ISSN 1330-9862
(FTB-3448)

scientific note

Immobilization of Organic Solvent-Tolerant Lipase from *Pseudomonas mendocina* M-37 with Potential Synthetic Activities

Praveen Dahiya[1§], *Subhash Chand*[2] *and Neeraj Dilbaghi*[1]*

[1]Department of Bio- and Nanotechnology, Guru Jambheshwar University of Science and Technology, Hisar-125001, Haryana, India

[2]Department of Biochemical Engineering and Biotechnology, Indian Institute of Technology, Hauz Khas, New Delhi-110016, India

[§]Present address: Amity Institute of Biotechnology, Amity University, Sector 125, Gautam Buddha Nagar, Noida-201303, Uttar Pradesh, India

Received: May 27, 2013
Accepted: March 17, 2014

Summary

A thermostable solvent-tolerant lipase was isolated from *Pseudomonas mendocina* M-37. The lipase production medium was optimized for cost-effective production. Olive oil as a carbon source, and glycine as a nitrogen source were selected as the best for maximum lipase production. Medium optimization led to 3.75-fold increase in the lipase production. The extracellular lipase was purified 42.2-fold to homogeneity by precipitation using polyethyleneglycol, ultrafiltration and hydrophobic interaction chromatography. Its molecular mass, determined with sodium dodecyl sulphate-polyacrylamide gel electrophoresis, was 32 kDa. The enzyme was further immobilized on microcrystalline cellulose. The lipase showed an optimal water activity of 0.53 for both, acidolysis and interesterification reactions. Six- to sevenfold increase in synthetic activity of immobilized lipase was observed when interesterification activity of 0.139 IU/mg and transesterification activity of 0.181 IU/mg, respectively, were obtained. This is the first report on *Pseudomonas mendocina* lipase with synthetic activity immobilized on microcrystalline cellulose.

Key words: acidolysis, immobilization, organic solvent-tolerant lipase, *Pseudomonas mendocina*, purification

Introduction

Lipases (triacylglycerol hydrolases, EC 3.1.1.3) are serine hydrolases which act at the lipid-water interface. The catalytic triad is composed of Ser-Asp/Glu-His, and usually also a consensus sequence (Gly-x-Ser-x-Gly) is found around the active site, serine. Three-dimensional structures of lipases reveal the characteristic α/β-hydrolase fold (*1*). Different genera of bacteria are known to produce lipase but *Achromobacter* spp., *Alcaligenes* spp., *Pseudomonas* spp. and *Chromobacterium* spp. have been well exploited. The bacterial genus *Pseudomonas* secretes a number of extracellular enzymes including lipases, in response to fluctuating external conditions. *Pseudomonas* lipases bear potential use in a variety of biotechnological applications because of their potential in organic synthesis for highly valuable chemicals.

There are a few reports on isolation, purification and characterization of solvent-tolerant lipases (*2–4*). Organic solvent-tolerant lipases are required in the synthesis of many useful products, such as production of biopolymeric materials, biodiesel and in the synthesis of fine chemicals (*5*). Immobilization of enzymes can improve their performance under optimal process conditions of acid-

*Corresponding author: Phone: +91 1662 263 500; Fax: +91 1662 276 240; E-mail: neeraj_dilbaghi@yahoo.co.in

ity, alkalinity, organic solvents and high temperatures. A hundredfold improvement in the enzyme activity during immobilization has been reported in the presence of organic solvents (*6*).

An organic solvent-tolerant lipase producer identified as *Pseudomonas mendocina* M-37 was previously isolated from a local source (*7*). The lipase was stable in the presence of various organic solvents such as *n*-hexane, cyclohexane, toluene, and 1-octanol. In this work, we report the production, purification, immobilization and synthetic activity of thermoalkaliphilic lipase produced by *Pseudomonas mendocina* M-37, isolated from an oil industry soil sample in Hisar, Haryana, India.

Materials and Methods

Isolation of lipase from Pseudomonas mendocina M-37

The solvent-tolerant strain of *Pseudomonas mendocina* M-37 was used. Its isolation and characterization had been reported previously (*7*). The isolated bacterial strain was identified at the Microbial Type Culture Collection (MTCC), IMTECH, Chandigarh, India. Crude enzyme preparation was reported previously (*7*). Total protein was estimated using Folin-Ciocalteu method, using bovine serum albumin (BSA) as standard (*8*).

Optimization of the media

Effect of nutritional factors on lipase production

The effect of different lipids on the lipase production was studied by adding the following sources to the basal medium at 1 % (by mass per volume): olive oil, palm oil, soya bean oil, apricot oil, groundnut oil, mustard oil, sunflower oil, glucose, sucrose, fructose and maltose. Similarly, nitrogen sources were added to the basal medium at 1 % (by mass per volume). Various organic and inorganic nitrogen sources used include glycine, urea, soya bean meal, tryptone, ammonium nitrate, potassium nitrate, ammonium chloride, ammonium sulphate and sodium nitrate. Cultures were incubated at 30 °C with rotary shaking at 250 rpm for 24 h.

