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Purification and characterization of a new cold active lipase, *En*L A from *Emericella nidulans* NFCCI 3643

Suseela Lanka and J. Naveena Lavanya Latha*

Department of Biotechnology, Krishna University, Machilipatnam, A.P. India.

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A mesophilic fungi producing an extracellular cold-active lipase was isolated from the soil samples of palm oil mill effluent dump sites, Pedavegi, West Godavari Dist, A.P. India and was identified as *Emericella nidulans*. The enzyme was purified by ammonium sulfate fractionation followed by hydrophobic interaction chromatography using phenyl sepharose. The enzyme was 35 fold pure compared to crude with a specific activity of 1494.51 U/mg. SDS PAGE analysis revealed that the protein is monomeric with a MW of ˜54 kDa and zymogram analysis showed that the purified protein was active. Characterization studies revealed that the temperature optimum was at 30°C and an optimum pH of 5. The K_m and V_{max} values were found to be 0.61 mM and 322.58 mM/min.mg, respectively. Sequencing of the purified protein by MALDI TOF-MS analysis followed by BLAST P analysis indicated that the protein is a putative secretary lipase from *E. nidulans*. Search of lipase engineering data base (LED) revealed that this protein belongs to a newly introduced super family of *Candida antarctica* lipase A like and to the homologous family of *Aspergillus* lipase like.

Key words: Cold active lipase, *Emericella nidulans*, hydrophobic interaction chromatography, *Candida antarctica* lipase A like.

INTRODUCTION

A large part of the earth's biomass is occupied by lipids; hence the role of lipolytic enzymes in the turnover of these compounds became significant. Lipases (EC 3.1.1.3) (triacylglycerol acyl hydrolases), are α/β hydrolases and are mainly involved in the hydrolysis and synthesis of esters from glycerol and long chain fatty acids (Gilbert, 1993). The general reaction catalyzed by the lipases is shown in Figure 1. The applicability of lipases in various bioprocess applications mainly depends upon their availability and stability in both organic and as well as in aqueous media (Aulakh and Prakash, 2010; Kumar and Kanwar, 2012). Microbial lipases are receiving much attention of the industrialists due to the advancements in enzyme technology (Hasan et al., 2006). They possess broad substrate, pH, temperature specificities and exhibit good chemo-, regio- and enantio selectivity. All these features make lipases as versatile biocatalysts (Kademi et al., 2005). Lipases as biocatalysts can be effectively used for biodiesel production (Delos Rios et al., 2011; Yan et al., 2011) and also for various other industrial applications. Most of the fungal lipases that were reported so far are derived from

*Corresponding author. E-mail: jnlavanyalatha@yahoo.co.in. Tel: 91-9502245034. Fax: 91-8672225960.

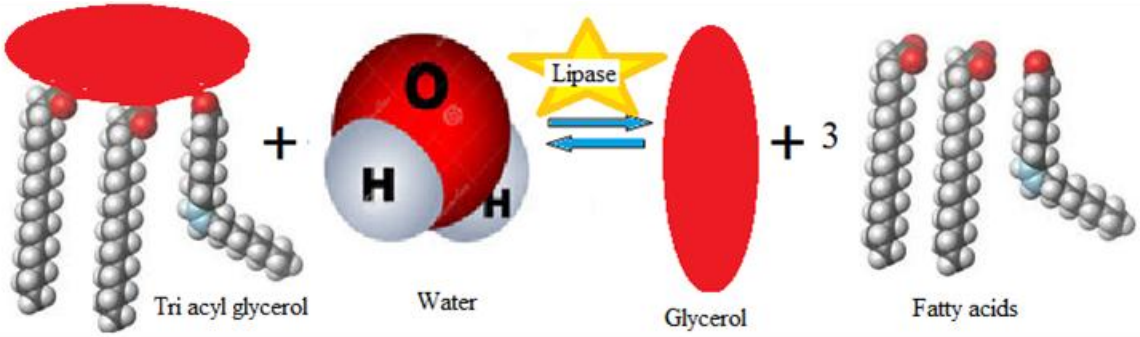


Figure 1. General reaction catalyzed by the lipases, depicting the lipase catalyzed reversible hydrolysis of triacyl glycerol to glycerol and fatty acids.

the genus *Aspergillus* (Contesini et al., 2010). Lipases purified from *Aspergillus* spp. were reported to have good thermal stability, specificity and stability in organic solvents etc.

Cold active enzymes with their unique kinetic and molecular properties along with their ability to serve as catalysts for enthalpy deficient conditions are widely employed in the organic synthesis of potentially useful compounds. These enzymes with their marked thermo lability and activity at low temperatures (Cavicchioli et al., 2002) have found numerous applications (Lo Giudice et al., 2006). Hence cold active lipases can be employed in various industrial applications which require substrate/ product stability and energy savings (Gerday et al., 1997; Marshall, 1997).

New lipases isolated from novel sources with potential industrial applications are paving way for the use of these enzymes for various bioprocess reactions (Islam et al., 2008). In the present study, a new cold active lipase, *Emericella nidulans* Lipase A (*En*L A), belonging to the new super family of *Candida antarctica* lipase A like was purified and characterized from a mesophilic fungus *E. nidulans* NFCCI 3643, screened and isolated from Palm Oil Mill Effluent (POME) dump sites.

MATERIALS AND METHODS

Microorganism and culture conditions

The fungal culture in the present study was screened and isolated from Palm Oil Mill Effluent (POME) dump sites, Pedavegi, West Godavari District. The strain was identified as *E. nidulans* DAOM 222012 by National fungal culture collection of India (NFCCI), Agarkhar Research Institute, Pune and was also deposited at NFCCI with an accession number 3643. Since then the strain was called *E. nidulans* NFCCI 3643.

Lipase production medium

Purification of extracellular lipase from *E. nidulans* NFCCI 3643 was carried out with the lipase production medium optimized using Response surface methodology (Suseela and Naveena Lavanya Latha, 2015) consisting of (gm/L), Olive oil, 18.98 mL; ammonium sulphate, 14.38; KH_2PO_4, 1; $MgSO_4$, 0.5; Gum Arabic, 5; and pH 5.17. The Fermentation was carried out at 25°C for a period of 4 days at 150 rpm.

Extracellular lipase assay

The culture filtrate after 4 days of growth was collected by centrifugation for 15 min at 10,000 *g*. The supernatant (crude enzyme) was tested for enzyme activity. The assay mixture (1 ml) consisted of 100 µl of sample and 900 µl of substrate solution containing 10 mg of pNPP in Tris–HCl pH 7.0 containing 40 mg of Triton X-100 and 10 mg of gum arabic. The mixture was incubated at 30°C for 30 min and the p-nitrophenol released was measured at 410 nm. One unit of enzyme activity was defined as the amount of enzyme that liberated 1 µ mol of p-nitrophenol per min under the assay conditions (Maia et al., 2001).

