Appl Biochem Biotechnol (2018) 185:619–640
https://doi.org/10.1007/s12010-017-2670-6

The Purification and Characterization of Lipases from *Lasiodiplodia theobromae*, and Their Immobilization and Use for Biodiesel Production from Coconut Oil

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Received: 3 July 2017 / Accepted: 29 November 2017 /
Published online: 18 December 2017

Abstract The coconut kernel-associated fungus, *Lasiodiplodia theobromae* VBE1, was grown on coconut cake with added coconut oil as lipase inducer under solid-state fermentation conditions. The extracellular-produced lipases were purified and resulted in two enzymes: lipase A (68,000 Da)—purified 25.41-fold, recovery of 47.1%—and lipase B (32,000 Da)—purified 18.47-fold, recovery of 8.2%. Both lipases showed optimal activity at pH 8.0 and 35 °C, were activated by Ca^{2+}, exhibited highest specificity towards coconut oil and *p*-nitrophenyl palmitate, and were stable in iso-octane and hexane. Ethanol supported higher lipase activity than methanol, and *n*-butanol inactivated both lipases. Crude lipase immobilized by entrapment within 4% (*w*/*v*) calcium alginate beads was more stable than the crude-free lipase preparation within the range pH 2.5–10.0 and 20–80 °C. The immobilized lipase preparation was used to catalyze the transesterification/methanolysis of coconut oil to biodiesel (fatty acyl methyl esters (FAMEs)) and was quantified by gas chromatography. The principal FAMEs were laurate (46.1%), myristate (22.3%), palmitate (9.9%), and oleate (7.2%), with minor amounts of caprylate, caprate, and stearate also present. The FAME profile was comparatively similar to NaOH-mediated transesterified biodiesel from coconut oil, but distinctly different to petroleum-derived diesel. This study concluded that *Lasiodiplodia theobromae* VBE1 lipases have potential for biodiesel production from coconut oil.

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Keywords Endophytic plant seed oil-associated fungus · Coconut cake/meal · Solid-state fermentation · Transesterification/methanolysis · Calcium alginate-immobilized lipases · Fatty acyl methyl esters

Introduction

Lipases (EC 3.1.1.3) catalyze the hydrolysis of triacylglycerols to free fatty acids and glycerol and can also synthesize esters from the hydrolysis products [1]. Interest in employing lipases to catalyze reverse synthesis reactions (esterification, transesterification) in non-aqueous environments, and the enzymes' *regio*-selectivities and *stereo*-selectivities, has increased markedly over the past decade in view of their diverse applications that can include biodiesel production [2, 3]. For commercial purposes, economically viable methods for the cost reduction of microbial lipases is an essential approach adopted to screen different environmental niches, agro-industrial residues as renewable sources, and through biomolecular engineering of microorganisms [2, 4]. Submerged fermentation (SmF) is widely used in the enzyme industry having advantages of process control to achieve high titers of exocellular enzymes. As an alternative, solid-state fermentation (SSF) has been developed and has proved to be an economical means to produce various enzymes including lipases [3, 5].

Several filamentous fungi have been studied in depth to produce lipases by SSF. Some examples include *Penicillium simplicissimum* [6], *Rhizopus oligosporus* [7], *Mucor racemosus* [8], *Aspergillus niger* [9], *Yarrowia lipolytica* [10], and *Aspergillus niger*, *Chalaropsis thielavioides*, *Colletotrichum gloeosporioides*, *Phoma glomerata*, and *Lasiodiplodia theobromae* [11]. An economic analysis of lipase production under SmF and SSF conditions revealed that for a production scale of 100 m^3 (100,000 L)/year, SSF was estimated to be more economical than SmF [3, 12].

A broad spectrum of agro-industrial residues has been studied as substrates in the production of lipases by SSF and has included seed oil-expended *cakes* derived from the extraction of plant oils, e.g., coconut [13], gingelly [14], jatropha [15], macauba [4], mustard [16], pongamia [17], soybean [6], as well as wheat bran [9] and sugarcane bagasse [18]. In some cases, the use of mixed-solid substrates, such as coconut kernel cake and wheat bran [19] or a mixture of wheat bran, coconut kernel cake, and wheat rava [20], has also proved advantageous. The use of fermented solids as inoculum in SSF appears to be a viable option, especially for large-scale fermentations where generation of large volumes of spore suspensions is a difficult and an expensive proposition [21].

SSF using agro-industrial wastes as substrates to reduce enzyme production costs has been reported for *Colletotrichum gloeosporioides* [17] grown on pongamia oil cake and *Lasiodiplodia theobromae* [13] on coconut kernel cake, as cheap fermentation substrates, and resulted in high lipase titers (983 U/g dry solids (DS) and 698 U/g DS, respectively).

Although *L. theobromae* (teleomorph of *Botryosphaeria rhodina*) is a good source of lipases for commercial applications, this fungal species constitutes a virulent phytopathogen that causes diseases of economically important agricultural crops. They colonize a broad range of plant species, including coconut, *Cocos nucifera*, the plant source from where the test fungus was isolated.