Effect of physical environment on lipase production

Lipase production by *P. mendocina* M-37 was optimized by varying the pH (6.5–8.0), temperature (26–38 °C), agitation (150–350 rpm) and inoculum size (5–15 %). The crude enzyme was obtained by centrifugation at 9168×*g* and 4 °C for 20 min. Lipase activity was assayed using olive oil as substrate. All the experiments were done in triplicate and repeated twice.

Enzyme assay

Lipase assay was performed using the procedure of Watanabe *et al*. (*9*). The reaction mixture contained olive oil emulsion composed of olive oil and polyvinyl alcohol. The biomass was estimated by absorbance measurement at 590 nm using distilled water as a blank. Dry cells were calibrated and mass concentration was expressed in g/L. A known volume of fermented broth was taken in duplicate, centrifuged at 825×*g* for 15 min and washed three times to remove any extraneous matter. This biomass was transferred to dried and preweighed aluminium cups, which were then dried at 50–80 °C for 24 h. After drying the cells, the cups were cooled to room temperature in a desicator and weighed again. Various dilutions were prepared and the absorbance was measured. The biomass was calculated as g of dry cells per L.

Purification with hydrophobic interaction chromatography

Lipase was purified to homogeneity by polyethylene glycol 6000 (PEG) precipitation, followed by hydrophobic interaction chromatography using Octyl Sepharose® CL-4B (18 cm×2.5 cm) column (Pharmacia, Uppsala, Sweden). PEG was added to the culture supernatant to reach 2 to 20 % (by mass per volume) saturation. The mixture was kept in a cold chamber with constant stirring for 4 h and then the precipitate was collected followed by ultrafiltration to remove the PEG from the concentrate. The obtained concentrate was collected and lyophilized overnight. A mass of 100 mg of lyophilized enzyme was added to 5 mL of preactivated Octyl Sepharose in 50 mL of binding buffer (0.01 M phosphate buffer, pH=7.0, and 1.0 M ammonium sulphate). The enzyme was eluted by reducing the ionic strength of the system by using decreasing salt gradient of ammonium sulphate (0.01–1.0 M), followed by 1 % buffered Triton X-100 with a flow rate of 0.5 mL/min. Fractions of 2.0 mL were collected and assayed for lipase activity. A UV detector monitored the protein in each of the fractions. Fractions with high lipase activity were pooled and concentrated by lyophilization. The relative molecular mass of the purified lipase was estimated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) as described by Maniatis *et al.* (*10*).

Immobilization of lipase

Microcrystalline cellulose (approx. 50 μm particle size) was used as a support for lipase immobilization. For activation, 2 g of microcrystalline cellulose were activated using 2.5 mL of epichlorohydrin and 30 mL of 0.6 M solution of hexamethylenetetramine. Activated microcrystalline cellulose was filtered and dried in vacuum desicator and the dried product was kept at –20 °C until use.

Partially purified *P. mendocina* M-37 lipase dissolved in Tris-HCl (pH=8.0) was made to react with the activated microcrystalline cellulose on the basis of different molar ratios. Six different molar ratios (on mass basis) of lipase and activated microcrystalline cellulose were prepared. The mixture was incubated at 40 °C and 90 rpm for 90 min. Buffer containing unbound enzyme was removed by decantation (1st washout) and the remaining pellet was washed with 100 mM phosphate buffer (pH= 7.0) by centrifugation (2nd washout). The immobilized lipase was recovered through centrifugation, dried in vacuum desiccators, and stored at –20 °C. Immobilized *P. mendocina* lipase was tested for hydrolytic activity in water, and acidolysis and interesterification activity in organic solvent hexane.

Acidolysis and interesterification activity

Acidolysis was performed using triolein and myristic acid, while substrates used for carrying out interesterification reactions were triolein and lauric acid methyl

ester. The enzyme and the substrate solutions were equilibrated separately with saturated solutions of salts of known water activities. The saturated solutions used for maintaining the desirable water activities were: LiCl (a_w= 0.17), LiI (a_w=0.17), $MgCl_2 \cdot 6H_2O$ (a_w=0.33), $Mg(NO_3)_2$ (a_w=0.53), NaCl (a_w=0.75) and K_2SO_4 (a_w=0.97). Equilibrium was maintained for at least 18 h. The substrates were prepared in 1:2 molar ratio and final volume was made up to 3 mL with hexane. Then, the immobilized lipase (10 % of the mass of the reactants) was added and incubated at 35 °C on a shaker at 200 rpm for 120 min. For interesterification, aliquots (100 μL) were withdrawn every 30 min until 120 min, centrifuged at 9168×*g* for 10 min at 4 °C for gas chromatography (GC) analysis.

