Journal of Molecular Catalysis B: Enzymatic 72 (2011) 256–262

Contents lists available at ScienceDirect

Journal of Molecular Catalysis B: Enzymatic

journal homepage: www.elsevier.com/locate/molcatb

Purification and characterization of an extracellular lipase from a novel strain *Penicillium* sp. DS-39 (DSM 23773)

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ARTICLE INFO

Article history:
Received 23 September 2010
Received in revised form 15 June 2011
Accepted 21 June 2011
Available online 28 June 2011

Keywords:
Penicillium sp. DS-39
Extracellular lipase
Purification
Characterization
Organic solvent-tolerant lipase

ABSTRACT

A newly isolated fungal strain, *Penicillium* sp. DS-39 (DSM 23773), was found to produce an inducible extracellular lipase when grown in a medium containing 1.0% (v/v) olive oil. Maximum lipase activity was obtained after 120 h of incubation at 28 °C. The lipase was purified 129-fold with a final specific activity of 308.73 IU/mg. The molecular weight of the homogeneous lipase was 43 kDa as determined by SDS-PAGE. It was optimally active at pH 5.5 and 45 °C. The lipase was most active on triolein and exhibited a broad substrate range with a preference for triacylglycerols containing long chain unsaturated fatty acids. It showed no regio-specificity for the ester bond in triolein. It was activated by Ca^{2+} and Mn^{2+}, while significant inhibition was observed with Hg^{2+} and Zn^{2+}. The lipase showed significant stability and activation in the presence of organic solvents with log $P \geq 2.0$. These features render *Penicillium* sp. DS-39 lipase (PEL) a potential biocatalyst for applications such as biodiesel production, enzymatic restructuring, by interesterification of different oils and fats, and biodegradation of oil spills in the environment.

1. Introduction

Lipases (EC 3.1.1.3) are ubiquitous hydrolytic enzymes that catalyze the breakdown of fats and oils into free fatty acids, monoacylglycerols, diacylglycerols and glycerol and operate at the interface of emulsified lipid substrates [1]. They constitute one of the most important groups of industrial enzymes due to their unique ability to hydrolyze fatty acid ester bonds in aqueous environments and synthesize them in non-aqueous medium [2]. Lipases have proven to be efficient and selective catalysts in many industrial applications, most of them involving the modification of fats and oils to high added-value lipid-based products [3]. Industrial applications to date range from use in detergent and paper manufacturing to the production of structured lipids, bioesters and biosurfactants based on carbohydrate esters [4–6]. These industrial applications have stimulated interest in isolation of new lipases from novel sources.

A considerable number of bacterial and fungal lipases have been commercially produced, the latter being preferable because fungi generally produce extracellular enzymes, which facilitates easy recovery of the enzyme from the fermentation broth [7]. Among fungi, the genus *Penicillium* contains many lipase producers [8–11]. *Penicillia* are widely used as a source of lipases [12]. The presence of lipolytic enzymes in moulds of the genus *Penicillium* are employed in the dairy industry, since these lipases play a major role in developing characteristic flavors in ripened cheeses [13]. Also lipases from *Penicillia* are useful in a number of bioconversions of industrial importance [14–20].

We have isolated a lipase producing fungal strain identified as *Penicillium* sp. DS-39 through 18S rRNA gene sequencing. In this study, we report production, purification and characterization of an inducible extracellular lipase secreted by *Penicillium* sp. DS-39. In addition, the purified lipase has been shown to be of potential use in non-aqueous biocatalysis owing to its broad substrate range coupled with its significant stability and activation in non-polar organic solvents.

2. Experimental

2.1. Materials

Analytical reagent grade chemicals were obtained from commercial sources at the purest grade available. Unless otherwise

Abbreviations: PEL, *Penicillium* sp. DS-39 lipase; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; DEAE, diethylaminoethyl; MUF-oleate, 4-methylumbelliferyl oleate; PUFA, polyunsaturated fatty acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; EDTA, *N,N,N′,N′*-ethylenediaminetetraacetic acid; PMSF, phenylmethylsulfonyl fluoride.

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doi:10.1016/j.molcatb.2011.06.013

mentioned, all chemicals were purchased from Sigma–Aldrich Ireland Ltd. (Dublin, Ireland).

2.2. Isolation and identification

The fungal strain used in this study was isolated by screening a soil sample collected from Phoenix Park, Dublin, Ireland. The soil sample (1 g) was suspended in 9 ml of sterile one quarter strength Ringer's solution (Oxoid, Basingstoke, UK) and serial dilutions were made. Aliquots (0.1 ml) of appropriate dilutions were surface plated on tributyrin agar. Plates were then incubated at 28 °C and periodically examined for 4–5 days. The fungal strain DS-39 was identified as a lipase producer on tributyrin agar through zone of clearance. The micromorphology of the isolate was studied by viewing lacto-phenol cotton blue wet mount preparations. Based on the morphological data, the isolate was assigned to the genus *Penicillium*. Confirmation of the assigned taxon was carried out by 18S rRNA gene sequence analysis. PCR amplification of this gene was carried out using primers NS1 (5′-GTAGTCATATGCTTGTCTC-3′) and NS4 (5′-CTTCCGTCAATTCCTTTAAG-3′). The amplified product was sequenced using ABI 3730 at Geneius Laboratories Ltd., Newcastle, UK. Partial 18S rRNA gene sequence thus obtained was submitted to GenBank database at NCBI (GeneBank accession No. HM579932). This sequence was submitted as a query to BLASTn search at NCBI server to identify the nearest neighbor sequences. A phylogenetic tree was constructed by neighbor-joining algorithm using maximum composite likelihood method, with18S rRNA gene of *Septofusidium herbarum* CBS 265.58 (GenBank accession No. AY526480) as an outgroup and bootstrap based on 1000 replicates, in MEGA 4.1 [21]. The identified strain *Penicillium* sp. DS-39 was deposited in the culture collection at the DSMZ – Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany, under the accession No. DSM 23773.