A recent study showed the existence of a hormone-sensitive lipase-like protein in the proteome of *Lasiodiplodia theobromae* [22]. The putative hormone-sensitive lipase gene showed ~90% sequence identity to the another species of Botryosphaeriales that resulted

from a BLAST(p) search, and revealed a 43.3 Mb draft genome sequence of *L. theobromae* CSS-01S that encoded 12,902 predicted genes (WGS genome; BioProject accession number PRJNA339237.

Recent advances and attention gained from using agricultural wastes for enzyme production and improvement in designing efficient bioprocess models have resulted in making SSF more desirable than SmF for producing enzymes. Nevertheless, there are still challenges in scaling-up SSF processes, such as efficient recovery of product, purification of targeted products, separation of biomass and its disposal and/or its recycling, and maintaining water activity, and the composition of the solid substrate itself can play a major influence in fermentation methods. In addition, heat removal in a SSF bioreactor is a crucial problem and difficult to overcome as the generated metabolic heat in the fermented solid bed contains an inter-particle phase occupied by air, which is a poor conductor of heat. Rapid mixing promotes reduction of heat through solid media contacting the surfaces of a cooled bioreactor [23, 24]. Solid mixing and sensitivity of fungal strains to withstand the mixing process is also a critical problem. Heat and mass transfer are major influential factors in SSF that can be overcome by designing bioreactors that can control the temperature and water activity suitable for microbial growth. Major factors influencing the efficiency of a SSF bioreactor when operated in a continuous mode are transfer of metabolic heat, O_2 consumption, temperature control, agitation/mixing rate, carbon source homogeneity, and residual nutrients in the substrate [23]. SSF conducted within a non-airflow box can resolve many of the challenges associated with the SSF process, which considers uniform distribution of culture and solid substrates to promote high yields and high reproducibility of industrially important enzymes [24]. An efficient SSF bioprocess development therefore will enable the economic production of industrially important enzymes.

Biodiesel (monoalkyl esters of long-chain fatty acids) can be produced by environmentally friendly process technologies employing enzymes (lipases) in transesterification/alcoholysis reactions from plant seed oils, and is a promising renewable resource for biofuels. Biodiesel has environmental benefits as a motor fuel that include its non-toxic nature and its lower exhaust emissions of particulate matter and greenhouse gases when combusted in motor vehicles [2, 25, 26]. The process to produce biodiesel by transesterification can be conducted chemically using acid or alkali, or enzymatically with lipases, in the presence of an alcohol (methyl or ethyl alcohol) [3].

The commercial scale production of biodiesel is limited by the cost of plant oil feedstocks. There are conflict issues associated between energy and food security in using edible oils (soybean, maize, canola, palm). The exploration of waste cooking oils, greases, animal fats, and tallow, among others, as alternative sources for biodiesel has received attention due to the effective disposal of these oils and cost reduction [27]. Non-edible oils such as castorbean oil (*Ricinus communis*) [28, http://www.castoroil.in/uses/fuel/castor_oil_fuel.html] and jatropha and karanja oils [29] are more preferable, but require some form of pretreatment to reduce the high free fatty acid contents by acid treatment during esterification, prior to their transesterification to fatty acyl alkyl esters [30]. Much interest in algae as a source of biofuels including biodiesel has been generated during the past decade. Presently, the high cost of producing algal biomass still precludes algal biofuels from being economical, but algae have potential as a future renewable and sustainable source to produce biofuels and chemicals [31].

Several commercially viable methods and advances in enzymatic production of biodiesel have tested lipases to overcome the major limitations of the enzyme-based transesterification/alcoholysis reaction, viz., the cost of enzyme and methanol-induced enzyme inhibition. A recent novel approach to biodiesel production to offset high enzyme costs and enzyme stability

investigated a solid-supported microbial whole-cell biocatalyst system producing lipase by directly employing *Burkholderia cenocepacia* [32]. The operational parameters affecting the biodiesel yield were optimized by statistical design and resulted in yields of > 90%. Successive batch operations after 288 h of repeated use presented 66.9% retention of the original enzyme activity. In addition, it was noted that strong and vigorous enzyme activity and thermostability were necessary properties of the lipases in enzymatic biodiesel production [32, 33].

The potential of lipase as biocatalyst for biodiesel can be improved through techniques such as metagenomics, directed evolution, and protein engineering [34]. These can improve the catalytic efficiency of lipases as biocatalysts and their tolerance to alcohols, while recombinant DNA technology allows the production of large quantities of lipases that can lower the overall cost of biodiesel. An example of a novel engineered lipase from *Proteus mirabillis*, called *Dieselzyme* 4, possessed industrially important features such as thermostability and solvent (alcohol) tolerance than compared with the wild-type enzyme [35]. Because the spatial organization of an enzyme can play a crucial role in the function and efficiency of biocatalysis, a recombinant lipase from *Photobacterium lipolyticum* was designed by incorporating a N-terminal amphipathic peptide and a C-terminus coiled coil peptide into its structure. The protein engineered (lipase NKC-M37-MAT) exhibited a 54-fold increase in catalytic activity compared with the wild-type lipase, and this feature resulted in reducing the time to produce biodiesel from 30 to 6 h [36]. Recombinant lipase-produced enzymes sand lipases engineered can therefore offer distinct advantages over the wild-type enzymes, which will push the technology for their use in biodiesel production.

A commercially viable enzymatic biodiesel production plant (see http://vieselfuel.com/) capable of producing ~ 19 million liters of biodiesel uses a two-stage recovery process with addition of fresh enzyme in each new batch with pretreated feedstock, and is performed under the optimal conditions of the biodiesel reactions [37].