For acidolysis, free fatty acids were separated, from which triglycerides (TG) were isolated by silica gel column chromatography (16 mm i.d. and 8 cm height) containing 100–200 mesh (75–150 μm) silica gel. The obtained TG fraction was weighed, methylated by the addition of chloroform/methanol (3:7) followed by the addition of 0.3 mL of thionyl chloride. The mixture was incubated at room temperature for 1 h. The obtained supernatant was analyzed using GC. Acidolytic activity was expressed in μmol of myristic acid incorporated into triolein per min per mg of the enzyme.

Gas chromatography analysis

The estimation of fatty acid composition of triglycerides was measured using an HP 5890 Series II gas chromatograph (Hewlett-Packard, Palo Alto, CA, USA), equipped with an HP-5 fused silica capillary column and flame-ionization detector (FID). The column temperature was kept at 150 °C for 3.5 min, then programmed to 225 °C at 30 °C/min and maintained for 13 min. The injector and detector temperatures were 330 and 360 °C, respectively. Nitrogen was used as a carrier gas at a flow rate of 40 mL/min.

Results and Discussion

Optimization of cultural conditions for maximum lipase production

Effect of carbon sources

The effect of different carbon sources (carbohydrates and lipid sources) on the lipase production was studied. Considerable amount of lipase was produced in all the media containing the tested carbon sources (Table 1). Maximum lipolytic activity ((4.05±0.02) IU/mL) was obtained with olive oil as a carbon source, followed by groundnut (3.5±0.06) and mustard oil ((3.5±0.08) IU/mL). In the presence of soya bean, palm and apricot oil, maximum lipolytic activity of 2.5 IU/mL was obtained after 24 h of incubation (Table 1). These results show that *Pseudomonas mendocina* M-37 prefer long-chain unsaturated fatty acids, which is in agreement with the earlier reports of use of olive oil as a lipid source (*4*,*11*,*12*). In contrast, low lipolytic activity was obtained when glucose and sucrose were added to the medium as carbon source, followed by fructose and maltose, suggesting that the low lipase production might be due to catabolite repression. There are earlier reports on glucose exhibiting catabolite repression of enzyme synthesis by other authors (*13*,*14*). Based on these results, optimal mass per volume ratio of olive oil in the range of 0.5 to 1.0 % for lipase production and cell growth was studied. It was observed that with the increase in olive oil ratio from 0.5 to 1 %, there was a 2.28-fold increase in the lipolytic activity ((8.00±0.05) IU/mL), which decreased to (2.70±0.02) and (0.80±0.06) IU/mL when olive oil ratio was increased to 1.5 and 2 %, respectively, which indicates inhibitory effect of high carbon ratio on lipase production. However, no correlation between biomass concentrations and lipase production was evident from the results shown in Table 2. Olive oil ratio of 2 % resulted in maximum biomass yield but in poor lipase yield. The inhibition of the lipase synthesis at higher olive oil ratio could be due to poor oxygen transfer into the medium or due to the accumulation of oleic acid, which represses lipase production. Similar results were reported by some researchers (*11*,*12*); however, higher ratio (2 %) of olive oil was reported by Gombert *et al.* (*15*).

Table 1. The effect of various carbon sources (1.0 % by mass per volume) on the lipase production by *Pseudomonas mendocina* M-37

Carbon source	Lipase activity* IU/mL	γ(biomass) g/L	Final pH
Nutrient broth + olive oil	4.05±0.02	3.58	7.42
Nutrient broth + palm oil	2.50±0.05	2.50	8.01
Nutrient broth + soya bean oil	2.50±0.03	4.53	6.29
Nutrient broth + apricot oil	2.50±0.03	3.54	6.72
Nutrient broth + groundnut oil	3.50±0.06	3.75	6.7
Nutrient broth + mustard oil	3.50±0.08	4.07	7.07
Nutrient broth + sunflower oil	2.30±0.07	2.04	6.84
Nutrient broth + glucose	1.00±0.08	2.66	6.74
Nutrient broth + sucrose	1.00±0.05	1.22	8.04
Nutrient broth + fructose	0.50±0.07	2.79	7.30
Nutrient broth + maltose	0.25±0.03	2.13	7.21

*Values are expressed as mean±standard deviations

Effect of nitrogen sources

To study the effect of nitrogen sources, peptone present in the medium was replaced by various organic and inorganic nitrogen sources. It was found that the removal of peptone from media resulted in the twofold increase in lipase activity. A similar response was reported by Kamini *et al.* (*16*). In contrast, it was found that the addition of peptone into the medium increased the enzyme production in *Penicillium restrictum* (*17*). An addition of glycine in place of peptone resulted in maximum lipolytic activity ((13.20±0.03) IU/mL), followed by soya bean meal and urea (Table 3). Similar results on good

Table 2. The effect of various ratios of olive oil and glycine on the lipase production by *Pseudomonas mendocina* M-37