2.3. Microorganism and culture conditions

The strain was maintained on potato dextrose agar (PDA, Scharlau Chemie, Spain) at 4 °C. For spore production PDA was used. After 5 days of incubation at room temperature (~20 °C), spores were collected using a sterile aqueous solution containing 0.02% (v/v) Tween 80 and 0.8% (w/v) NaCl. The liquid medium for lipase production consisted of (g/l): bacto-peptone, 5.0; yeast extract, 1.0; $NaNO_3$, 0.5; KCl, 0.5; $MgSO_4{\cdot}7H_2O$, 0.5; KH_2PO_4, 2.0 and olive oil, 10.0. The pH was adjusted to 7.0 ± 0.2 with 1.0 M NaOH. The cultivation was performed in 1 l Erlenmeyer flask containing 0.5 l medium at 28 °C, under orbital agitation (180 rpm) for 168 h using an inoculum of 1.8 ml spore suspension (10^8 spores/ml). The cultures were filtered (Whatman® No. 1 filter paper) at different time intervals and the mycelia harvested were dried (90 °C, 48 h) to constant weight for growth studies. The culture filtrate obtained after 120 h incubation was passed through a 0.22 μm pore size filter (Millipore® Stericup™ filter unit) and the "cell-free" filtrate thus obtained was used as a source of extracellular lipase.

2.4. Lipase assay

Lipase activity was assayed using *p*-nitrophenyl palmitate (*p*-NPP) as substrate according to Winkler and Stuckmann [22] with some modifications as described [23]. The assay was typically run for 10 min at 45 °C before termination by addition of 2 ml of 0.2 M Na_2CO_3. Liberated *p*-nitrophenol was determined at 410 nm ($\varepsilon_{410\,nm}$ = 0.0169/μM cm) using a UNICAM UV2 2000E UV-VIS spectrophotometer (Cambridge, UK). Appropriate controls (blanks without enzyme) were used to subtract the absorbance corresponding to the reaction mixture other than that produced by the specific hydrolysis of *p*-NPP. One international unit (IU) was defined as the amount of enzyme needed to liberate 1 μmol of *p*-nitrophenol per minute under the assay conditions.

2.5. Protein assay

Protein concentrations were determined by Bradford protein microassay [24] using bovine serum albumin (BSA) as a standard. During chromatographic purification steps, protein concentrations were monitored by measuring the absorbance of fractions at 280 nm ($A_{280\,nm}$).

2.6. Purification of PEL

The cell-free culture filtrate, from a 120 h-old culture grown in an olive oil based medium, was incubated for 20 min at 55 °C. After rapid cooling, insoluble denatured proteins were removed by centrifugation at 15,000 × *g* for 30 min at 4 °C. The resulting supernatant, containing PEL activity, was loaded on a DEAE-cellulose column (1.5 × 20 cm) equilibrated with buffer A (20 mM Tris–HCl, pH 8.0, 50 mM NaCl, 2 mM benzamidine). Under these conditions, PEL was not adsorbed onto the support and was eluted during washing by buffer A. PEL-active fractions eluted from DEAE-cellulose were pooled and subjected to ammonium sulfate protein precipitation at 70% saturation, with constant stirring for 30 min at 4 °C, followed by centrifugation at 15,000 × *g* for 30 min at 4 °C. The pellet obtained was resuspended in buffer A (10 ml) and the insoluble material was removed by centrifugation at 15,000 × *g* for 30 min at 4 °C. The supernatant obtained was concentrated (Centricon Plus-70, Biomax 5000 MWCO, Millipore, UK) and loaded on a Sephacryl® 100-HR column (1.0 × 100 cm) equilibrated with buffer A. Elution of PEL from Sephacryl® 100-HR was performed with buffer A at a flow rate of 10 ml/h. The fractions, containing PEL activity, eluting between 1.2 and 1.3 void volumes, were pooled and applied to a Q Sepharose® HP column (2.5 × 20 cm) equilibrated with buffer B (20 mM Tris–HCl, pH 8.0, 10 mM NaCl, 1 mM benzamidine). Unbound proteins from Q Sepharose® HP were washed out with buffer B and the adsorbed proteins were eluted with a linear gradient of NaCl. During gradient elution with 10–250 mM NaCl, PEL emerged at 150 mM NaCl. The fractions, containing PEL activity, were pooled, concentrated (Centricon Plus-70, Biomax 5000 MWCO, Millipore, UK) and loaded on a Sephacryl® 100-HR column (1.0 × 100 cm) equilibrated with buffer A. Elution of PEL from Sephacryl® 100-HR was performed with buffer A at a flow rate of 10 ml/h. The fractions containing PEL activity were eluted as a single protein peak at 1.3 void volumes. PEL-active fractions were pooled and purity of PEL was determined by SDS-PAGE.