The lipase-producing ability of different plant oilseed-associated endophytic fungi (Coelomycetes) grown on various oilseed cakes was previously reported by us [11, 13]. Among the 1279 fungi isolated and screened for lipolytic activity, highest lipase titers were produced by *Lasiodiplodia theobromae* isolate VBE-1 [13]. Herein, we describe the purification and characterization of two lipases produced when *L. theobromae* VBE-1 was grown on coconut kernel cake by SSF, the immobilization of crude lipases by entrapment within calcium alginate beads, and the use of the immobilized lipase preparation in the transesterification/methanolysis of coconut oil to produce biodiesel.

Materials and Methods

Materials

Commercial coconut oil-extracted cake produced by the pressing of coconut kernels to extract the oil was obtained from Sri Venkateswara Oil Mill Pvt. Ltd., Veppanapalli, India. All vegetable oils used in this study were obtained in supermarkets in Chennai, Tamil Nadu (India), and included almond oil (*Prunus dulcis*, syn. *P. amygdalus*), castorbean oil (*R. communis*), coconut oil (*Cocos nucifera*), peanut (synonym of groundnut) oil (*Arachis hypogaea*), mustard oil (*Brassica nigra*), neem oil (*Azadirachta indica*), olive oil (*Olea europaea*), pongamia oil (*Pongamia glabra*, now renamed *P. pinnata*), and sunflower oil (*Helianthus annuus*). The synthetic substrates of several *p*-nitrophenyl esters (acetate,

caproate, laurate, myristate, palmitate, and stearate) were obtained from Sigma-Aldrich, St. Louis, MO, USA. The commercial diesel sample used in this study was obtained from Hindustan Petroleum Corporation Limited, Saidapet, Chennai, Tamil Nadu, India.

Isolation of Fungi from Coconut Kernel and Screening for Lipase Activity

The oilseed-associated endophytic fungus, *Lasiodiplodia theobromae* strain VBE-1, was isolated from coconut kernels collected in the state of Tamil Nadu, India, according to the following procedure: coconut kernels were first thoroughly washed in running tap water and then sliced into small segments and surface-sterilized sequentially in 70% ethanol (left for 5 s) and sodium hypochlorite solution (4% available chlorine, 60 s), and finally rinsed with distilled water for 10 s, and then air-dried in a sterile cabinet. The surface-sterilized coconut kernel segments were placed equidistantly on the surface of potato dextrose agar (PDA) media amended with 25 μg/mL streptomycin (antibiotic) in petri dishes, and were sealed (Parafilm M®) and left for 7 days at 25 ± 1 °C. Growth on the agar surfaces was observed periodically, and the fungi emerging out of the coconut kernel segments were isolated and maintained as axenic cultures. The fungi were screened for extracellular lipases by a developed zymogram procedure on tributryin agar [11], and the lipase-positive isolate was maintained and used for further study. The lipase-producing fungus isolated from coconut kernels was identified by molecular genomic sequence analysis based on the ITS of 18S rDNA as *Lasiodiplodia theobromae* VBE-1 [11].

Lipase Production by Solid-State Fermentation

L. theobromae VBE-1 was cultivated by SSF on coconut kernel cake (1 kg) mixed with Czapek-Dox medium (ratio of 1:1) under the optimized conditions reported by us for maximal lipase production (pH_i 8.0, 30 °C, 66.4% moisture content, coconut oil (0.985%, *v*/*v*) added as lipase inducer, and 1.5%, *w*/*v*, Triton X-100) as validated by the statistical design model developed using the response surface method [13]. An Erlenmeyer flask was inoculated with 50 7-mm diameter agar plugs colonized with fungal mycelium, and left under stationary conditions at 25 °C for 10 days. Following SSF, 2500 mL of 50 mM Tris-HCl buffer (pH 8.0) was added to the flask's contents, and placed on a rotary shaker (200 rpm) for 1 h at 25 °C. The flask's contents were filtered (Whatman #1 filter paper) and the filtrate collected. Next, 1250 mL of buffer solution was added to the extracted residue, and the contents mixed for a further 1 h at 25 °C, and filtered. This step was repeated once more. All of the wash filtrates were pooled with the first extract (~3750 mL total volume) and used as the source of crude lipase.

Lipase Assay

Lipase activity present in the filtrates resulting from extracting the expended coconut kernel cakes following SSF was assayed against *p*-nitrophenyl palmitate (*p*NPP) as substrate for 15 min at 37 °C as previously reported [11].

Immobilized lipase activity was determined against *p*NPP as substrate by incubating the immobilized enzyme in 50 mM Tris-HCl (pH 8.0) buffer at 37 °C for 15 min. A 1 mL sample, free of the immobilized enzyme preparation, was withdrawn, and its absorbance at 410 nm measured to determine immobilized lipase activity.

One unit (U) of lipase activity is defined as the amount of enzyme that liberates 1 μmol of *p*-nitrophenol per minute. Lipase activities in SSF were expressed as unit per gram DS. Specific activity (U/mg) is expressed in units of lipase activity per milligram of protein.