$\frac{m(\text{substrate})}{V(\text{medium})}$/%	Olive oil			Glycine		
	Lipase activity* IU/mL	γ(biomass) g/L	Final pH	Lipase activity* IU/mL	γ(biomass) g/L	Final pH
0.5	3.50±0.05	3.0	6.2	5.00±0.02	3.54	7.2
1.0	8.00±0.05	3.0	6.3	13.20±0.06	3.10	7.0
1.5	2.70±0.02	3.6	6.2	7.00±0.05	1.77	7.0
2.0	0.80±0.06	3.6	6.3	2.00±0.08	1.10	7.1

*Values are expressed as mean±standard deviations

Table 3. The effect of various nitrogen sources (1.0 % by mass per volume) on the lipase production by *Pseudomonas mendocina* M-37

Nitrogen source	Lipase activity* IU/mL	γ(biomass) g/L	Final pH
NBWP + olive oil (C)	8.00±0.05	2.70	6.36
NBWP + ammonium sulphate	6.00±0.08	4.44	4.89
NBWP + ammonium chloride	2.50±0.07	3.81	5.56
NBWP + ammonium nitrate	4.00±0.06	3.77	5.98
NBWP + potassium nitrate	8.00±0.07	3.91	6.40
NBWP + sodium nitrate	2.20±0.02	2.42	6.23
NBWP + urea	8.50±0.05	1.74	6.00
NBWP + soya bean meal	9.10±0.03	6.44	6.34
NBWP + glycine	13.20±0.03	3.21	7.15
NBWP + tryptone	4.20±0.06	3.62	7.05

*Values are expressed as mean±standard deviations, NBWP=nutrient broth without peptone, C=control

growth and moderate lipase activity in the presence of glycine were reported in *Antrodia cinnamomea* (*18*) and in *Pseudomonas fluorescens* 2D (*19*).

Optimal mass per volume ratio of glycine in the range of 0.5 to 1.0 % for lipase production and cell growth was studied and it was observed that glycine at ratio of 1 % showed maximum lipolytic activity (Table 2). Above and below 1 % of glycine, lipase production decreased drastically. Similar observation was made in *Aeromonas liquefaciens* (*20*). It was found that biomass concentration decreases with an increase in the concentration of glycine, which may be due to the inhibitory effect of glycine on bacterial growth (*20,21*).

Effect of agitation and inoculum on lipase production

To determine the effect of agitation on lipase production by *Pseudomonas mendocina* M-37, cultures were incubated on reciprocating shaker at agitation rates of 150, 200, 250, 300 and 350 rpm. As shown in Fig. 1, it is evident that the agitation rate affects the growth as well as lipase activity. Maximum lipase activity ((13.20±0.03) IU/mL) was obtained at 250 rpm, while it decreased drastically as the agitation rate increased or decreased. However, biomass yield was found to be maximum (3.45 g/L) at 350 rpm but no lipase activity was detected

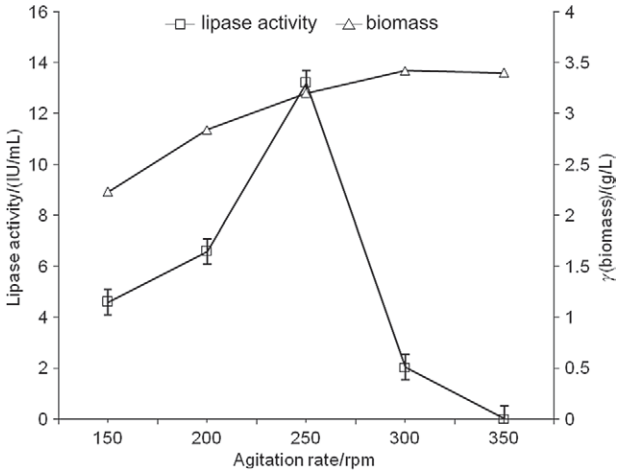

Fig. 1. Effect of agitation on the cell growth and lipase production by *Pseudomonas mendocina* M-37

at this agitation rate. Thus, biomass yield and lipase activity are not correlated. This may be due to oxidation or as a result of shearing stress on an organism. These results are in agreement with the findings of Long *et al.* (*22*) and Yu *et al*. (*23*).

Pseudomonas mendocina M-37 supported maximum enzyme production and biomass yield at 10 % inoculum levels ($40 \cdot 10^9$ CFU/50 mL). Further increase in inoculum size (15 %) resulted in drastic drop in lipase activity ((5.10±0.09) IU/mL). However, high biomass yield (4.01 g/L) was obtained with 15 % inoculum, which may be due to limitation of olive oil, which was utilised for the production of lipase (Table 4). Gulati *et al.* (*24*) reported a high inoculum density for maximum lipase production in *Aspergillus terreus*. However, lipase production was reduced at high inoculum density in *Pseudomonas* Lip35 (*23*).