Purification of extracellular lipase

Crude enzyme extract was subjected to ammonium sulphate precipitation (40 to 60% saturation) at 4°C, and dialyzed against three changes of same buffer for overnight. This was followed by centrifugation at 10,000 x *g* for 15 min to collect the precipitated protein which was then dissolved in 50 mM phosphate buffer, pH 6.5. The sample was applied to phenyl sepharose (Pharmacia Biotech) column pre equilibrated with 3 bed volumes of 50 mM phosphate buffer (pH 6.5). The column was washed with 2 bed volumes of 1 M ammonium sulfate in the equilibration buffer and then with 2 bed volumes of 50 mM Tris-HCl buffer (pH 7.5) until the absorbance of the eluent at 280 nm was zero. Finally, lipase was eluted with 2 bed volumes of 5 mM Tris-HCl buffer (pH 7.5) containing 20% 2-propanol (Mayordomo et al., 2000). The eluent was collected in 5 ml fractions and concentrated by freeze-drying. The fractions were assayed for lipase activity by using pNP-palmitate as substrate. The protein content of the collected fractions was estimated using the method of Bradford (Bradford, 1976).

Gel electrophoresis and zymography

The molecular weight of the purified lipase was determined by SDS-PAGE using Laemmli method (Laemmli, 1970). Zymographic analysis was done following native PAGE (Davis, 1964). For zymographic analysis, the gel was rinsed three times with distilled water and equilibrated in 50 mM citrate phosphate buffer (pH 6.0)

for 30 min at room temperature. The gel was overlaid with molten chromogenic substrate prepared by using phenol red (0.01%) along with 1% lipidic substrate (olive oil), 10 mM $CaCl_2$, and 2% agar. The pH was adjusted to 7.3 to 7.4 by using 0.1 N NaOH. The over laid gel was then allowed to solidify and incubated at 30°C. The lipase activity can be observed within 5 to 15 min as yellow band over a pink background (Rajni et al., 2006).

Effect of temperature and pH on enzyme activity and stability

The effect temperature and pH on enzyme activity and stability was studied by using pNP-palmitate as substrate. The temperature optima was determined by incubating the purified lipase at different temperatures ranging from 0 to 70°C in 50 mM citrate phosphate buffer of pH 6 followed by enzyme estimation. The temperature stability of the enzyme was determined by incubating the assay mixture at respective temperatures for 1 h followed by enzyme estimation. For the optimum pH, the enzyme was incubated with substrate at 30°C over a varied pH range (from 2 to 10.0) followed by estimation of enzyme activity. The pH stability was determined by incubating the purified enzyme in the buffers of different pH. The reaction mixtures were then incubated at 30°C for 1 h and the residual enzyme activities were then determined.

Effect of organic solvents on enzyme activity and stability

The effect of organic solvents on enzyme activity and stability was studied by incubating the purified enzyme with substrate in the presence of various organic solvents *viz*., methanol, ethanol, isopropanol, diethyl ether, DMSO, butanol and acetone at a concentration of 10 to 50% (10, 30 and 50%) for a period of 24 hrs followed by enzyme estimation.

Kinetic studies

The kinetic parameters of the purified lipase were determined by incubating the enzyme with different concentrations of pNP-palmitate (0 to 50 µM), pH 5 for 30 min at 30°C followed by enzyme estimation. The K_m and V_{max} for the purified enzyme were determined by using Line weaver-Burk equation plot.

Bioremediation of simulated oil effluents using partition gravimetric method

The potential of the purified lipase in the bioremediation was studied by incubating the enzyme (1%v/v) with simulated oil effluents containing 10% each of palm oil, dalda, grease, butter, olive oil, ghee etc. at 30°C for 24 h followed by the estimation of oil and grease content using partition gravimetric method (Kirschman and Pomeroy, 1949).

Biodiesel production

Analysis of FAME's (fatty acid methyl esters)

FAME's production was carried out by incubating the purified lipase (2.6 mL) with Olive oil (7.89 mL) and methanol (0.99 mL) at 30°C with shaking at 150 rpm for 48 h (Yoo et al., 2011). After incubation, 200 µL of sample was taken from the reaction mixture and diluted with 1 mL of *n* hexane for 2 min followed by centrifugation at 10,000 rpm for 15 min. The upper layer separated was used for FAME's analysis using GC-MS and Fourier transform infrared (FTIR) spectroscopy.

GC-MS analysis for FAME's

Fatty acid methyl esters (FAME) content in the reaction mixture was analyzed using GCMSQP2010, SHIMADZU instrument equipped with a ZB-5ms column with 30 m length, 0.25 µm thickness and 0.32 mm diameter. The column temperature was held at 150°C for 2 min and then raised to 300°C at the rate of 8°C /min and maintained at this temperature for 10 min. The injector temperature was set at 250°C and that of detector at 1000 kV. Helium was used as carrier gas and the total GC scan time was 44 min. The FAME's produced were identified using GC-MS library.

FTIR analysis for FAME's

The infrared absorption spectrum of the sample was obtained in a Fourier transform infrared spectrometer (Attenuated Total Reflectance (ATR), Bruker) using KBr tablets in the range of 4000 to 400 cm^{-1}.

Determination of the amino acid sequence of the purified lipase

The amino acid sequence of the purified lipase was determined by MALDI TOF-MS analysis of the tryptic digested peptides using MALDI-TOF/TOF MS Bruker Daltonics, ULTRAFLEX III instrument and further analysis was done with Flex analysis software for obtaining the peptide mass fingerprint. The masses obtained in the peptide mass fingerprint were submitted for Mascot search engine for the identification of the protein.

*En*L A and lipase engineering database (LED)

Since LED (http://www.led.uni-stuttgart.de/) is a repository of lipase sequences from all possible sources, it was searched for the presence of *En*L A sequence.

RESULTS

Purification of extracellular lipase

The extracellular lipase from *E. nidulans* NFCCI 3643 was purified using ammonium sulphate precipitation followed by Hydrophobic Interaction Chromatography (HIC) using Phenyl Sepharose. The elution profile of the protein by HIC was shown in Figure 2. A 35 fold purified lipase with a specific activity of 1494.5 U/mg was achieved with this method (Table 1).

Gel electrophoresis and zymogram analysis

The apparent molecular weight of the purified lipase as determined by SDS PAGE was found to be around ˜54 kDa (Figure 3a). A single band appeared on native PAGE gel and the purified enzyme showed activity on Zymogram (Figure 3b).

Effect of temperature, pH and organic solvents on enzyme activity and stability

The purified lipase exhibited temperature optima at 30°C.