Zymogram Staining of Lipases on Polyacrylamide Gels by the Tributyrin Method

For lipase activity staining of polyacrylamide gels following electrophoresis (see below), the lipase samples applied to the gel were not denatured, and following sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; see below), the SDS was removed from the gels by washing the gels once with 20% isopropanol for 20 min and twice each with distilled water for 10 min. For detection of lipase activity, the polyacrylamide gels were transferred to a petri dish onto the surface of an agar tributyrin layer comprising 1.67% (*w*/*v*) agar and 1% (*v*/*v*) tributyrin (as lipase substrate) in 50 mM Tris-HCl (pH 8.0) containing 5 mM $CaCl_2$. After incubation for 6 to 12 h at room temperature, lipase activity was visualized by clearance bands appearing in the agar as a consequence of tributyrin hydrolysis as seen by the precipitated fatty acid calcium salts around the protein bands displaying hydrolytic (lipase) activity [38].

Protocol for the Purification of Extracellular Lipases from *Lasiodiplodia theobromae* VBE-1 Cultivated by Solid-State Fermentation

Lipase Fractionation by Ammonium Sulfate Precipitation

L. theobromae VBE-1 was grown on coconut oil-coconut kernel cake mixture (1 kg) for 10 days at 25 °C by SSF, and the culture filtrate extracted (3750 mL) as described above served as the source of crude enzyme. Protein was measured by the Bradford method [39]. The culture filtrate was subjected to ammonium sulfate precipitation at different levels of saturation at 4 °C. Proteins from the SSF extract were precipitated using increasing ammonium sulfate saturations within the range (20–80%), and commenced with 20% saturation and followed stepwise by increases to 30, 40, 50, 60, 70, and 80% saturation. The precipitated proteins at each of the different ammonium sulfate saturations were collected by centrifugation (12,000×*g* for 20 min at 4 °C), dissolved in 50 mM Tris-HCl buffer (pH 8.0), and dialyzed against the same buffer for 48 h at 4 °C to remove residual ammonium sulfate. The dialyzates were used to assay for lipase activity and protein. They were also subjected to SDS-PAGE to determine protein homogeneity through staining with silver nitrate and to visualize lipase-staining bands on the developed polyacrylamide gels by the zymogram procedure employing tributyrin agar. Each of the ammonium sulfate-extracted enzyme fractions collected were lyophilized and stored at 4 °C. Protein fractions with relatively high lipase activity (50–80% ammonium sulfate saturations) were combined and chosen for enzyme purification.

Ion Exchange Chromatography

Fast-flowing sulfonic quaternary ammonium anion exchanger, Q Sepharose (Sigma-Aldrich, USA), was used for ion exchange chromatography. A known amount of protein in the 50–80% ammonium sulfate saturation sample selected for purification (100 mg/mL in 50 mM Tris-HCl buffer, pH 8.0; specific activity 137.26 U/mg of protein) was applied to a column of Q Sepharose (15.0 × 1.5 cm), and the column was eluted with a linear concentration gradient of 0.005–500 mM NaCl in 50 mM Tris-HCl buffer (pH 8.0). Five-milliliter fractions were

collected on an automated fraction collector (LKB Broma 7000 Ultra Fraction Collector, Sweden). Each fraction was assayed for protein (A_{280}), lipase activity (*p*NPP as substrate), and by the zymogram procedure employing tributryin agar to confirm lipase activity. Lipase-active fractions (22–25) were pooled, dialyzed against 50 mM Tris-HCl buffer (pH 8.0) to remove NaCl, and were chosen for further enzyme purification.

Gel Filtration Chromatography

Fractions containing lipase activity resulting from ion exchange chromatography on Q Sepharose (22–25, pooled) were further fractionated by gel filtration chromatography (GFC) using Sephadex G-100 (Sigma-Aldrich, USA). A 1 mL sample containing 50 mg protein in 50 mM Tris-HCl buffer (pH 8.0) (specific activity of 381.46 U/mg protein) was loaded onto the Sephadex G-100 column (90.0 × 2.5 cm), and eluted with 50 mM Tris-HCl buffer (pH 8.0). Two-milliliter fractions were collected, and the absorbance at 280 nm (protein) and lipase activity measured. Each fraction was checked for lipase activity employing the tributryin agar zymogram procedure. Two peaks of lipase activity (Lip A: fractions 19–28 and Lip B: 43–53) resulted, and were collected and pooled separately, lyophilized, and stored at − 20 °C.

Sodium Dodecylsulfate-Polyacrylamide Gel Electrophoresis

Protein-containing fractions resulting from the purification steps were analyzed for homogeneity by SDS-PAGE on slab polyacrylamide gels employing a 10% separating gel and a 5% stacking gel [40]. Gels were stained with silver nitrate to visualize protein bands [41]. Finally, the gels were transferred to a 3% acetic acid (*v*/*v*) solution, and photographed.

Native Polyacrylamide Gel Electrophoresis

Polyacrylamide gel electrophoresis under non-denaturation conditions of the purified lipase was carried out as follows: a known amount of enzyme sample (5 mg/mL; specific activity 77.95 U/mg of protein) was mixed with sample buffer (50 mM Tris HCl, pH 8.0) and 1% (*w*/*v*) sucrose solution (1:1), and the samples loaded into the sample wells. Bromophenol blue was added into the upper tank buffer as a tracking dye [42]. Electrophoresis was carried out at room temperature with a 20 mA current applied until the tracer dye reached 0.5 cm from the bottom end of the gel.