Effect of temperature and pH on lipase production

Lipase production by *Pseudomonas mendocina* M-37 was studied at different temperatures, *i.e.* 26, 30, 34 and 38 °C. Optimum temperature for maximum lipase production ((13.20±0.06) IU/mL) was found to be 30 °C, however, the lipolytic activity obtained at temperatures of 26 and 34 °C was about 49 and 48 %, respectively. At a temperature above 34 °C, the recoverable lipase activity was very low (Table 5). With an increase in temperature from 26 to 38 °C, the cell growth decreased and

Table 4. The effect of inoculum density on the lipase production by *Pseudomonas mendocina* M-37

Inoculum density	Lipase activity*	γ(biomass)	Final pH
%	IU/mL	g/L	
5	8.00±0.06	3.02	6.4
8	10.50±0.05	3.35	6.8
10	13.50±0.08	3.67	7.2
12	10.00±0.03	3.70	6.6
15	5.10±0.05	4.01	6.6

*Values are expressed as mean±standard deviations

Table 5. The effect of various temperatures on the lipase production and biomass by *Pseudomonas mendocina* M-37

Temperature	Lipase activity*	γ(biomass)	Final pH
°C	IU/mL	g/L	
26	6.50±0.02	3.20	7.3
30	13.20±0.06	3.10	7.1
34	6.40±0.05	3.05	6.8
38	0.80±0.03	2.80	6.2

*Values are expressed as mean±standard deviations

consequently did the pH of the culture medium. Most authors reported lipase production at an optimum temperature of 28 and 29 °C (*12,25*), whereas a higher optimum temperature of 37 °C was reported by Hasan *et al.* (*26*).

The effect of pH of the medium on lipase production by *Pseudomonas mendocina* M-37 was studied at various pH values ranging from 6.5 to 8.0 using various buffers at 30 °C after 24 h of incubation. From the obtained results, it was evident that both growth and lipase production were influenced by initial pH of the production media. The lipase activity at pH=6.5 was found to be (5.6±0.1) IU/mL, which increased gradually with the increase in the initial pH, exhibiting the highest growth and lipase yield ((15.20±0.05) IU/mL) at pH=7.5 (Fig. 2). Further increase in pH to 8.0 resulted in a drastic drop in both cell growth and enzyme yield, which may be due to increased production of protease at an pH higher than 7.5. These results are in agreement with the findings of Thomas *et al.* (*25*) and Hasan *et al.* (*26*), where majority of lipases are produced at neutral or alkaline

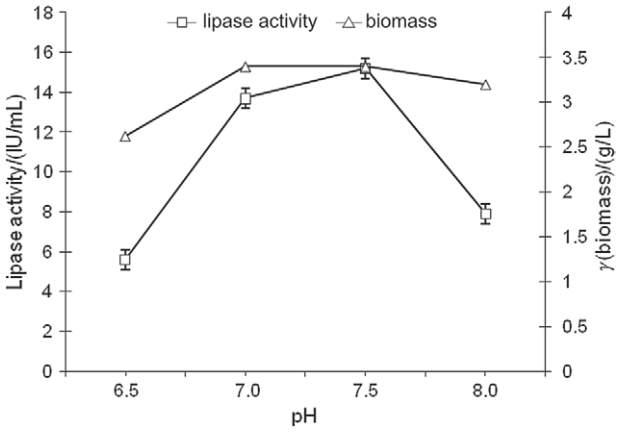

Fig. 2. Effect of pH of the medium on the cell growth and lipase production by *Pseudomonas mendocina* M-37

pH. However, few acidic lipases have also been reported by various researchers (*16,18*).

Purification of lipase

The extracellular lipase from *Pseudomonas mendocina* M-37 was subjected to purification involving precipitation by polyethylene glycol (PEG), and ultrafiltration followed by hydrophobic interaction chromatography (HIC) using Octyl Sepharose CL-4B (Table 6). Precipitation by PEG 6000 followed by ultrafiltration resulted in 18.4-fold purification. The lyophilized fraction was applied to HIC with Octyl Sepharose CL-4B column. The enzyme was then eluted using 1 % buffered Triton X-100. Many protein peaks were observed (Fig. 3), and one prominent activity peak was detected (fractions 60–82). Active fractions were pooled and a final recovery (yield) of 32.1 % and a 42.2-fold purification were achieved (Table 6). Higher recovery of lipase from 52 to 67.5 % was reported when using *Pseudomonas* sp. strain S5 (*2*) and *Pseudomonas fluorescens* P21 (*4*). A single band of the lipase was obtained by SDS-PAGE analysis. The molecular mass of *P. mendocina* M-37 lipase was determined to be 32 kDa (Fig. 4). Molecular masses of solvent-tolerant lipases from different P*seudomonas* sp. mainly belong to two groups: 30–45 and 50–60 kDa (*3*). Therefore, the present lipase could be related to the lower-molecular mass lipases.