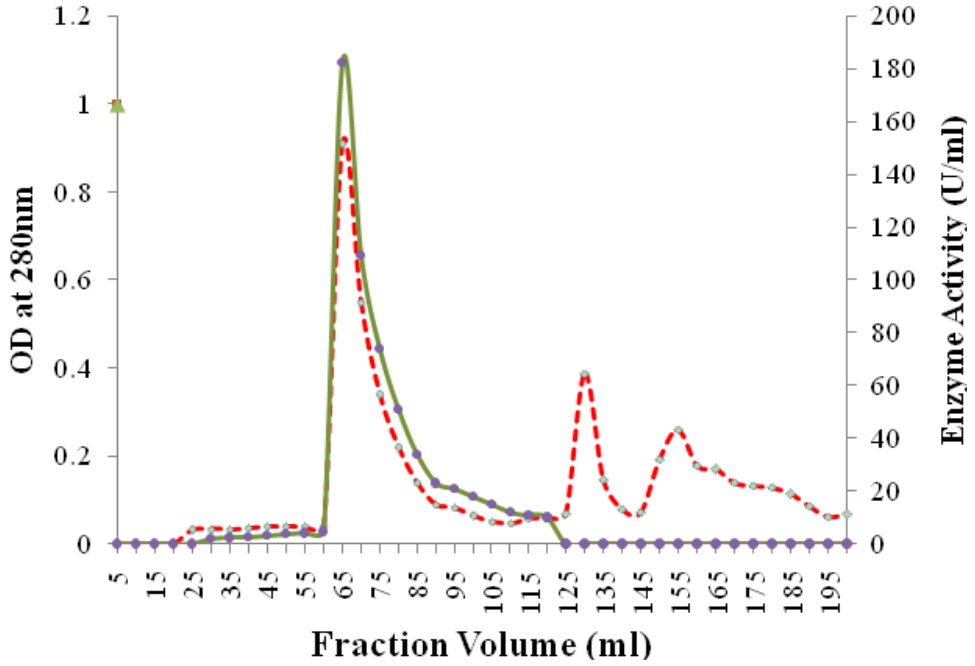


Figure 2. Elution profile of Enzyme fractions obtained from HIC using Phenyl sepharose. Elution of proteins from the column was followed by measuring absorbance at 280 nm using UV absorbance at 280 nm and by spectro photometric estimation of the enzyme activity using pNP-palmitate as substrate.

Table 1. Purification table.

Sample	Volume (ml)	Total protein (mg)	Total Activity (U)	Specific Activity (U/mg)	Yield of protein (%)	Yield of Activity (%)	Fold purification
Crude extract	700	980	61,789	63.05	100	100	1
Ammonium sulphate precipitation (40-60%)	15	12	15055.16	1254.59	1.22	24.36	19.96
Hydrophobic Interaction Chromatography (HIC)	60	4.2	9266	1494.51	0.428	14.996	35.037

Table showing the summary of the fold purification of extracellular lipase from *E. nidulans* NFCCI 3643.

The enzyme was active over a wide temperature range between 10 to 30°C and the enzyme even exhibited ~40% activity at 0°C. Further, the temperature stability of the purified lipase was determined by incubating the enzyme with substrate at different temperatures for a period of 1 h and found that the enzyme is relatively stable between 4 to 30°C (Figure 4a). The optimum pH for pure lipase was found to be 5, however; the enzyme is relatively active over a broad pH range from 5 to 7. The lipase was stable in the pH range of 4 to 7 after incubation for 1 h suggesting that the lipase is acidic lipase (Figure 4b). Enzyme was active and stable towards all the organic solvents tested at 10% concentration and even the enzyme retained more than 70% of its activity at higher concentrations (30 and 50%) with an exception to butanol (Figure 4c).

Determination of kinetic parameters

The Michaelis-Menten constant (K_m) and the maximum velocity of the reaction (V_{max}) were determined from the Lineweaver-Burk plot (Figure 5). The K_m was found to be 0.61 mM and V_{max}, the maximal velocity was found to be 322.58 mM/min.mg.

Bioremediation of simulated oil effluents using purified lipase from *E. nidulans*

From the results obtained (Figure 6), it can be inferred that there was 84.84% reduction in oil/grease content after treatment of simulated oil effluent containing olive oil with *E. nidulans* lipase, followed by palm oil, grease,

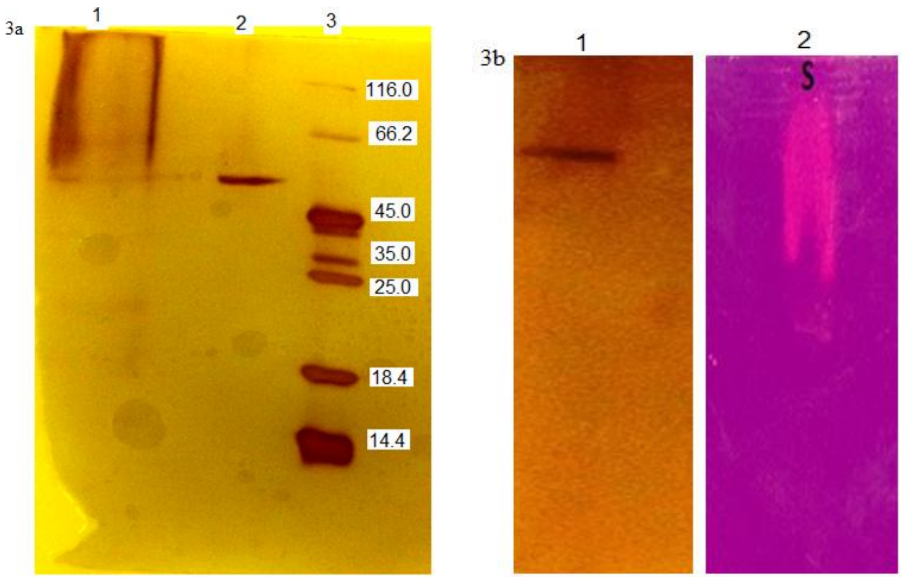


Figure 3. SDS-PAGE, Native PAGE and Zymographic analysis of purified lipase from *Emericella nidulans* NFCCI 3643. **a.** SDS PAGE of purified lipase from *E. nidulans*. The 40-60% ammonium sulphate precipitate and the purified lipase from HIC and molecular weight marker were electrophoresed on 12% SDS-PAGE followed by silver staining to visualize the bands. Lane 1, 40 to 60% ammonium sulphate precipitate; lane 2 Purified protein from HIC; lane 3 molecular weight markers (the makers are β-galactosidase (116.0 kDa), bovine serum albumin (66.2 kDa), ovalbumin (45.0 kDa), lactate dehydrogenase (35.0 kDa), REase Bsp 981 (25.0 kDa), β-lactoglobuin (18.4 kDa) and lysozyme (14.0 kDa)). **b.** Native PAGE (Silver stained gel) and Zymogram of purified lipase. The purified lipase was electrophoresed on 10% Native page gel. Zymography of the purified lipase on native PAGE gel was done by overlaying the gel with the molten chromogenic substrate, which was then allowed to solidify and incubated at 30°C. The lipase activity can be observed within 5 to 15 min as yellow band over a pink background. Lane 1**,** Purified Lipase on Native PAGE Gel; lane 2, Activity stained Native Gel.