Determination of Molecular Mass

The molecular masses of proteins separated by SDS-PAGE, and the purified isoforms Lip A and Lip B, were determined with a Bio-Rad, Gel DocTM EZ Imaging system using Image Lab™ software version 5.1. The MWs correlated to 68,000 Da for Lip A and 32,000 Da for Lip B. The molecular mass of the purified lipases was determined by SDS-PAGE run concurrently with standard protein markers consisting of phosphorylase B (97,400 Da), bovine serum albumin (66,000 Da), ovalbumin (43,000 Da), carbonic anhydrase (29,000 Da), and lactoglobulin (18,400 Da) obtained from GeNei Laboratories Pvt. Ltd., Bangalore, India. After protein separation, the gel was stained with silver nitrate and analyzed with a Bio-Rad, Gel DocTM EZ Imaging system using Image Lab™ software version 5.1 to determine the molecular mass of the proteins.

Physicochemical Properties of the Purified Lipases from *Lasiodiplodia theobromae* VBE-1

The physicochemical characterization of the purified *L. theobromae* VBE-1 lipases (isoforms: Lip A and Lip B) was determined as follows. Optimum pH was determined by incubating the enzyme (50 μg of each purified lyophilized lipase isoform/mL of 50 mM Tris-HCl buffer, pH 8.0) and substrate (1 mM *p*NPP) with the various buffers over different pH ranges: [50 mM citrate phosphate buffer (pH range 2.5–3.5), 50 mM sodium acetate buffer (pH 4.0–5.5), 50 mM sodium phosphate buffer (pH 6.0–7.5), and 50 mM Tris-HCl buffer (pH 8.0–10.0)] for 1 h at 37 °C, and then assayed for lipase activity. The optimal temperature was determined by incubating the purified lipases in 50 mM Tris-HCl buffer at optimal pH (8.0) with substrate (1 mM *p*NPP) for 1 h at temperatures within the range of 10 to 80 °C followed by measuring lipase activity. In the pH and temperature stability experiments, the purified lipases were preincubated at the optimum temperature (30 °C) in the various buffer solutions either at different pHs ranging from 2.5 to 10.0 or at different temperatures (10–80 °C) in 50 mM Tris-HCl buffer (pH 8.0), respectively, for 1 h, and then, substrate (1 mM *p*NPP) was added, and lipase activity determined.

To test the stability of each of the purified lipase isoforms to various activating/inhibiting compounds, each lipase isoform (50 μg/mL) was preincubated with the various compounds under study in 50 mM Tris-HCl buffer (pH 8.0) for 30 min at 30 °C, followed thereafter by adding 1 mM *p*NPP and assaying for lipase activity. Solvents included: (25% *v*/*v* final concentration) acetone, methanol, ethanol, butanol, ethylene glycol, hexane, isooctane, DMSO, xylene. Inhibitors: (10 mM) ETDA, iodine, potassium permanganate, silver nitrate, mercuric chloride. Surfactants: (1%, *w*/*v*) SDS, Triton X-100. Reducing agents: (1%, *w*/*v*) DTT, β-mercaptoethanol. The effect of various metal cations on lipase activity was evaluated using 5 mM metal salts in 50 mM Tris-HCl buffer (pH 8.0) for 30 min at 30 °C. The metal cations as salts in the chloride and sulfate forms included Na^{+}, K^{+}, Ca^{2+}, Mg^{2+}, Mn^{2+}, Ba^{2+}, Mo^{2+}, Zn^{2+}, Co^{2+}, Fe^{2+}, and Cu^{2+}.

The substrate specificity of the purified lipase isoforms was determined using the following substrates contained in 50 mM Tris-HCl buffer (pH 8.0) and incubated for 30 min at 30 °C: vegetable oils at 1% (*w*/*v*) concentrations (almond, castorbean, coconut, gingelly, groundnut (peanut), mustard, neem, olive, pongamia, sunflower), and synthetic substrates of *p*-nitrophenyl esters (1 mM concentration) of acetate, caproate, laurate, myristate, palmitate, and stearate.

Immobilization of Crude Lipase by Entrapment within Calcium Alginate Beads

The immobilization by entrapment of crude lipase from *L. theobromae* VBE-1 was performed by gently blending the enzyme solution obtained by SSF with 100 mL of sterile sodium–alginate solution (4%, *w*/*v*) and then withdrawing the mixture aseptically into a sterile 5-mL syringe barrel. The mixture was then allowed to flow out of the syringe via a 22-gauge fitted needle under gravity with the droplets falling into a solution of 0.2 M $CaCl_2$, which gave rise to beads of 2.0–3.0-mm diameter. One hundred grams of beads was collected. The beads formed instantly upon contact with the $CaCl_2$ solution and were left to harden in $CaCl_2$ overnight, after which $CaCl_2$ solution was removed and the beads were washed twice in distilled water, and then stored under water at 4 °C.

In experiments with the immobilized lipase preparation, the temperature and pH stability was determined by incubating the enzyme preparations in 50 mM Tris-HCl buffer at optimal

pH (8.0) for 1 h at temperatures within the range of 10 to 80 °C, or with various buffers over the pH range 2.5 to 10 at 37 °C for 1 h, respectively, and then, substrate (1 mM *p*-PNP) was added and lipase activity determined. The same protocol was used to determine these properties of the crude-free lipase preparation.