2.7. Gel electrophoresis and zymography

SDS-PAGE was carried out in 12.5% (w/v) gels according to Laemmli's method at room temperature (ATTO AE-6450, Tokyo, Japan) as previously reported by Dheeman et al. [23]. For activity staining zymographic analysis was performed using MUF-oleate as a substrate essentially as described by Prim et al. [25].

2.8. Deglycosylation

In order to analyze for the presence of carbohydrate moieties in the purified PEL, deglycosylation was carried out using endoglycosidase (Endo H, NEB, UK) according to the manufacturer's instructions. Twenty microliters of purified PEL (5.0 μg) was incubated in 0.05% SDS and 0.1% β-mercaptoethanol at 100 °C for 10 min. Then 1 μl of Endo H and 2 μl 50 mM sodium citrate (pH 5.5) was added to the reaction mixture and incubated at 37 °C for

3 h. Deglycosylation was analyzed by mobility shift on SDS-PAGE gel together with the untreated control.

2.9. Effect of pH and temperature

The effect of pH and temperature on purified PEL was investigated by using *p*-NPP as the substrate. The optimal pH of the purified enzyme was determined at 45 °C over a $pH^{45\,°C}$ range of 2.5–9.5 at constant molarity (50 mM) in different buffers: glycine–HCl (pH 2.5–3.5), citrate-phosphate (pH 3.5–5.5), sodium phosphate (pH 6.5–7.5), Tris–HCl (pH 8.0 and 9.5). The pH stability was studied by incubating the purified PEL in selected buffers of $pH^{20\,°C}$ range 2.5–9.5 for 24 h at 20 °C. The residual enzyme activity was measured using *p*-NPP spectrophotometric assay at 45 °C, pH 5.5. The optimum temperature of the purified enzyme was determined by measuring the enzyme activity at various temperatures (20–60 °C) in 50 mM citrate-phosphate buffer, pH 5.5. Thermostability was determined by incubating purified lipase in 50 mM citrate-phosphate buffer, pH 5.5 at various temperatures (20–60 °C) for 3 h and residual activity was analyzed using *p*-NPP spectrophotometric assay at 45 °C, pH 5.5.

2.10. Substrate specificity

Specificity of PEL towards various oils, triacylglycerols and methyl esters of varying fatty acid chain lengths was determined using a titrimetric method as described by Burkert et al. [26]. Activity of PEL on 5% (w/v) natural oils (olive oil, soybean oil, sunflower oil, linseed oil, rapeseed oil, cottonseed oil, corn oil, castor oil), 20 mM triacylglycerols (triacetin (C2:0); tributyrin (C4:0), tricaprylin (C8:0), triolein (C18:1, *cis*-9), trivaccinin (C18:1, *trans*-9), trilinolein (C18:2, *cis*-9,12), trilinolenin (C18:3, *cis*-9,12,15)) and 20 mM methyl esters (C6:0, C12:0 C18:0, C18:1, *cis*-9) was studied using substrate emulsions prepared in 50 mM citrate-phosphate buffer (pH 5.5) containing 7% (w/v) gum acacia. Reactions were incubated at 45 °C for 10 min with orbital shaking. The enzymatic reaction was initiated by the addition of 1 ml of appropriately diluted enzyme solution to the reaction mixture (5 ml) and stopped by the addition of 15 ml of acetone–ethanol mixture (1:1, v/v). The liberated fatty acids were titrated with 0.05 N NaOH. A control was carried out similarly, except that the enzyme solution was added after the addition of acetone–ethanol mixture. Relative lipase activity was calculated taking that on olive oil emulsion as 100%.

2.11. Regio-specificity

Regio-specificity of purified PEL was examined by thin-layer chromatography (TLC) of the reaction product obtained by using pure triolein as described by Sugihara et al. [27]. A reaction mixture composed of 20 mM triolein, 2 ml of 50 mM citrate-phosphate buffer (pH 5.5), and 25 IU of the purified PEL were incubated at 30 °C for 30 min with magnetic stirring. After incubation, the reaction products were extracted with 8 ml of ethyl ether. Aliquots (10 μl) of the ether layer were applied to a Silica Gel 60 plate (Merck KgaA, Darmstadt, Germany) and developed with a 95:4:1 (v/v/v) mixture of chloroform, acetone, and acetic acid. The spots were visualized using a saturated iodine chamber and compared with commercially available standards.

2.12. Effect of metal ions, EDTA and PMSF

The effect of various metal ions, EDTA and PMSF on purified PEL activity was analyzed by incubating the purified enzyme in 1 mM or 10 mM of these effectors for 1 h at 30 °C in 50 mM citrate-phosphate buffer (pH 5.5). Residual activity was measured using *p*-NPP spectrophotometric assay at 45 °C, pH 5.5.

2.13. Effect of organic solvents

The effect of various organic solvents (50%, v/v) on purified PEL was determined by incubating purified enzyme solution in different organic solvents in airtight vials at 30 °C, 200 rpm for 24 h. The control used was the enzyme sample without organic solvent under the same experimental conditions. Residual activity was measured using *p*-NPP spectrophotometric assay at 45 °C, pH 5.5.