Immobilization of lipase and synthetic activities

The lipase obtained from *Pseudomonas mendocina* M-37 was immobilized on activated microcrystalline cellulose. No reports are available regarding the use of microcrys-

Table 6. Summary of the purification of the lipase from *Pseudomonas mendocina* M-37

Purification step	γ(protein)	Lipase activity	Total activity	Specific activity	Yield	Purification fold
	mg/mL	IU/mL	U	U/mg	%	
Culture filtrate	4.20	8.5	2550	2.02	100.00	1.0
Precipitation by PEG 6000	1.37	30.0	1800	21.89	70.58	10.8
Ultrafiltration	0.57	21.0	1050	37.10	41.17	18.4
HIC (Octyl Sepharose CL-4B)	0.35	30.0	840	85.70	32.10	42.2

HIC=hydrophobic interaction chromatography

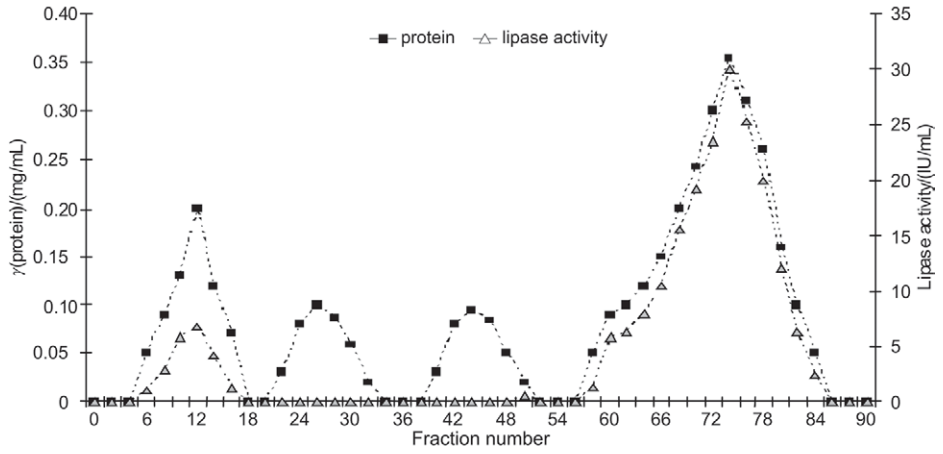

Fig. 3. Chromatogram of *Pseudomonas mendocina* M-37 on Octyl Sepharose® CL-4B

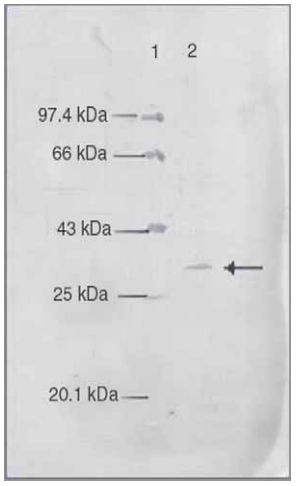

Fig. 4. SDS-PAGE of purified *Pseudomonas mendocina* M-37 lipase. Lane 1: protein markers: soya bean trypsin inhibitor 20.1 kDa, carbonic anhydrase 25 kDa, ovalbumin 43 kDa, bovine serum albumin 66 kDa, phosphorylase b 97.4 kDa; lane 2: lipase enzyme

talline cellulose for activation with modifying agents, whereas epichlorohydrin was used for immobilization as reported by Kumar and Bandyopadhyay (*27*). Similarly, hexamine was used for modification of *Humicola lanuginosa* lipase (*28*). The immobilized lipase was tested for hydrolytic activity in water and transesterification and interesterification activity in organic solvent hexane. Considering the stability of lipase in hexane and its high substrate specificity in triolein, the *P. mendocina* M-37 lipase was possibly suitable for modification of fats and oils. *P. mendocina* lipase had a profound effect on acidolysis and interesterification activity after immobilization, as evident in Table 7. Optimal water activity for *P. mendocina* M-37 lipase was 0.53 in both interesterification and transesterification reactions (results not shown). Beyond this, a drastic decrease in synthetic activity was observed and this may be due to the aggregation of the enzyme. Similarly, Ma *et al.* (*29*) reported optimal water activity (a_w=0.53). However, *Pseudomonas* lipase has been reported to have maximal activity at a water activity close to 1.0 (*30,31*). Interesterification activity of 0.139 IU/mg was obtained at 1:5 molar ratio of modified lipase as compared to 0.022 IU/mg in the case of unmodified lipase. The sixfold increase of interesterification activity was observed with immobilized lipase in comparison with a free lipase. Acidolysis activity of 0.181 IU/mg was obtained with 1:5 molar ratio as compared to 0.025 IU/mg of free enzyme, where a sevenfold increase was observed (Table 7). This increase could be attributed to the improvement of lipase solubility and dispersability in the hydrophobic environments in the non-polar solvent, which made

Table 7. Catalytic activities of *Pseudomonas mendocina* M-37 lipase immobilized on microcrystalline cellulose (MCC)