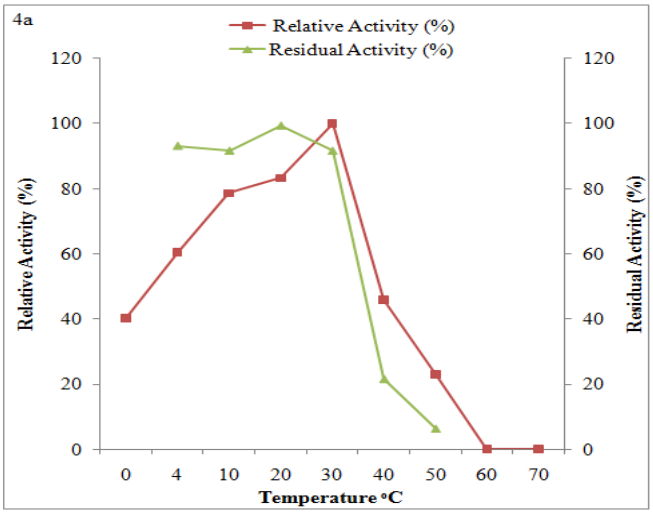


Figure 4 Characterization of purified lipase from *Emericella nidulans* NFCCI 3643. **a,** Effect of Temperature on lipase activity and stability. The effect of temperature on lipase activity was determined by incubating the purified lipase with substrate at different temperatures ranging from 0 to 70°C followed by enzyme estimation. The temperature stability of the enzyme was determined by pre incubating the enzyme extract at indicated temperatures for a period of 1 h followed by estimation of remaining activity.

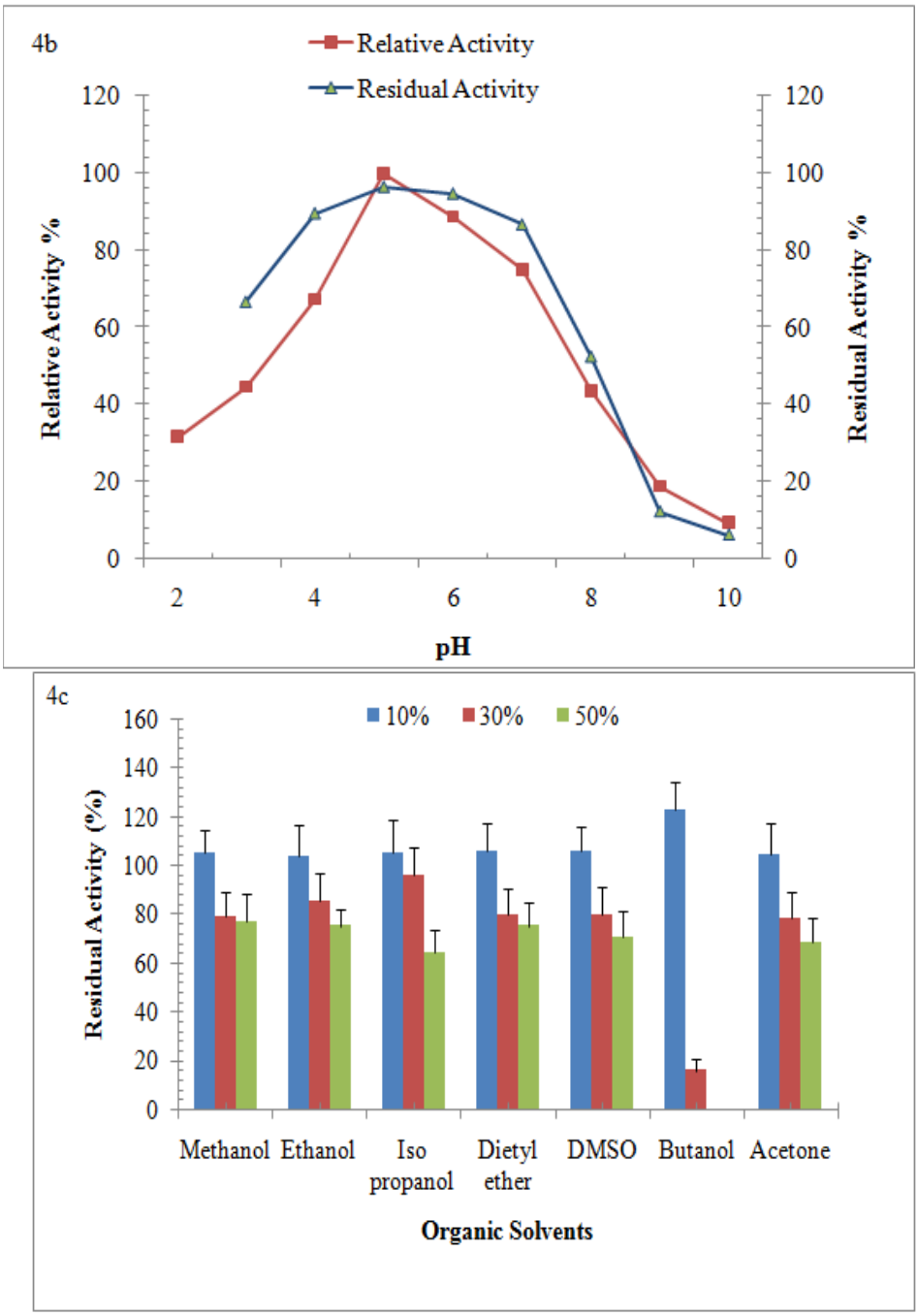


Figure 4 Contd. b, Effect of pH on enzyme activity and stability. The effect of pH on lipase activity was studied by incubating the enzyme with substrate at 30°C over different pH ranges followed by estimation of enzyme activity. The pH stability of the lipase was determined by incubating the enzyme at each desired pH for 1 h at 30°C followed by enzyme estimation. **c,** Effect of organic solvents on enzyme activity and stability. The effect of organic solvents on enzyme activity was studied by incubating the purified enzyme with substrate containing each of various organic solvents Viz., methanol, ethanol, isopropanol, diethyl ether, DMSO, butanol and acetone at a concentration of 10 to 50% (10, 30 and 50%) for a period of 24 h followed by enzyme assay.

ghee, butter and dalda with 71.82, 57.2, 47.5, 41.7, 29.8% reduction in oil/grease content suggesting that the purified lipase from *E. nidulans* can be effectively used for bioremediation of oil effluents.

