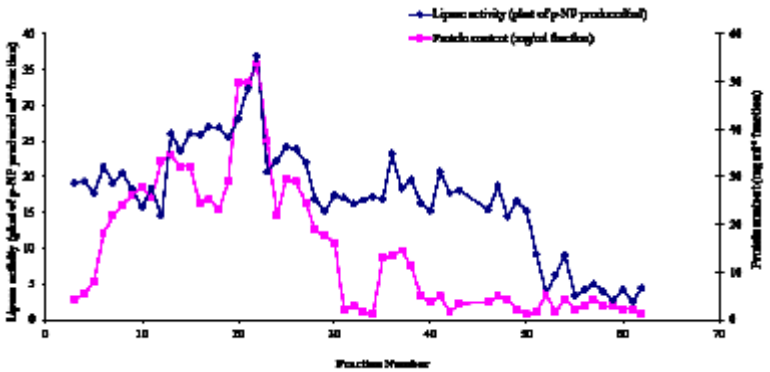

Fig. 2. Elution profile of extracellular lipase from *P. aeruginosa* using DEAE cellulose column chromatography

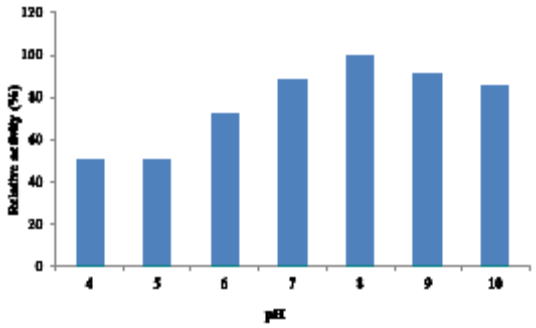

Fig. 3. Effect of pH on the activity of purified lipase from *P. aeruginosa*

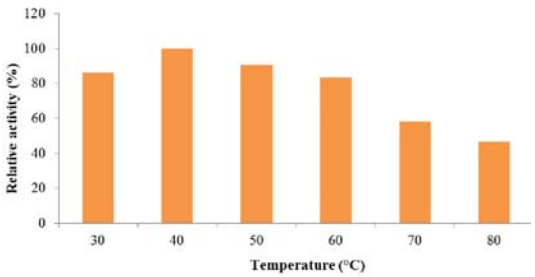

Fig. 4. Effect of temperature on the activity of purified lipase from *P. aeruginosa*

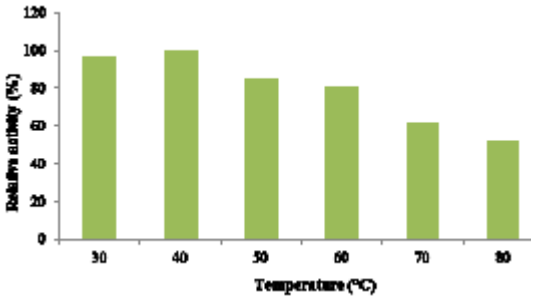

Fig. 5. Effect of temperature on the stability of purified lipase from *P. aeruginosa*

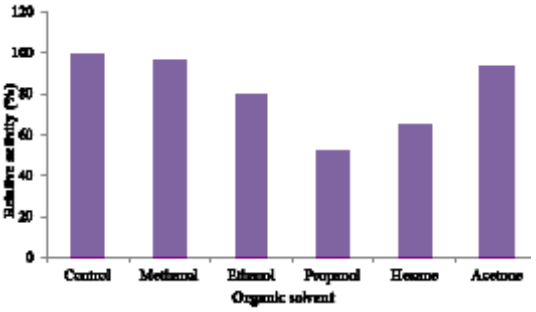

Fig. 6. Effect of different organic solvents on the activity of purified lipase from *P. aeruginosa*

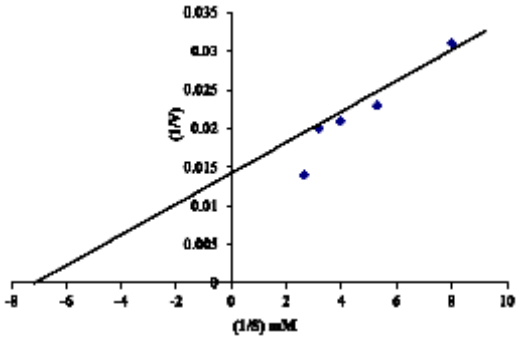

Fig. 7. Lineaweaver-Burk plot of *P. aeruginosa* lipase at its optimum pH and temperature

Effect of temperature on the activity and thermostability of lipase

The effect of temperature on the lipase activity was studied within the range of 30-80°C (Fig. 4). Lipase showed maximum activity at 40°C, whereas at higher temperatures, i.e. 70 and 80°C, the enzyme activity decreased up to 58.3 and 46.5 per cent of its maximum activity.