Alkali and Lipase-Catalyzed Biodiesel Production from Coconut Oil

The fatty acid content of coconut oil was determined by hydrolysis with NaOH (2.2 g of solid NaOH for 1 h at 60 °C), and the fatty acids released methylated using methanol and analyzed by gas chromatography (GC) (see below). Transesterification/methanolysis reactions using alkali and immobilized lipase were carried out with coconut oil as the substrate, and the production of fatty acyl methyl esters (FAMEs) evaluated by GC.

The immobilized crude lipase-catalyzed transesterification/methanolysis reaction was carried out over 6 h in a 500-mL screw-capped bottle on a reciprocal shaker (150 oscillations/min). The reaction mixture comprised 2 g of calcium alginate beads containing the immobilized lipase (100 U/g), 300 g of coconut oil, and 60 g methanol. The reaction commenced and was left at 35 °C for 5.30 h, and the temperature then raised to 60 °C and left for 30 min.

Chemical transesterification/methanolysis was carried out with NaOH as catalyst for 1 h at 60 °C. The reaction mixture contained 2.2 g of solid NaOH, 300 g of coconut oil, and 60 g methanol.

Following the transesterification reactions, the mixture was transferred to a separatory funnel and enough 26% (*w*/*v*) NaCl solution added at room temperature until the fatty acyl methyl esters separated out of solution. The reaction mixture was then left overnight, and the FAME-containing upper layer separated from the lower glycerol layer. Residual glycerol in the crude FAME fraction was removed by centrifugation, and the collected fraction enriched in FAMEs was washed several times with hot water to remove further glycerol, and finally rinsed with 50 mL of petroleum ether and transferred into a separatory funnel, and the FAME layer separated. Petroleum ether was removed using a rotary evaporator. The crude FAME fraction was further purified by distilling off unreacted methanol under atmospheric pressure, centrifuged, and traces of water removed by adding anhydrous sodium sulfate.

Gas Chromatographic Analysis of Coconut Oil-Produced FAMEs

The FAMEs constituting biodiesel from transesterification/methanolysis of coconut oil by the immobilized lipase preparation and NaOH, as well as a commercial diesel sample used as standard, were analyzed by GC either at a constant temperature or according to a given temperature regimen. To the fatty acid methyl esters (1.0 g), sufficient acetone was added and 1 μL samples injected into the injection port. The chromatograph consisted of a CHEMITO GC 8610 instrument equipped with a flame ionization detector and a BPX-70 (50% cyanopropyl 50% methylsiloxane) column. The carrier gas was nitrogen and hydrogen, and oxygen was used for ignition purposes. The temperature regimen maintained during the operation was as follows: injection port 250 °C and detector port 260 °C; oven starting temperature was 160 °C and then increased by 7.5 °C per minute intervals to a final temperature of 240 °C. A Winchrom Software package of the GC instrument analyzed the data of the methyl ester peaks (http://www.indtechinstruments.com/chromatography-interface-development.html).

Results and Discussion

There is much interest globally in plant-derived seed oils (esp., soybean, corn, palm) as feedstocks for the production of biodiesel as an alternative to petrochemical-based diesel [3, 5, 11]. Microbial lipases constitute one of the most sought-after groups of industrial enzymes, and fungi provide one of the most important sources of lipases for commercial applications that can include biodiesel production. To completely convert plant-derived seed oils into biodiesel by enzymatic transesterification processes relies upon enzymes possessing industrially robust catalytic features such as temperature stability, solvent tolerance, extreme pH range stability, and substrate specificity [3, 5, 11, 13].

In search of potential lipase catalysts for biodiesel production, the crude lipase preparation from *L. theobromae* VBE-1 was immobilized by entrapment within calcium alginate beads and was tested for its ability to produce fatty acyl methyl esters (biodiesel) from coconut oil in a transesterification/methanolysis reaction. The findings of our study showed that endophytic fungi (Coelomycetes) associated with oilseed plants constitute a potential and alternative source for industrially important exocellular lipases that can find application to produce biodiesel.

Purification of Two Lipases from *Lasiodiplodia theobromae* VBE-1

The extracellular lipases produced by *L. theobromae* VBE-1 grown by SSF on coconut oil-extracted cake supplemented with coconut oil as lipase inducer were extracted from the oil-expended coconut cake fermented solids, and the crude enzyme preparation obtained was purified. Figure 1a, b shows coconut kernels used to isolate the endophytic fungus *L. theobromae* VBE-1, and its colonization on agar media on which the kernel segments were placed. Figure 1c shows a developed zymogram of the extracted extracellular enzyme from *L. theobromae* VBE-1 exhibiting lipase activity on tributyrin agar indicated by clearance (hydrolytic) zones around the agar wells where the cell-free enzyme solution was applied.