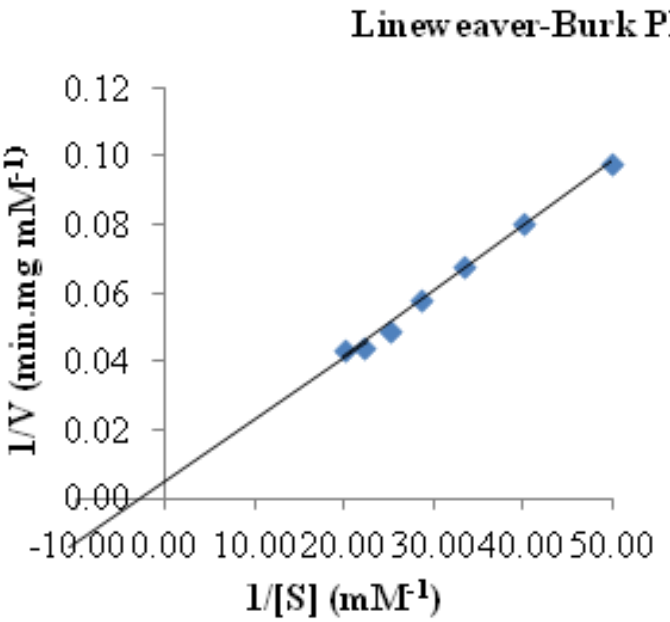


Figure 5. Lineweaver-burk plot of purified lipase from *E. nidulans* NFCCI 3643. The K_m and V_{max} values were determined by incubating the purified enzyme with different concentrations of the substrate (pNP-palmitate).

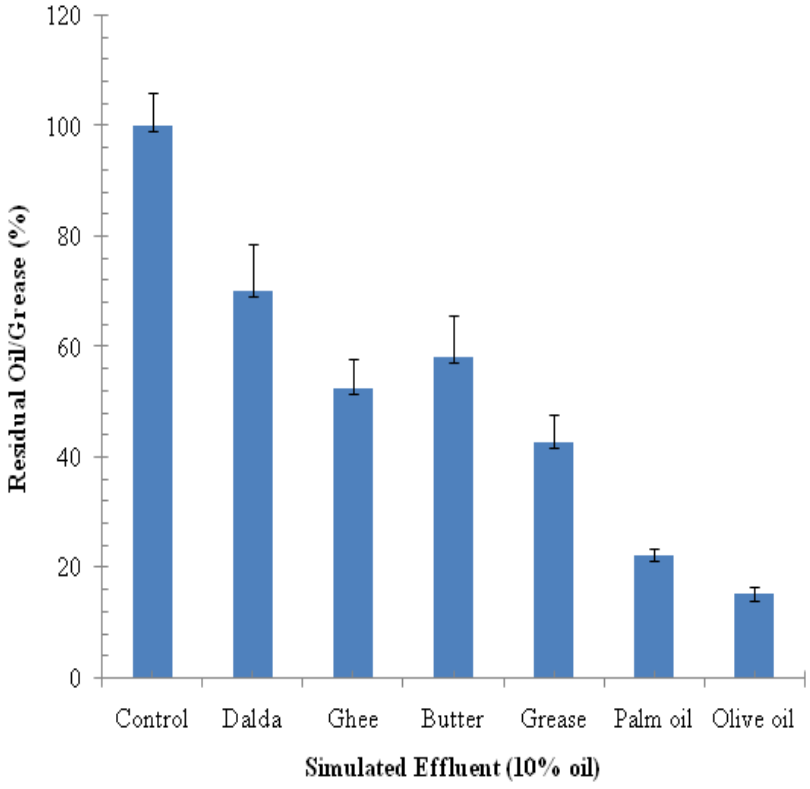


Figure 6. Bioremediation of simulated oil effluents using purified lipase from *E. nidulans* NFCCI 3643. Pure enzyme (1% v/v) from *E. nidulans* NFCCI 3643 was incubated with simulated oil effluents containing 10% each of Palm oil, Dalda, Grease, Butter, Olive oil, Ghee etc at 30°C for 24 h followed by the estimation of oil and grease content using partition gravimetric method.