3. Results and discussion

3.1. Strain isolation, identification and production of PEL

Fungal strain DS-39, isolated from soil was primarily identified to be a lipolytic strain on tributyrin agar. Micromorphological studies of the isolate showed terverticillate penicilli. Sequencing of 18S rRNA gene and phylogenetic analysis confirmed that the newly isolated strain DS-39 is a *Penicillium* species and is closely related to *Penicillium olsonii* isolate PenI (Fig. 1). The true lipase secretion from *Penicillium* sp. DS-39 was confirmed through fluorescence zone formation on olive oil-rhodamine B agar under UV irradiation [28]. Since, supplementation of olive oil as an inducer was required for enhanced lipase production; it was concluded to be an inducible lipase secreted into the culture medium (Fig. 2). The highest lipase activity (3.25 IU/ml) was observed at the late logarithmic phase of the culture (120 h). The decrease in lipase activity after 120 h of incubation suggests release of proteases into the medium at late stationary phase. After 144 h, the lipase activity decreased signifi-

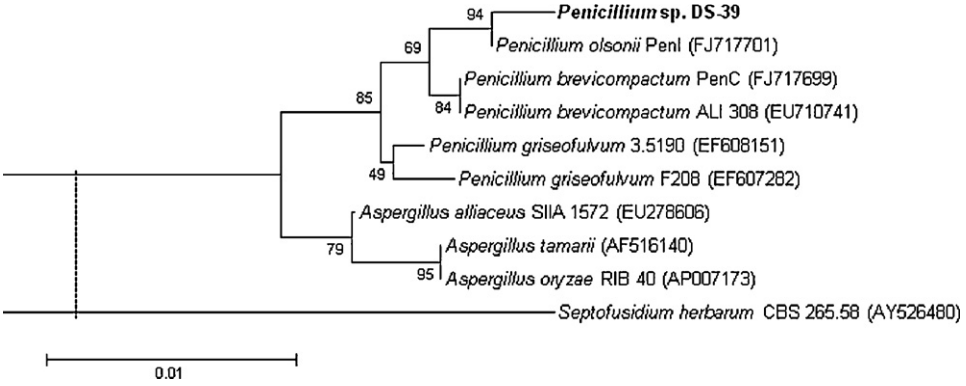

Fig. 1. Neighbor joining phylogenetic tree derived from partial 18S rRNA gene sequences of representative mitosporic Trichocomaceae. The *Penicillium* sp. isolate DS-39 (GeneBank accession No. HM579932) is shown in bold. The evolutionary distances were computed using the maximum composite likelihood method in MEGA 4.1. The numbers at the nodes represent percentage bootstrap values based on 1000 replicates. The horizontal scale bar indicates a distance of 0.01.

Table 1
Purification of PEL.

Purification step	Total activity[a] (IU)	Total protein[b] (mg)	Specific activity (IU/mg)	Purification (fold)	Yield (%)
Culture filtrate (120 h)	1190.0	498.0	2.38	1.00	100.0
Heat treatment (20 min at 55 °C)	952.00	99.6	9.55	4.01	80.00
DEAE-cellulose	634.60	9.98	63.58	26.71	53.27
0–70% ammonium sulfate precipitation	396.60	4.97	79.79	33.52	33.32
Sephacryl® 100-HR–I	237.98	1.49	159.71	67.10	19.99
Q Sepharose® HP	158.63	0.54	293.75	123.42	13.33
Sephacryl® 100-HR–II	104.97	0.34	308.73	129.72	8.82

[a] Lipase activity was determined using *p*-NPP as a substrate as described in Section 2.4.
[b] Protein concentration was measured by Bradford protein microassay [24].

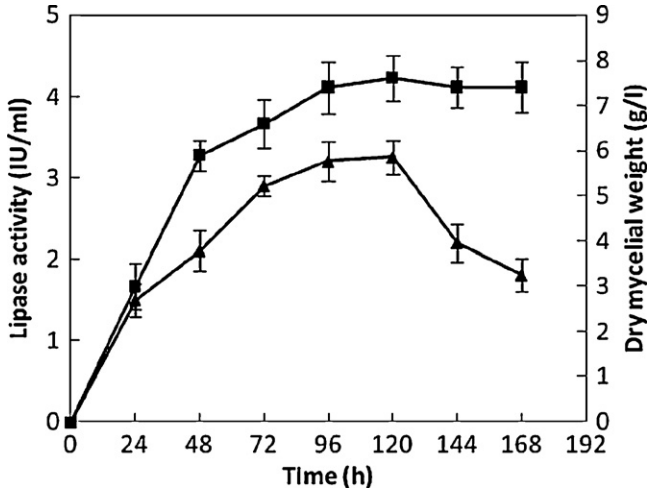

Fig. 2. Time course of lipase production by *Penicillium* sp. DS-39. Cultivation was performed as described in Section 2.3. Batches were harvested at 24 h intervals to monitor dry mycelial weight (■) and lipase activity (▲).

cantly to about 1.81 IU/ml which might be attributed to proteolytic degradation.