Similarly, Chakraborty and Paulraj (2009)[5] reported that the purified extracellular lipase from *P. fluorescens* MTCC 2421 was active in the temperature range of 35-50°C with maximum activity at 40°C. The activity was found to be reduced to 50.7 and 93.0 per cent of its initial value at 30°C and 20°C, respectively. The lipase activity declined rapidly above 55°C with only 13 per cent of the activity remained at 60°C. However, the purified

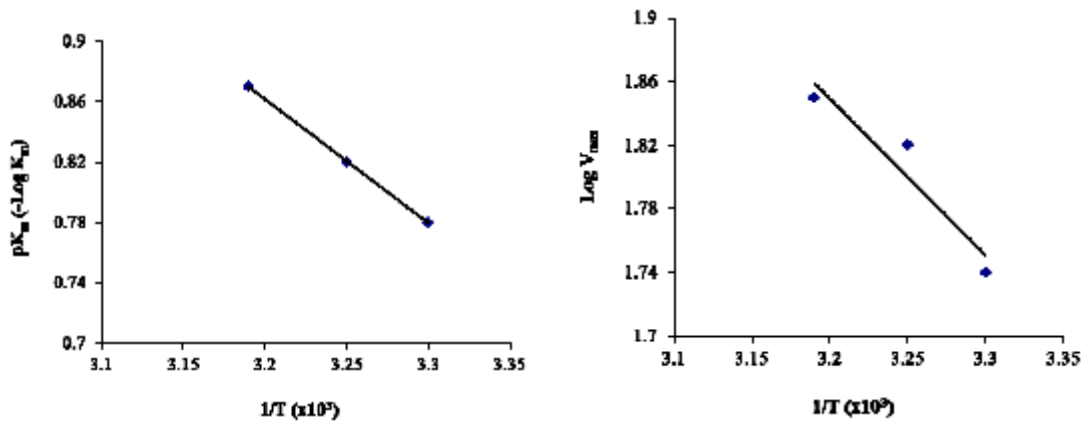

Fig. 8. Effect of temperature on K_m and V_{max} of purified lipase from *P. aeruginosa*

lipase from *P. aeruginosa* SRT 9 exhibited maximum activity at 55°C and the enzyme was found to be stable up to 65°C. As per Borkar *et al* (2009)[13], the activity of enzyme declined above 65°C, thus, indicating the thermal denaturation of enzyme.

The thermostability of lipase was examined by incubating the enzyme at different temperatures (30-80°C) for 2 h at optimum pH (Fig. 5). The enzyme was relatively stable within the temperature range of 30-40°C and retained 97.2 and 100.0 per cent of its activity at 30 and 40°C, respectively. The enzyme retained about 61.9 and 51.9 per cent of its maximum activity after exposure at 70 and 80°C, respectively for 2 h. Similarly, Gaur *et al* (2008)[12] reported the temperature optima of *P. aeruginosa* PseA lipase to be 40°C and the enzyme was found be stable at this temperature for at least 4 h.

Effect of different organic solvents on the activity of lipase

The effect of various organic solvents at 30 per cent (v/v) concentration on the stability of lipase is shown in the Fig. 6. The enzyme retained about 96.5, 80.3 and 93.4 of its maximum activity in the presence of methanol, ethanol and acetone, respectively. However, the relative activity of enzyme was comparatively less in the presence of hexane and propanol (64.8 and 52.8%). The stability of this enzyme in methanol indicated the potential use of methanol for transesterification reaction and biodiesel production.

The low relative activity of enzyme in the presence of propanol and hexane indicated that the long chain alcohols and non-polar organic solvents may have inhibitory effect on the enzyme activity[14]. Similarly, Sharon *et al* (1998)[11] reported that *P. aeruginosa* KKA-5 was found to be stable in the presence of methanol, ethanol and acetone. The study conducted by Ugur *et al* (2014)[14] on stability of lipase Lip SB 25-4 isolated from *Streptomyces bambergiensis* OS 25-4 in hydrophilic and hydrophobic solvents revealed that the enzyme was stable in methanol and ethanol retaining more than two-third of its activity for 60 h.