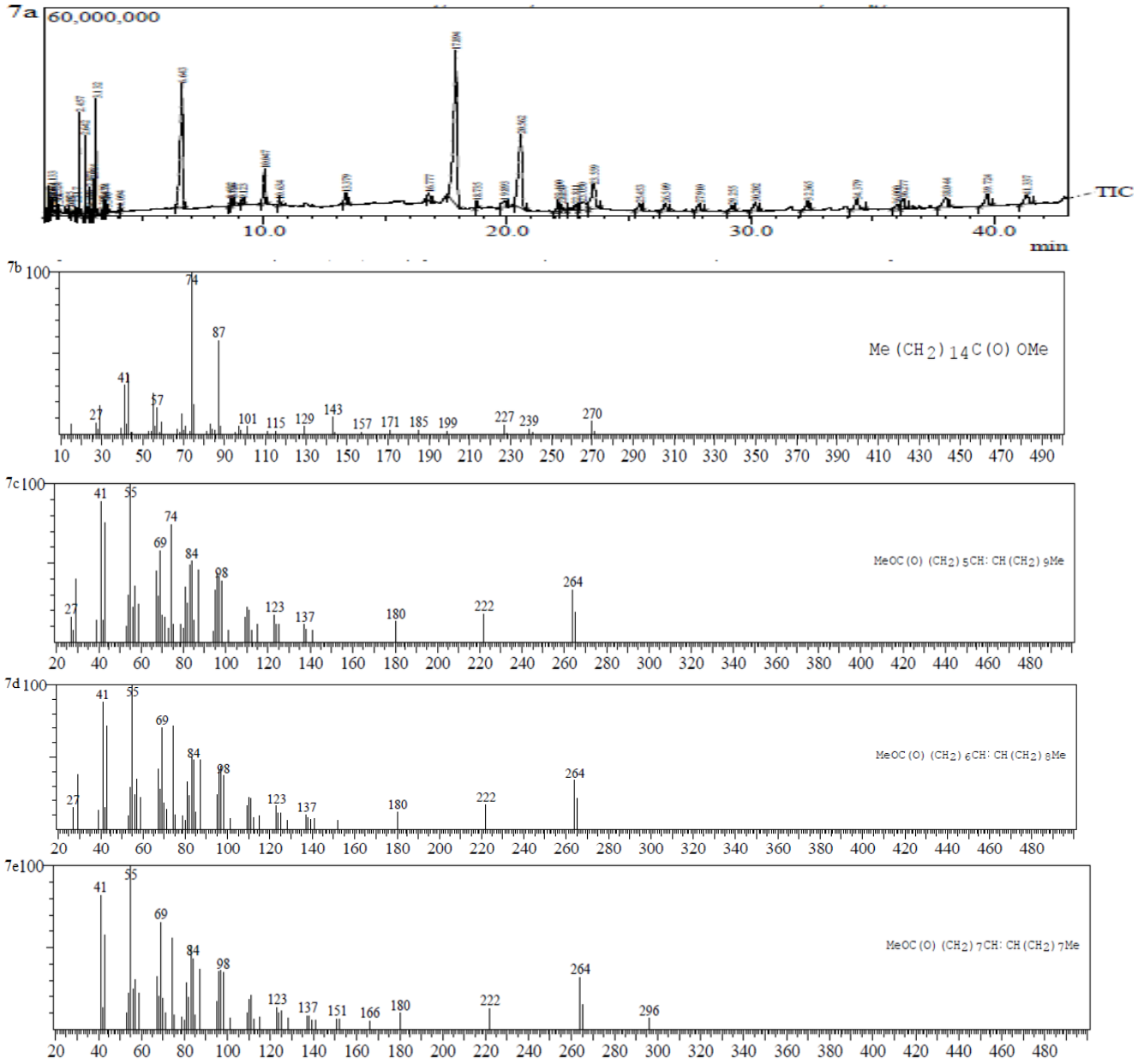


Figure 7. FAME's (Biodiesel) production using purified lipase from *Emericella nidulans*. **a,** Full scan GC-MS chromatogram of FAME's synthesized by incubating purified lipase with olive oil and methanol at 30°C with shaking at 150 rpm for 48 h followed by extraction of products (methyl esters) with n-hexane. **b,** mass spectra of hexadecanoic acid ethyl ester. **c,** 7-octadecenoic acid methyl ester. **d,** 8-octadecenoic acid methyl ester. **e,** 9-octadecenoic acid methyl ester.

Biodiesel (FAME's) production

GC-MS analysis

GC-MS analysis of the n-hexane extracted products of the olive oil, purified lipase and methanol reaction mixture showed peaks related to the retention time (RT): 18.735 min, for the production of hexa decanoic acid methyl ester, the peak with RT: 22.100 min, for the production of 7-Octadecenoic acid methyl ester and the peak with relation to the RT: 22.250 min, for the production of 9-Octadecenoic acid methyl ester, indicating the potential of the purified lipase in the biodiesel production (Figure 7a to e).

FTIR spectroscopic analysis of FAME's

The Fourier transform infrared spectrum of the biodiesel from the olive oil is shown in Figure 8. The spectrum

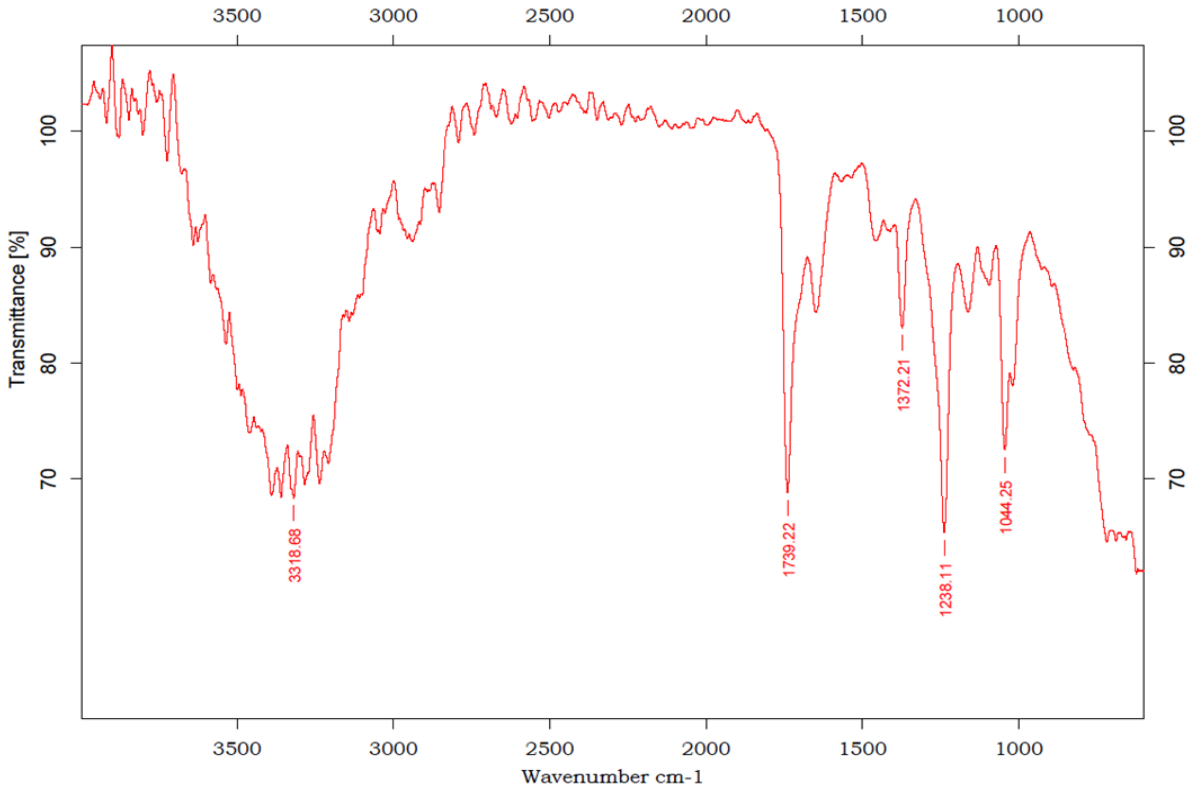


Figure 8. FTIR spectrum of transesterified olive oil. The n-hexane extracted products were used for spectral analysis which were obtained by incubating the purified enzyme with olive oil and methanol at 30°C with shaking at 150 rpm for 48 h followed by extraction with *n*-hexane.

showed a peak at 3318 cm^{-1}, assigned to the -OH stretch of carboxylic acid; the strong peak at 1739.22 cm^{-1}, due to –C=O stretch of ester; the peak at 1372.21 cm^{-1}, due to –C=C- stretch of alkenes; a peak at 1238.11 cm^{-1}, due to C-O single bond; and the peak at 1044.25 cm^{-1}, due to C-O stretch of ester indicating the formation of FAME's.