3.2. Purification of PEL

The PEL containing cell-free culture filtrate was incubated at 55 °C for 20 min. After rapid cooling, denatured proteins were separated to recover 80% of initial activity in the culture supernatant. The PEL from heat-treated supernatant was purified by a five step procedure (Table 1). The patterns of Q Sepharose® HP anion exchange chromatography and Sephacryl® 100-HR gel exclusion chromatographies are shown in Fig. 3A. The pure lipase was recovered as a single peak in void volume (Fig. 3A-c). About 129-fold purification with 8.82% recovery was achieved. PEL is the only *Penicillium* lipase to be purified by this combination of chromatographies [12] resulting in a highly pure homogeneous preparation (Fig. 3B-a). The zymographic analysis using MUF-oleate revealed an activity band that coincided with the migration of purified protein (Fig. 3B-b). The molecular mass of purified lipase was estimated to be 43 kDa on SDS-PAGE. Most of the known lipases from the genus *Penicillium* have been reported to have molecular mass in the range of 25–40 kDa [12] with a few reports on higher

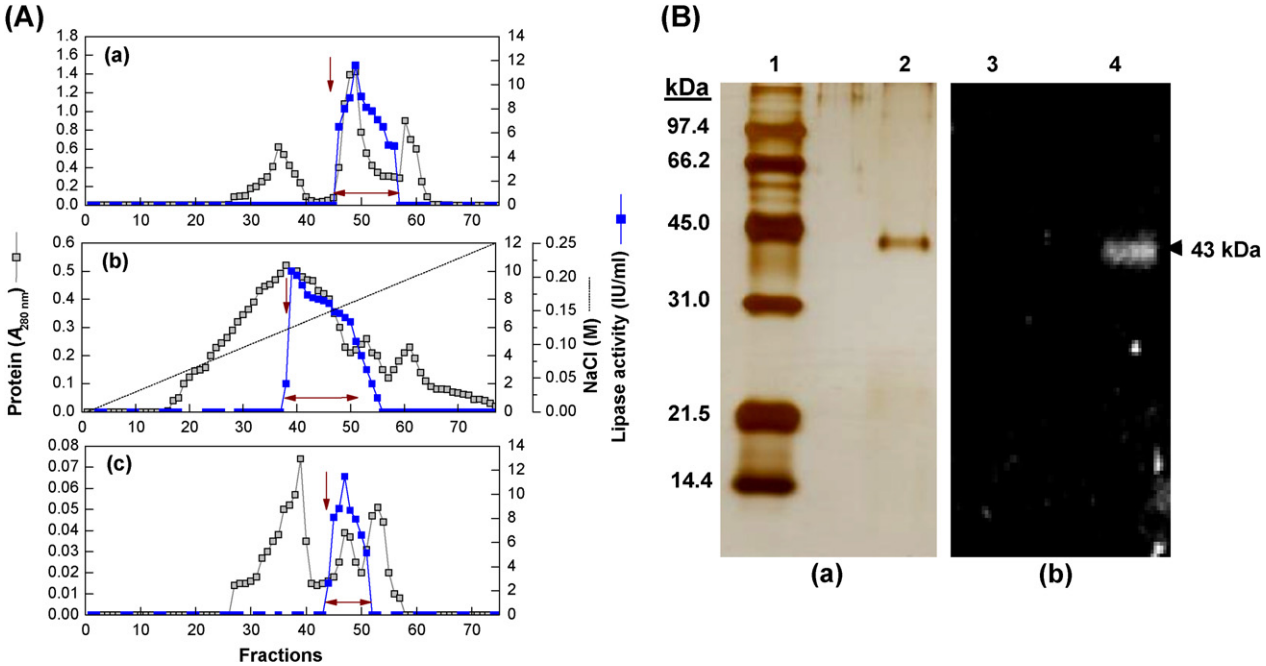

Fig. 3. Purification of PEL. (A-a) Chromatography on Sephacryl® 100-HR. (A-b) Chromatography on Q Sepharose® HP. (A-c) Chromatography on Sephacryl® 100-HR. In each step of chromatography, elution of proteins was followed by measuring absorbance at 280 nm (-■-) and PEL activity was followed by a spectrophotometric assay using *p*-NPP as a substrate (-■-). The downward arrows indicate the initiation of elution of PEL. The dotted line (. . .) indicates NaCl concentration of the eluting buffer. The transverse left-right arrows indicate the fractions collected for further studies. (B) Analysis of purified PEL on a 12.5% SDS-PAGE gel (a) and zymography using MUF-oleate as a substrate (b). The SDS-PAGE gel was silver stained after zymographic analysis. Lanes 1 and 3, low molecular weight markers; lane 2, 100 ng of purified PEL; lane 4, activity of 100 ng purified PEL on MUF-oleate.

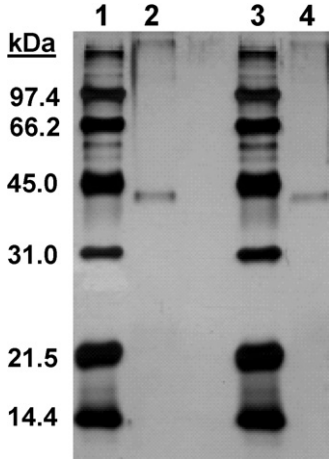

Fig. 4. Effect of deglycosylation on the mobility of purified PEL under denaturing conditions. Lane 1 and 3, low molecular weight markers; lane 2, PEL treated with Endo H; lane 4, untreated purified PEL. The gel was stained with PhastBlue R-350 (GE Healthcare, UK).

molecular masses of 40 and 93 kDa [8,29]. The present lipase, PEL can be rated among the higher molecular mass lipases.