Protein precipitation using ammonium sulfate was used as the first step in the protocol to purify the fungal lipases. Lipase activity was mostly found in the 50–80% saturated ammonium sulfate fraction and was subjected to native PAGE and checked for enzyme activity by the tributyrin agar zymogram procedure (Fig. 1d). Two distinct lipase-staining bands were observed on the gel (Fig. 1e, f) and designated as Lip A and Lip B. Attempts to separate the two lipases in the crude extracellular enzyme preparation could not be achieved by precipitation with ammonium sulfate at different saturations. Furthermore, lipase activity was found over a wide range of ammonium sulfate saturations (30–80%), and those with highest lipase activity (50–80% saturation) were pooled and fractionated by ion exchange chromatography on Q Sepharose resulting in a broad peak (fractions 22–25) containing two possible lipolytic enzymes that were not separated (Fig. 2). These were pooled and subjected to gel filtration chromatography on Sephadex G 100 and were resolved into two somewhat asymmetrical-shaped peaks (fractions 19–28, designated Lip A, and 43–53, Lip B) containing lipase activity (Fig. 3). Each lipase peak tested positive by zymogram staining on tributyrin agar (see Fig. 1d). The electrophoretic patterns of protein separation of the two lipases (Lip A and Lip B) on polyacrylamide gels stained with silver nitrate indicated homogeneity (see Fig. 1e, f). The two lipase-active fractions from Fig. 3 (Lip A and Lip B) were separately pooled, and used for further characterization.

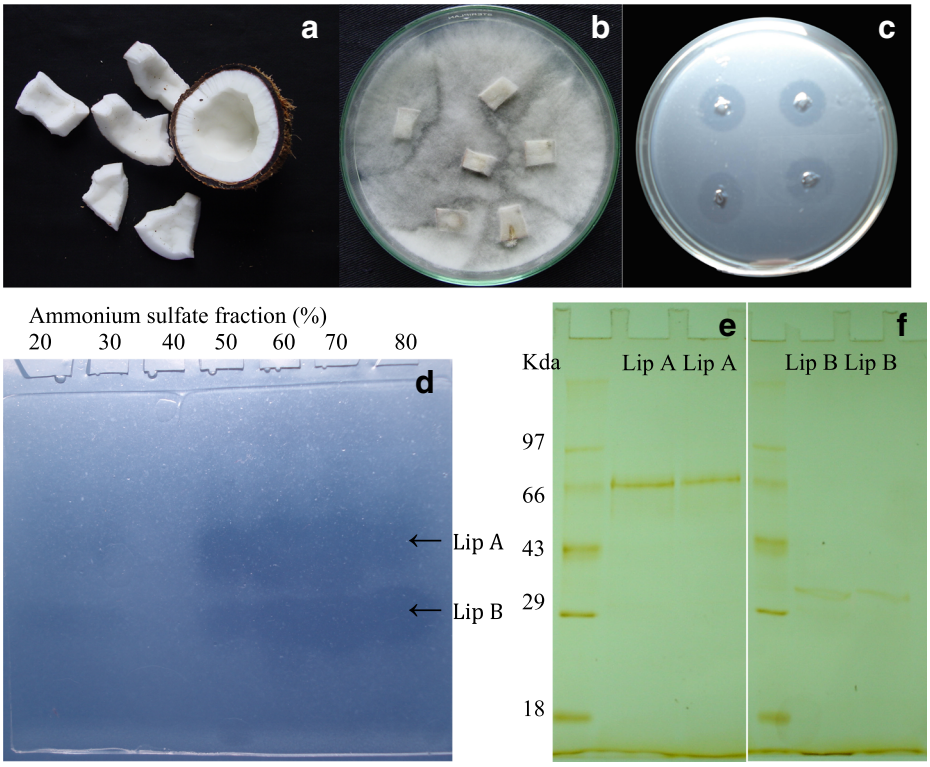

Fig. 1 The isolation, identification, and purification of lipases from *Lasiodiplodia theobromae* VBE-1. **a** Coconut kernel. **b** Coconut kernel-associated fungus, *L. theobromae* VBE-1. **c** Zymogram of extracellular lipase activity on tributyrin agar showing clearance (hydrolytic) zones around the agar wells. **d** Native PAGE of ammonium sulfate precipitated fractions (50–80%) showing lipase A and B activity following staining with tributyrin agar. **e**, **f** Gel filtration chromatography fractions of purified lipases [Lip A (**e**), Lip B (**f**)] at two protein concentrations applied. Gels were stained by the silver nitrate method. The first lanes of **e** and **f** correspond to molecular weight (M) protein markers

Details of the different steps in the purification protocol of the lipases from *L. theobromae* VBE-1 showing the yields and purification folds are summarized in Table 1.

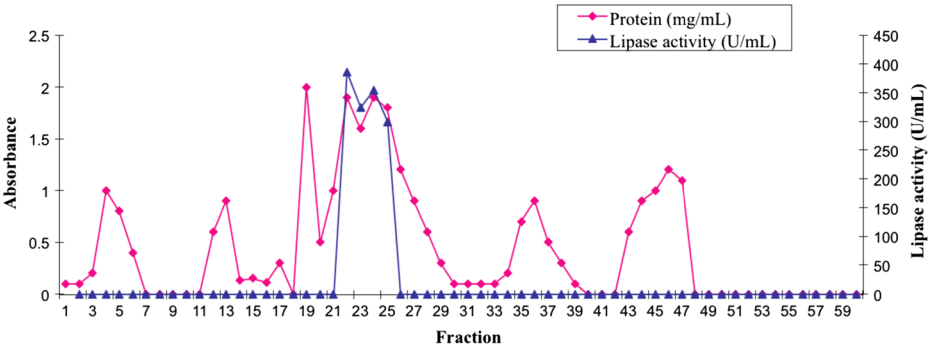

Fig. 2 Elution profile of lipases from *Lasiodiplodia theobromae* VBE-1 on Q Sepharose