$\frac{n(\text{lipase})}{n(\text{MCC})}$	$\frac{w(\text{protein adsorbed on MCC})}{\text{mg/g}}$	Hydrolytic activity / IU per mg of protein	Interesterification activity / IU per mg of protein	Acidolysis activity / IU per mg of protein
Novozyme 435 (standard)		NT	0.056	0.030
Free enzyme		8.0	0.022	0.025
Immobilized enzyme				
1:1	0.078	4.4	0.031	0.047
1:2	0.052	5.7	0.040	0.073
1:3	0.064	5.3	0.047	0.081
1:4	0.033	7.0	0.115	0.137
1:5	0.035	7.9	0.139	0.181
1:6	0.025	6.8	0.062	0.083

NT=not tested

the contact between the enzyme and dissolved substrates, and therefore the reaction, easier. Similarly, an increase in the synthetic activity (*32*,*33*) was observed; whereas a decrease in hydrolytic activity was detected after immobilization. Various researchers have reported significant losses of enzymatic hydrolytic activity (*32*,*34*). This phenomenon has usually been related to the enzyme conformational changes.

Conclusions

An organic-solvent-stable lipase was produced by a newly isolated *Pseudomonas mendocina* M-37. The lipase production by this bacterium was optimized, which resulted in 3.75-fold increase in the yield. The lipase was purified using a three-step purification procedure with an overall yield of 32.1 % and 42.2-fold purification, and its molecular mass was found to be 32 kDa. For immobilization, microcrystalline cellulose activation using epichlorohydrin and hexamine was found to be a simple and cost-effective process. The acidolysis and interesterification efficiency of *P. mendocina* M-37 lipase in the immobilization in microcrystalline cellulose was improved by six- to sevenfold. Although many authors have reported solvent-tolerant lipases, *P. mendocina* M-37 lipase showed unique features. The solvent tolerance property and the ability of the lipase to catalyze synthetic reactions in organic solvents suggests its application for the production of novel compounds in nonaqueous organic synthesis used in various biotechnological processes.