Lipases from different sources have significant variation in properties especially with respect to carbohydrate content, regio-specificity, fatty acid specificity, thermostability, pH optimum and kinetics in non-aqueous system [30]. A broad spectrum of substrate utilization coupled with enantioselectivity and enhanced efficiency in nonaqueous medium is always desirable in new lipases [31]. With these considerations, the purified PEL was examined for novel characteristics.

3.3. Deglycosylation

Deglycosylation of PEL exhibited unaltered electrophoretic mobility on SDS-PAGE gel (Fig. 4). Increasing incubation time and endoglycosidase concentration in the reaction mixture did not change the results, indicating PEL has no endoglycosidase (Endo H) sensitive glycosyl moities as previously reported in the case of *Penicillium simplicissimum* lipase [32].

3.4. Effect of pH and temperature

pH optimum of PEL was found to be 5.5, by standard *p*-NPP spectrophotometric assay as well as titrimetrically using triolein as substrate, which is similar to lipases reported from other *Penicillia* [11,32,33]. The PEL showed good pH stability retaining more than 95% activity in the pH range 5.0–6.0 after 24 h (Fig. 5A). Most of the lipases from *Penicillia* are reported to be most active and stable in neutral to alkaline pH range [8,29,34–37]. However, PEL showed optimum pH and stability towards slightly acidic range. Similar pH activity and stability profiles were observed in a few lipases from *Penicillia* [32,33]. The temperature optimum of PEL was observed to be 45 °C, in common with other lipases from *Penicillia* [9,13,37,38]. While studying the thermal stability profile, PEL was found to be stable at 45 °C at least up to 3 h and it retained 90% of its original activity at 50 °C after 3 h (Fig. 5B). The thermoactive lipase from *Penicillium watmanii* showed lower stability retaining 55% residual activity after 1 h at 50 °C [9]. Thus, the high activity and stability of PEL makes it potentially useful in biocatalytic processes operating at high temperatures.

Table 2
Substrate specificity of PEL.

Substrate	Concentration	Relative activity (% ±SD)[a]
Natural oils		
Olive oil	5% (w/v)	100 ± 2.1
Castor oil	5% (w/v)	88.4 ± 2.1
Corn oil	5% (w/v)	89.1 ± 1.8
Cottonseed oil	5% (w/v)	81.6 ± 1.0
Linseed oil	5% (w/v)	84.0 ± 1.6
Rapeseed oil	5% (w/v)	85.3 ± 2.2
Soybean oil	5% (w/v)	73.7 ± 1.1
Sunflower oil	5% (w/v)	89.5 ± 0.5
Triacylglycerols		
Triacetin (C2:0)	20 mM	34.8 ± 1.1
Tributyrin (C4:0)	20 mM	51.3 ± 2.2
Tricaprylin (C8:0)	20 mM	59.7 ± 2.0
Triolein (C18:1, *cis*-9)	20 mM	148.9 ± 2.9
Trivaccinin (C18:1, *trans*-9)	20 mM	91.8 ± 1.4
Trilinolein (C18:2, *cis*-9,12)	20 mM	94.4 ± 0.8
Trilinolenin (C18:3, *trans*-9,12,15)	20 mM	96.3 ± 3.1
Fatty acid methyl esters		
Caproic acid methyl ester (C6:0)	20 mM	4.1 ± 0.6
Lauric acid methyl ester (C12:0)	20 mM	4.3 ± 1.6
Stearic acid methyl ester (C18:0)	20 mM	10.8 ± 1.3
Oleic acid methyl ester (C18:1, *cis*-9)	20 mM	11.7 ± 2.1

[a] Experiments were performed in triplicate and relative activity was calculated by activity on olive oil. The average of relative value ($N = 3$) and standard deviations (SD) are shown.

3.5. Substrate specificity

Different substrate specificities within lipases are attributed to the differences in the geometry and size of their active sites [39]. The purified PEL showed significant activity on various emulsified oils and triacylglycerols (Table 2). Triolein was most efficiently

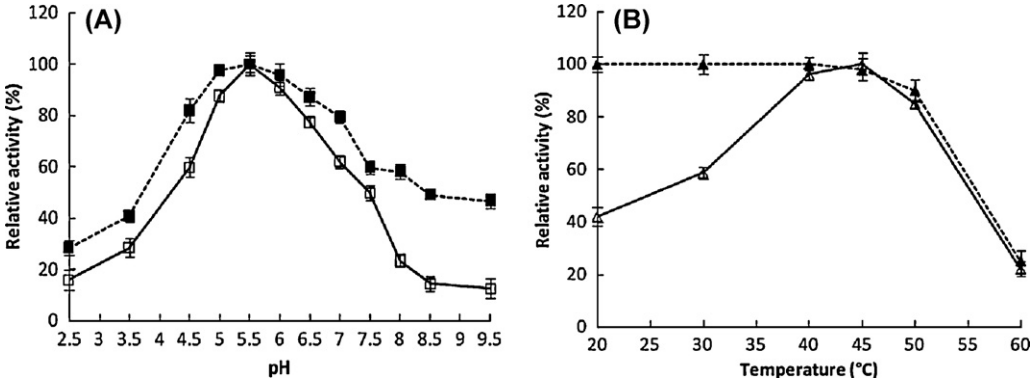

Fig. 5. (A) Effect of pH on activity (□) and stability (■) of PEL. Experiments were performed in triplicate and relative activity was calculated by activity at pH 5.5. The average of relative value ($N = 3$) and error bars are shown. (B) Effect of temperature on activity (△) and stability (▲) of PEL. Experiments were performed in triplicate and relative activity was calculated by activity at 45 °C. The average of relative value ($N = 3$) and error bars are shown.