Determination of the amino acid sequence of the purified lipase

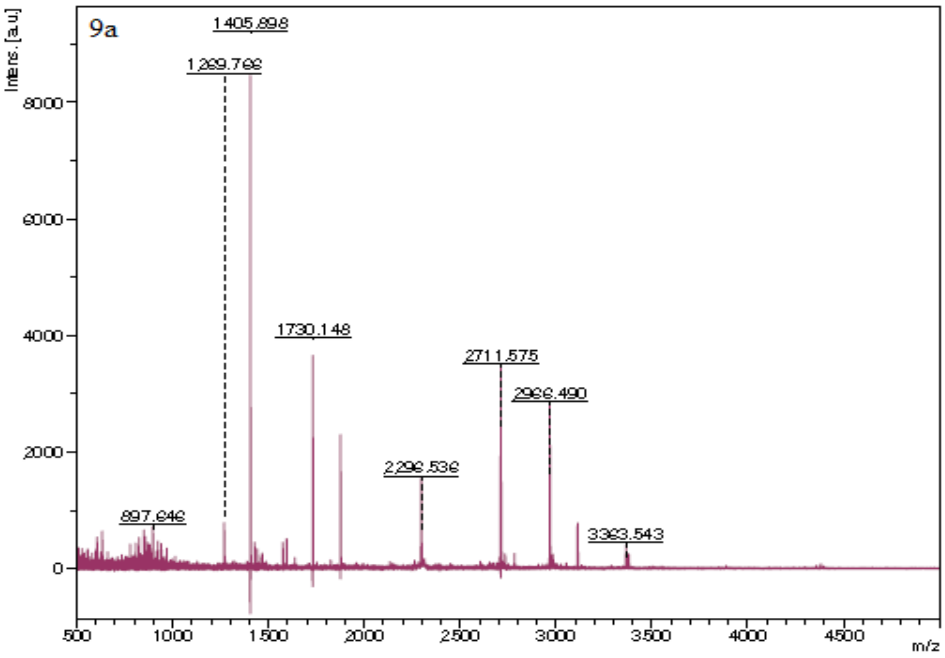
MALDI TOF-MS analysis followed by mascot search with the obtained sequence revealed that the sequence of the purified lipase showed high score with the sequence from *Aspergillus nidulans* with accession no gi|67522685|XP_659403.1 (Figure 9). The peptide mass spectrum is shown in Figure 9a and mascot search engine results with the obtained peptide sequences is shown in Figure 9b. Search of UNIPROT KB revealed that the protein is a putative secretary lipase from *E. nidulans* (Figure 10).

*En*L A and lipase engineering database (LED)

Lipase engineering database, a database of all lipases including putative ones from various organisms placed *En*L A under the *Aspergillus* lipase like homologous family of *Candida antarctica* lipase A like super family (Table 2a and 2b).

DISCUSSION

Cold active lipases are attracting industrial biotechnologists owing to their specific applications in various fields. Although a number of cold active lipase producing sources are available, only a few bacteria and yeasts have been exploited for the production of cold adapted lipases (Joseph et al., 2006). A very few reports regarding cold active lipases from fungal sources were available (Joseph et al., 2007). These include *C. antarctica* (Patkar et al., 1993); *Candida lipolytica, Penicillium roqueforti* and *Geotrichum candidum* (Alford and Pierce, 1961), *A. nidulans* WG 312 (Mayordomo et al., 2000), *Rhizopus* sp. and *Mucor* sp. (Coenen et al., 1997) etc. There were also some recent reports regarding cold active lipases from *Pichia lynferdii* Y- 7723 (Hak-Ryul Kim et al., 2010); *Yarrowia lipolytics* NCIM3639 (Yadav et al., 2011); *Penicillium expansum* (Suja et al., 2013). In addition, all the cold active lipases that were reported till date were mainly screened and



9b

qi|67522685 Mass: 48208 Score: 108 Expect: 4.6e-05 Matches: 8
hypothetical protein AN1799.2 [Aspergillus nidulans FGSC A4]

Observed	Mr(expt)	Mr(calc)	ppm	Start	End	Miss	Peptide
859.6439	858.6366	858.5578	91.8	341	347	0	K.IPILIYK.G
1405.8936	1404.8863	1404.7878	70.1	157	169	0	K.GFVVIVPDHLGPR.S
1596.0936	1595.0863	1594.9810	66.0	436	450	0	K.FLLSLLTLPVGPIGR.-
1730.1442	1729.1370	1729.0250	64.7	232	249	0	K.IAGAALGGTVPQIPPVIR.A
1876.1126	1875.1054	1874.9850	64.2	170	187	0	R.SAFLANTLSGQAVLDNVR.A
2711.5745	2710.5672	2710.4806	31.9	254	279	0	K.GIFTGLIPAGIQGLANEYPAAQQLIR.D
3111.7382	3110.7309	3110.6876	13.9	250	279	1	R.ASNKGIFTGLIPAGIQGLANEYPAAQQLIR.D
3363.5463	3362.5390	3362.5361	0.87	348	377	1	K.GVNDQISPVKDTDALYDTYCSNGANVEYVR.D

No match to: 825.6279, 851.6503, 897.6536, 1269.7696, 1427.8732, 1574.9035, 2296.5485, 2966.4942

Figure 9. MALDI TOF-MS analysis of peptide fragments of purified lipase from *E. nidulans* NFCCI 3643. **a,** Peptide mass spectra of the tryptic digested peptides obtained from MALDI TOF-MS. **b,** MASCOT search engine results for the obtained peptides.

MASLLYQLLFLLVPLLAAGLPANPVKKAGPQPPGEDPFYTPPDGWESTEPGAILRHRTPP
YPIAAFGLAEVNLDASYQILYRTTDSFGEPIATVTTILIPHNADYTKVLSYQVAQDAADP
NCSPSFAIQQFSDAGEALALVMPQLEYLFMSSALNKGFVVIVPDHLGPRSAFLANTLSGQ
AVLDNVRAALASTDITGISSQATVALWGYSGGSLASGFAAELQPSYAPELKIAGAALGG
TVPQIPPVIRASNKGIFTGLIPAGIQGLANEYPAAQQLIRDAILPDKWAEFNKTQELCLTG
NLIEYLGKDIYTYVNDPNVFESPLANSLTEPNAMGHNTPKIPILIYKGVNDQISPVKDTDA
LYDTYCSNGANVEYVRDLLAEHALMTITGAPDAFMWLTERLSGVPVKKGCRRKTQLT
GLQDPKALAALGTTVVKFLLSLLTLPVGPIGR

Figure 10. Amino acid sequence of the purified lipase from *E. nidulans* NFCCI 3643 obtained from the Uniprot KB based on annotation and gi number of the protein that showed high scores with peptide sequences analyzed by MALDI TOF-MS. Uniprot KB results (Q5BCD1_EMENI) indicated that the sequence is a putative secretary lipase from *Emericella nidulans*.

Table 2a. *Candida antarctica* Lipase A like super family and Aspergillus lipase like homologous family of LED (Lipase Engineering Database) containing *EnL* A sequence. Table showing number of proteins, their sequences and structures belonging to *Aspergillus* lipase like homologous family of *C. antarctica* superfamily.

Homologous family abH38.03 (*Aspergillus* lipase like)	
Superfamily	abH38 - Candida antarctica lipase A like
Proteins	9
Sequences	10
Structures	0 Alignment [annotated clustalW] Tree [rooted] Sequences [FASTA] HMMER [profile]

*Source: Lipase engineering database (LED)

Table 2b Table showing a hypothetical protein (AN 1799.2) from *A. nidulans* belonging to *Aspergillus* lipase like homologous family and *Candida antarctica lipase* A like super family.