Determination of kinetic constants

The K_m and V_{max} values for lipase at its optimum pH 8.0 and temperature 40°C from Lineaweaver-Burk plot were found to be 0.136 mM and 71.4 pkat ml^{-1}, respectively (Fig. 7). The lower K_m values indicated that the partially purified lipase had higher affinity for p-NPP. Borkar *et al* (2009)[13] determined the kinetic constants of purified lipase from *P. aeruginosa* SRT9. The K_m and V_{max} values for lipase catalyzed reaction using p-NPP as substrate were reported to be 0.037 mM and 188.6 mmol l^{-1} min^{-1}, respectively. The K_m values decreased with rise in temperature, which was a reflection of higher affinity of enzyme for substrate (Table 2). The values of log V_{max} and pK_m (-log K_m) at different temperatures were plotted against 1/T (Fig. 8). The energy of activation (E_a), enthalpy change (ΔH) and entropy change (ΔS) were calculated from these graphs using following formulae

E_a = -2.303×R×slope (Fig. 8b)

ΔH= -2.303×R×slope (Fig. 8a)

ΔG= -RT×2.303×log K_{eq}

$\Delta G = \Delta H - T\Delta S$

The free energy change (ΔG), enthalpy change (ΔH), entropy change (ΔS) and energy of activation (E_a) for the lipase catalyzed reaction were found to be -23.2 kJ mol^{-1}, 15.9 kJ mol^{-1}, 124.7 J K^{-1} mol^{-1} and 19.1 kJ mol^{-1}, respectively.

In the present work extracellular lipase from *P. aeruginosa* KF 853103 was produced, purified and characterized. The purified enzyme showed pH optima of 8.0 and temperature 40°C and was relatively stable within the temperature range of 30-40°C. The enzyme retained about 96.5, 80.3 and 93.4 of its maximum activity in the presence of methanol, ethanol and acetone, respectively. Due to its stability in a wide range of organic solvents, *P. aeruginosa* KF 853103 lipase could be of significant biotechnological potential, particularly in enzyme-catalyzed transesterification reactions for biodiesel production from plant oils.

REFERENCES

1. Joseph, B., Ramteke, P.W., Thomas, G. Cold active microbial lipases: Some hot issues and recent developments. *Biotechnol. Adv.*, 2008; **26**(5): 457-470.
2. Griebeler, N., Polloni, A.E., Remonatto, D., Arbter, F., Vardanega, R., Cechet, J.L., Luccio, M.D., Oliveira, D., Treichel, H., Cansian, R.L., Rigo, E., Ninow, J.L. Isolation and screening of lipase-producing fungi with hydrolytic activity. *Food Bioprocess Technol.*, 2011; **4**(4): 478-586.
3. Veerapagu, M., Narayanan, A.S., Ponmurugan,

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

4. Bajaj, A., Lohan, P., Jha, P.N., Mehrotra, R. Biodiesel production through lipase catalyzed transesterification: an overview. *J. Mol. Catal.*, 2010; **62**(1): 9-14.
5. Chakraborty, K., Paulraj, R. Purification and biochemical characterization of an extracellular lipase from *Pseudomonas fluorescens* MTCC 2421. *J. Agric. Food Chem.*, 2009; **57**(9)*:* 3859-3866.
6. Gokbulut, A.A., Arslanoglu, A. Purification and biochemical characterization of an extracellular lipase from psychrotolernt *Pseudomonas florescens* KE38. *Turk. J. Biol.*, 2013; **37**(5): 538-546.
7. Winkler, U.K., Stuckmann, M. Glycogen, hyaluronate and some other polysaccharides greatly enhance the formation of exolipase by *Serratia marcescens. J. Bacteriol.*, 1979; **138**(3): 663-670.
8. Lowry, O.H., Rosebrough, N.J., Faee, A.L., Randall, R.J. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.*, 1951; **193**(1): 265-275.
9. Saeed, H.M., Zaghloul, T.I., Khalil, A.I., Abdelbaeth, M.T. Purification and characterization of two extracellular lipases from *Pseudomonas aeruginosa* Ps-X. *Pol. J. Microbiol.*, 2005; **54**(3): 233-240.
10. Zouaoui, B., Bouziane, A. Isolation, optimisation and purification of lipase production by *Pseudomonas aeruginosa*. *J. Biotechnol. Biomater.*, 2011; **1**(7): 1-4.
11. Sharon, C., Furugoh, S., Yamakido, T., Ogawa, H., Kato, Y. Purification and characterization of a lipase from *P. aeruginosa* KKA-5 and its role in castor oil hydrolysis. *J. Ind. Microbiol. Biotechnol.*, 1998; **20**: 304-307.
12. Gaur, R., Gupta, A., Khare, S.K. Purification and characterization of lipase from solvent tolerant *Pseudomonas aeruginosa* PseA. *Process Biochem.*, 2008; **43**(10): 1010-1046.
13. Borkar, P.S., Bodade, R.G., Rao, S.R., Khobragade, C.N. Purification and characterization of extracellular lipase from a new strain – *Pseudomonas aeruginosa* SRT 9. *Braz. J. Microbiol.*, 2009; **40**(2): 358-366.
14. Ugur, A., Sarac, N., Boran, R., Ayaz, B., Ceylan, O., Okmen, G. New lipase for biodiesel production: Partial purification and characterization of LipSB 25-4. *ISRN Biochem.*, 2014; *doi: 10.1155/2014/289749*.