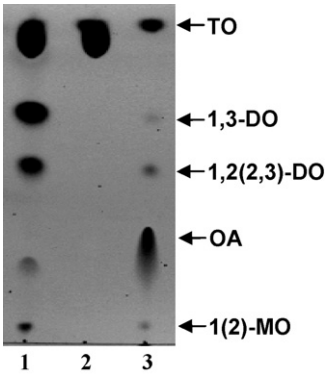

Fig. 6. Regio-specificity of PEL The specificity of PEL for ester bond positions was determined by hydrolysis of triolein. The hydrolysis products were analyzed by TLC as described in Section 2.11. Lane 1, standards (1(2)-monoolein (1(2)-MO), oleic acid (OA); 1,2(2,3)-diolein (1,2(2,3)-DO), 1,3-diolein (1,3-DO), triolein (TO)); lane 2, control (triolein without enzyme); lane 3, products of triolein hydrolysis by purified PEL (1,3- diolein; 1,2 or 2,3- diolein; oleic acid, 1 or 2- monoolein and residual triolein).

hydrolyzed compared to other substrates tested. A trend of preferential specificity toward oils and triacylglycerols, with longer chain length unsaturated fatty acids, is clearly evident. However, methyl esters of long chain fatty acids were found to be poor substrates for PEL. Most of the reported lipases from *Penicillia* prefer triacylglycerols with short chain fatty acids or their methyl esters [11,13,29,35,40,41]; however, similar to PEL, a lipase from *Penicillium expansum* showed specificity for triacylglycerols with long chain fatty acids [37]. High relative activity on various emulsified oils suggests that PEL may play a significant part in the biodegradation of oil spills in the environment [4]. Moreover, its broad hydrolysis of various oil substrates suggests that it can be potentially applied in the production of biodiesel [4,31]. The distinct specificity of PEL for long, unsaturated fatty acyl chains is a valuable property for enzymatic restructuring by interesterification of fats and oils with unsaturated fatty acids to improve the physical and nutritional properties of triacylglycerols for use in food industries [42].

3.6. Regio-specificity

Lipases are further categorized based on their preference for acyl group positions in triacylglycerol substrate *viz.*1,3-specific, 2-specific, and non-specific or random. This was investigated for PEL using triolein as substrate [27]. The products were analyzed by TLC (Fig. 6). PEL cleaved not only the 1,3-positioned ester bonds but also the 2-positioned ester bond of triolein leading to the formation of a mixture of products (1,3-diolein; 1,2 or 2,3-diolein; oleic acid and residual triolein). This indicated that PEL is a non-regio-specific lipase. This kind of non-regio-specificity of PEL has been reported earlier in lipases from *Penicillia* and other bacteria [2,27,32,41]. Thus, PEL may be useful in ester synthesis with both primary and secondary alcohol moieties and therefore, can be a potential candidate to synthesize PUFA-rich triacylglycerols. Since PUFAs such as EPA and DHA are very unstable, enzymatic synthesis of PUFA-rich triacyglycerols under mild conditions is preferred over chemical methods. Non-regio-specific lipases are favourable rather than 1,3-specific ones because all three hydroxyl groups should be esterified [43]. The specificity of PEL for long unsaturated fatty acids together with non-regio-specificity makes it a potential candidate for such applications.

3.7. Effect of metal ions, EDTA and PMSF

The effect of various metal ions, EDTA and PMSF on PEL activity and stability was tested at 1 or 10 mM in 50 mM citrate-phosphate buffer at pH 5.5 (Table 3). Lipase activity was found to be stimulated or stabilized in the presence of Ca^{2+} and Mn^{2+}. Stimulation of lipase activity by Ca^{2+} and Mn^{2+} has been reported for other lipases from *Penicillia* [11,32,37]. Hg^{2+} and Zn^{2+} significantly inhibited the activity by 73.2% and 45.6%, respectively as previously reported for lipases from *Penicillium aurantiogriseum*, *Penicillium abeanum* and *P. simplicissimum* [7,27,32]. Hg^{2+} was also reported to be a strong inhibitor of a lipase from *P. expansum* [37] and a mono- and diacylglycerol lipase (MDGL) from *Penicillium camembertii* U-150 [14]. The strong inhibition of PEL by Hg^{2+} suggests the presence of key cysteine residues in the enzyme [27]. A minor reduction in PEL activity was observed with Ag^{+} as reported in case of other lipases [32,37]. The enzyme activity remained unaffected by the metal chelator, EDTA at 1 and 10 mM concentration, indicating that PEL is not a metalloenzyme. The effect of PMSF, a serine inhibitor on PEL at 1 and 10 mM concentration showed only marginal reduction in lipase activity. This indicated that the serine residues in PEL may be buried or less accessible [2]. No significant change in the activity of *P. camembertii* U-150 MDGL was observed upon addition of EDTA and PMSF [14]. However, *Penicillium chrysogenum* 9′ lipase was drastically inhibited by PMSF and EDTA [11].