Organism	Protein	Source DB	NCBI accession code	Description	LED/3D
Aspergillus nidulans	Hypothetical Protein	gi	67522685	Hypothetical protein (AN 1799.2)	No 3D structure

*Source: Lipase engineering database (LED)

and isolated from organisms of deep sea sediments, frozen food samples and Antarctic habitats etc. (Joseph et al., 2007). In the present study, we reported the production of a new cold active lipase, *EnL* A from a mesophilic fungus *E. nidulans* NFCCI 3643, screened and isolated from POME effluent dump sites of Pedavegi palm oil industry, West Godavari Dist, Andhra Pradesh, India. A cold active lipase from *A. nidulans* WG 312 was already reported by Mayordomo et al. (2000), but that lipase was different from the one we are reporting now with respect to molecular weight and as well with other biochemical characteristics.

The extracellular lipase from *E. nidulans* NFCCI 3643 was purified by HIC using phenyl sepharose. Several different lipases were purified from *Aspergillus niger* using hydrophobic supports (Fernandez-Lorente et al., 2005). The apparent molecular weight of the purified lipase from *E. nidulans* NFCCI 3643 separated by SDS PAGE was found to be around ˜54 kDa whereas the molecular weight of the lipase sequence obtained from MALDI-TOF analysis of tryptic digested peptides of the purified lipase was 48 kDa. Similar differences in the molecular weights were reported by Tsuchiya et al. (1996) for *A. oryzae* lipase*,* Bihong et al. (2010) for *Aspergillus tamari* lipase*,* Yamaguchi et al. (1991) for *Penicillium camembertii* lipase*.* This could be due to glycosylation of the purified protein separated by SDS PAGE. Most of the lipases isolated from *Aspergillus* spp. were found to have molecular weights in the range of 25 to 70 kDa (Sharma et al., 2001; Contesini et al., 2010).

The optimum temperature of the purified lipase was found at 30°C. The enzyme is active over a broad temperature range between 10 to 30°C and the enzyme even showed up to 40% of its activity at 0°C indicating that the purified enzyme is a cold active enzyme. The enzyme was stable between the temperature range 4 to 30°C. The lipase exhibited pronounced heat lability above 30°C and this property is similar to many other cold adopted enzymes (Marshall, 1997; Gerday et al., 1997; Feller and Gerday, 1997). Microorganisms adapted to cold environment in general requires lower temperatures for their growth and metabolism which necessitates the use of energy consuming cooling systems to maintain lower temperatures during their fermentative production. Such problem will not arise with *E. nidulans* NFCCI 3643 as it is a mesophilic organism with optimum growth temperatures between 25 to 30°C. The optimum pH of the purified enzyme is 5 and the enzyme exhibited greater stability over a broad pH range 4 to 7. An optimum temperature and pH of 40°C and 6.5, respectively, were reported for the cold active lipase from *A. nidulans* WG 312 by Mayordomo et al. (2000) and 37°C, pH 8, respectively, for *Serratia marcescens* by Abdou et al. (2003).

The K_m and V_{max} for the cold active lipase *En*L A was found to be 0.61 mM and 322.58 mM/min.mg, respectively. The low K_m of the enzyme indicates that the enzyme is having good affinity with its substrate. K_m and K_{cat} values of 0.28 mM and 494 s^{-1} were reported for cold active lipase purified from *A. nidulans* by Mayordomo et

al. (2000). The enzyme exhibited pronounced activity and stability in the presence of various organic solvents and also proved its potential in biodiesel production. Biodiesel production by immobilized *Rhizopus oryzae* fungal cells was reported by Nagaraj et al. (2010) using GC-MS and FTIR analysis. Sunil Kumar et al. (2015) also reported biodiesel production by immobilized lipase from *Bacillus aerius* employing GLC and FTIR analysis. Biodiesel production from rice bran oil via immobilized lipase catalysis was reported by Ying et al. (2013) using GC-MS and FTIR analysis. Use of lipases is a new path in the field of bioremediation for waste disposal. Cold active lipases have also found numerous applications in the field of bioremediation, a technique of waste management involving the use of microorganisms to remove or neutralize pollutants from the harmful wastes disposed at contaminated sites. Cold-adapted lipases play significant role in the field of bioremediation especially in fat contaminated cold environment and waste water treatment (Buchon et al., 2000). Bioremediation studies of simulated oil effluents by the cold active lipase from *E. nidulans* revealed that this enzyme can be effectively used for the removal of oil from oil contaminated soils and water bodies.

Protein sequence determination using MALDI-TOF/MS analysis following search of protein databases with the sequence obtained revealed that the purified protein is a hypothetical protein from *E. nidulans* with a gi no. 67522685. Search of Lipase engineering database, a database that maintains information about all the lipases including putative lipases from various sources revealed that this protein belongs to newly introduced super family of *C. antarctica* lipase A like and to the homologous family of *Aspergillus* lipase like of Class Y lipases. This super family was introduced in the new release 3.0 (December 2009) of the Lipase Engineering Database (Widmann et al., 2010) based on the differences in the cap region of CAL-A (*C. antarctica* lipase A) which is unique among all other proteins of the α/β hydrolase fold. CAL -A was found to have wide spread applications in various fields (Joseph et al., 2008). The purified enzyme from *E. nidulans* was named as *En*L A (*E. nidulans* Lipase A) as this enzyme belongs to *C. antarctica* lipase A like super family.

Conclusion

Taking all the above characteristics into consideration, we conclude that the *En*L A from *E. nidulans* NFCCI 3643 reported in our study is a new cold active enzyme purified from a POME isolate and this enzyme could become a novel biocatalyst to suit for various industrial applications where cold active enzymes are needed.

Conflict of interests

The authors did not declare any conflict of interest.

Abbreviations: SDS PAGE, Sodium dodecyl sulfate polyacrylamide gel electrophoresis; **MALDI TOF-MS,** Matrix-Assisted Laser Desorption Ionization Time of Flight-Mass Spectrometry; **BLAST,** Basic Local Alignment Search Tool; **LED,** Lipase Engineering Data base; ***En*L A,** *Emericella nidulans* Lipase A; **NFCCI,** National Fungal Culture Collection of India; **POME,** Palm Oil Mill Effluent; **pNPP,** para-nitro phenyl palmitate; **DMSO,** Dimethyl sulfoxide; **FAME's,** Fatty Acid Methyl Esters; **GC-MS,** Gas Chromatography–Mass Spectrometry; **FTIR,** Fourier Transform Infrared Spectroscopy; **ATR,** Attenuated Total Reflectance; K_m, Michaelis-Menten constant; V_{max}, Maximum velocity of the reaction **RT,** Retention time; **CAL -A,** *Candida antarctica* lipase A.

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