References

1. M. Nardini, B.W. Dijkstra, α/β Hydrolase fold enzymes: The family keeps growing, *Curr. Opin. Struct. Biol. 9* (1999) 732–737.
2. R.N.Z. Rahman, S.N. Baharum, M. Basri, A.B. Salleh, High-yield purification of an organic solvent-tolerant lipase from *Pseudomonas* sp. strain S5, *Anal. Biochem, 341* (2005) 267–274.
3. Q. Ji, S. Xiao, B. He, X. Liu, Purification and characterization of an organic solvent-tolerant lipase from *Pseudomonas aeruginosa* LX1 and its application for biodiesel production, *J. Mol. Catal. B: Enzym. 66* (2010) 264–269.
4. B.H. Cadirci, I. Yasa, An organic solvents tolerant and thermotolerant lipase from *Pseudomonas fluorescens* P21, *J. Mol. Catal. B: Enzym. 64* (2010) 155–161.
5. L.D. Castro-Ochoa, C.R. Gomez, G.V. Alfaro, R.O. Ros, Screening, purification and characterization of the thermophilic lipase produced by *Bacillus thermoleovorans* CCR11, *Enzyme Microb. Technol. 37* (2005) 648–658.
6. R.A. Sheldon, Enzyme immobilization: The quest for optimum performance, *Adv. Synth. Catal. 349* (2007) 1289–1307.
7. P. Dahiya, P. Arora, S. Chand, A. Chaudhury, N. Dilbaghi, Characterization of an extracellular alkaline lipase from *Pseudomonas mendocina* M-37, *J. Basic Microbiol. 50* (2010) 1–7.
8. O.H. Lowry, N.J. Rosenbrough, A.L. Farr, R.J. Randall, Protein measurement with folin phenol reagent, *J. Biol. Chem. 193* (1951) 265–275.
9. N. Watanabe, Y. Ota, Y. Minoda, K. Yamada, Isolation and identification of alkaline lipase producing microorganisms, cultural conditions and some properties of crude enzymes, *Agr. Biol. Chem. 41* (1977) 1353–1358.
10. T. Maniatis, E.F. Fritsch, J. Sambrook: *Molecular Cloning – A Laboratory Manual III,* Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, NY, USA (1989).
11. P. Ellaiah, T. Prabhakar, B. Ramakrishna, A. Thaer Taleb, K. Adinarayana, Production of lipase by immobilized cells of *Aspergillus niger, Process Biochem. 39* (2004) 525–528.
12. H.M. Rifaat, A.A. El-Mahalawy, H.A. El-Menofy, S.A. Donia, Production, optimization and partial purification of lipase from *Fusarium oxysporum, J. Appl. Sci. Environ. Sanit. 5* (2010) 39–53.
13. A. Annibale, F.F. Sermanni, T. Petruccioli, Olive-mill wastewaters: A promising substrate for microbial lipase production, *Bioresour. Technol. 97* (2006) 1828–1833.
14. V. Brozzoli, S. Crognale, I. Sampedro, F. Federici, D. Annibale, A. Petruccioli, Assessment of olive-mill wastewater as a growth medium for lipase production by *Candida cylindracea* in bench-top reactor, *Bioresour. Technol. 100* (2009) 3395–3402.
15. A.K. Gombert, A.L. Pinto, L.R. Castilho, D.M.G. Freir, Lipase production by *Penicillium restrictum* using babassu oil cake as substrate, *Process Biochem. 35* (1999) 85–90.
16. N.R. Kamini, T. Fujii, T. Kurosu, H. Iefuji, Production, purification and characterization of an extracellular lipase from the yeast, *Cryptococcus* sp. S-2, *Process Biochem. 36* (2000) 317–324.
17. P. Pogaku, A. Suresh, P, Srinivas, S. Ram Reddy, Optimization of lipase production by *Staphylococcus* sp. Lp12, *Afr. J. Biotechnol. 9* (2010) 882–886.
18. E.S. Lin, C.C. Wang, S.C. Sung, Cultivating conditions influence lipase production, *Microbial Technol. 39* (2006) 98–102.
19. A. Makhzoum, J.S. Knapp, R.K. Owusu, Factors affecting growth and extracellular lipase production by *Pseudomonas fluorescens* 2D, *Food Microbiol. 12* (1995) 277–290.
20. Y. Ikura, Stimulatory effects of certain amino compounds, antibiotics and polyamines on production of alkaline phosphatase on *Aeromonas liquefaciens, J. Ferment. Bioeng. 69* (1990) 83–88.
21. W. Hammes, K.H. Schleifer, O. Kandier, Mode of action of glycine on the biosynthesis of peptidoglycan, *J. Bacteriol. 116* (1973) 1029–1053.
22. K. Long, H.M. Ghazali, A. Ari, K. Ampon, C. Bucke, Mycelium-bound lipase from a locally isolated strain of *Aspergillus flavus* link: Patterns and factors involved in its production, *J. Chem. Technol. Biotechnol. 67* (1996) 157–163.
23. H.W. Yu, J. Han, N. Li, X.S. Qie, Y.M. Jia, Fermentation performance and characterization of cold-adapted lipase produced with *Pseudomonas* Lip35, *Agric. Sci. 8* (2009) 956–962.
24. R. Gulati, R.K. Saxena, R. Gupta, Fermentation and downstream processing of lipase from *Aspergillus terreus*, *Process Biochem. 36* (2000) 149–155.
25. A. Thomas, M. Mathew, A.K. Valsa, S. Mohan, R. Manjula, Optimization of growth for the production of extracellular lipase by *Bacillus mycoides, Ind. J. Microbiol. 43* (2003) 67–69.
26. F. Hasan, A.A. Shah, A. Hameed, Industrial applications of microbial lipases, *Enzyme Microb. Technol. 39* (2006) 235–251.
27. U. Kumar, M. Bandyopadhyay, Sorption of cadmium from aqueous solution using pretreated rice husk, *Bioresour. Technol. 97* (2006) 104–109.
28. I.C. Omar, H. Saeki, N. Nishio, S. Nagai, Hydrolysis of triglycerides by immobilized thermostable lipase from *Humicola lanuginosa, Agric. Biol. Chem. 52* (1988) 99–105.
29. L. Ma, M. Persson, P. Adlercreutz, Water activity dependence of lipase catalysis in organic media explains success-

ful transesterification reactions, *Enzyme Microb. Technol. 31* (2002) 1024–1029.

30. R.H. Valivety, P.J. Halling, A.D. Pellow, A.R. Macrea, Lipases from different sources vary widely in dependence of catalytic activity on water activity, *Biochem. Biophys. Acta, 1122* (1992) 143–146.

31. G. Koller, M.J. Aris, Z. Ujang, A.M. Vaidya, Dependence of the synthetic activity of nine immobilized lipase on water activity, *Biocatal. Biotransform. 19* (2001) 37–49.

32. M. Basri, A. Kamaruzaman, W.M.Z. Wan Yunus, C.A.N. Razak, A.B. Salleh, Synthesis of fatty esters by polyethylene glycol-modified lipase, *J. Chem. Technol. Biotechnol. 64* (1995) 10–16.

33. Y. Mine, K. Fukunaga, M. Yoshemoto, K. Nakao, Y. Sugimura, Modification of lipases with polyethylene glycol and polyoxyethylene detergents and their catalytic activities in organic solvents, *J. Biosci. Bioeng. 92* (2001) 539–543.

34. V.V. Mozhaev, N.S. Melik-Nubarov, V.Y.U. Levitsky, V.A. Siksnis, K. Matrinek, High stability to irreversible inactivation at elevated temperatures of enzymes covalently modified by hydrophilic reagents: α-Chymotrypsin, *Biotechnol. Bioeng. 40* (1992) 650–652 (doi: 10.1002/bit.260400603).