Table 3
Effect of metal ions and additives on PEL.

Compound	Concentration (mM)	Relative activity (% ±SD)[a]
Control	–	100 ± 3.3
$AgNO_3$	1	81.9 ± 1.3
$BaCl_2$	1	94.4 ± 2.4
$CaCl_2$	1	117.7 ± 1.9
	10	123 ± 2.2
$CoCl_2$	1	96.3 ± 2.7
$CuCl_2$	1	97.9 ± 1.4
$FeCl_2$	1	98.7 ± 2.4
$HgCl_2$	1	26.8 ± 3.4
LiCl	1	97.4 ± 1.1
$MgCl_2$	1	94.3 ± 2.2
$MnCl_2$	1	104 ± 1.4
$ZnCl_2$	1	54.4 ± 2.6
EDTA	1	99.2 ± 1.1
	10	98.3 ± 1.2
PMSF	1	91.1 ± 2.3
	10	87.0 ± 2.3

[a] Experiments were performed in triplicate and relative activity was calculated by activity of untreated control. The average of relative value ($N = 3$) and standard deviations (SD) are shown.

3.8. Effect of organic solvents

High activity and stability of lipases in organic solvents is an essential prerequisite for applications in organic synthesis [31,44], hence activity and stability in organic solvents are considered novel attributes in a lipase [2]. Effect of various organic solvents on the stability of PEL is shown in Fig. 7. After 24 h, PEL was stable and activated in the presence of non-polar hydrophobic solvents such as toluene, *n*-xylene, *n*-hexane, *n*-heptane, isooctane and dodecane. However, exposure to polar solvents (dimethylsulfoxide (DMSO), dimethylformamide (DMF), methanol, ethanol, 2-propanol and *n*-butanol) showed drastic reduction in residual activity. Similarly, *P. simplicissimum* lipase was very tolerant towards non-polar hydrophobic solvents with significant instability in polar solvents [32]. Similar results were obtained for a lipase from *P. aurantiogriseum* [7]. The significant deactivation in polar solvents is due to the stripping-off of crucial bound-water monolayer from the enzyme molecule essential for its activity [44]. The high stability and stimulation of PEL with residual activities of 114.5% for toluene, 137.4% for *n*-hexane, 120.6% for isooctane and 112% for dodecane are in agreement with the general behaviour of lipases in non-polar

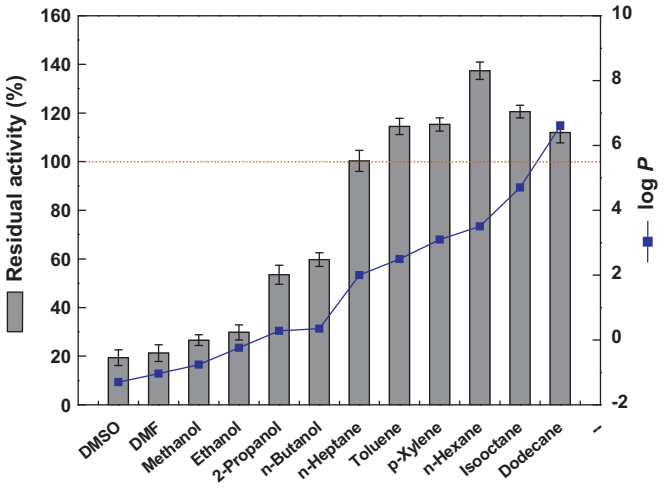

Fig. 7. Stability of PEL in organic solvents. The purified PEL was incubated in various organic solvents at 50% (v/v) concentration at 30 °C for 24 h. Residual activities were measured using *p*-NPP as substrate as described in Section 2.13. Experiments were performed in triplicate and relative activity was calculated by activity at 0% (v/v) organic solvent. The average of relative value ($N = 3$) and error bars are shown.

hydrophobic solvents [24,32,45]. Activation is due to the residues of carried-over non-polar hydrophobic solvent providing an interface, thereby keeping the enzyme in an open conformation and thus resulting in the observed activation [46,47]. The reasonably high stability of PEL in non-polar solvents makes it potentially useful for practical application in synthetic reactions in non-conventional media.

4. Conclusions

In this study PEL produced by a newly isolated *Penicillium* sp. DS-39 was purified to homogeneity with 129-fold purity and a specific activity of 308.73 IU/mg. It showed a high thermostability which is uncommon in lipases from mesophilic filamentous fungi. It exhibited a broad substrate range with distinct specificity for oils and triacylglycerols of long unsaturated fatty acids and was found to be significantly stable in the presence of non-polar hydrophobic solvents. These features render PEL suitable for potential applications in non-aqueous biocatalysis such as biodiesel production, enzymatic restructuring, by interesterification of different oils, and fats and biodegradation of oil spills in the environment

Acknowledgement

This research work was financially supported by DIT ABBEST Research Scholarship to DSD (PB 03557/2007).

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