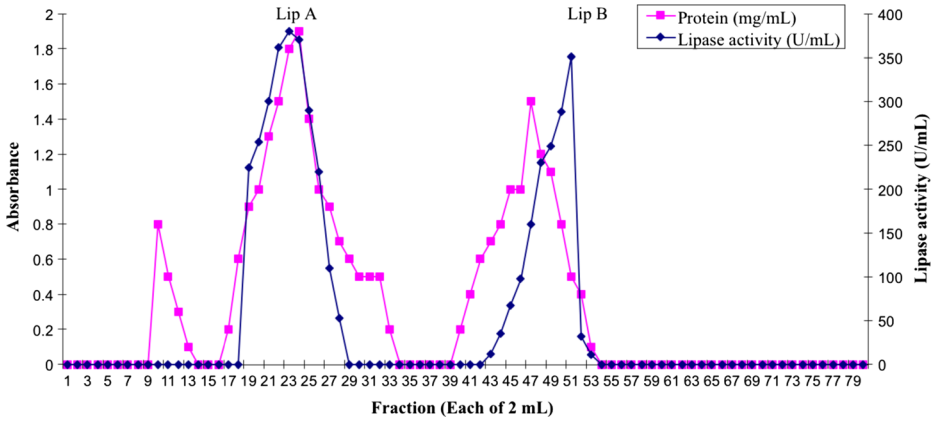

Fig. 3 Elution profile of lipases from *Lasiodiplodia theobromae* VBE1 on Sephadex G-100

The crude lipase resolved into two pure lipase fractions. Lip A was purified 25.4-fold with a recovery of 47.1% based upon the original lipase activity in the crude extracellular enzyme preparation, and a specific activity of 1981 U/mg protein. Lip B was purified 18.5-fold resulting in a specific activity of 1440 U/mg protein with a final recovery of 8.2%.

SSF of *L. theobromae* VBE-1 on coconut kernel oil-extracted cake produced two separate lipases on purification (Lip A and Lip B), each having different molecular masses, 68,000 and 32,000 Da, respectively, calibrated by SDS-PAGE (Fig. 1e, f). When this fungus was grown on coconut oil by SmF, only one lipase component was detected, as demonstrated by a single band on PAGE stained by the zymogram procedure [11]. It is possible that some ingredients in the coconut cake used to produce lipases by SSF may have induced the synthesis of another lipase, as only one lipase was present in the extracellular fluid arising from SmF. It is quite possible that the two lipases (A and B) produced by *L. theobromae* may exist as subunits (dimeric and monomeric forms, resp.) because of the large MW difference between the two enzymes.

Two lipases termed "isoforms" of different molecular masses (60 and 52 kDa) were reported from *Ophiostoma piliferum* [43]. In another study, three distinct isoforms of lipases (Lip A, Lip B, Lip C) were produced by SSF from *Candida rugosa* grown on coconut cake and wheat bran, and the purified lipases had molecular masses of 64, 62, and 60 kDa, respectively [44]. By contrast, two purified lipases from *Rhizopus homothallicus* were reported to have identical protein structure, each with the same molecular mass (29.5 kDa), but some of the properties of the two lipases differed [45].

Table 1 Summary of the purification steps for two extracellular lipases from *Lasiodiplodia theobromae* VBE-1

Purification step	Total protein (mg)	Total lipase activity (U)	Specific activity (U/mg)	Yield (%)	Purification fold
Culture filtrate	1347	105,000	77.95	100	1
$(NH_4)_2SO_4$ (50–80%)[a]	600	82,359	137.26	78.43	1.7
Q Sepharose (fractions 22–25)	142	54,168	381.46	51.58	4.8
Sephadex G-100					
Lip A	25	49,527	1981	47.1	25.41
Lip B	6	8641	1440	8.2	18.47

[a] % saturation

Physicochemical Characterization of *Lasiodiplodia theobromae* VBE-1 Lipases A and B

Lipase enzymes from various microorganisms have been purified and characterized in terms of different environmental parameters [1]. The important variables against which these enzymes were characterized included pH, temperature, substrate specificity, metal cations, inhibitors, and solvents among others. Both purified lipases from *L. theobromae* VBE-1 showed a pH optimum of around 8.0. The pH optimum of Lip A occurred over a broad pH range (6.0–10.0), whereas that of Lip B was optimal at pH 8.0. This attribute may have advantages for the enzyme's application in detergent formulations. An alkaline lipase (pH 8.0) from *Penicillium aurantiogriseum* has been reported [46]. In our study, Lip A was stable over a broad pH range of 7.0–10.0, while Lip B showed stability over a narrower range, pH 7.0–8.0. A lipase from *Aspergillus carneus* showed a similar stability trend over pH 8–10 [47], while a purified acidic lipase from *A. niger* was optimal at pH 4.4 with a half-life of 42 h that could be prolonged to 138 h in the presence of calcium [48]. The pH stability of an enzyme is an important attribute as this confers a buffering ability on the enzyme so as to render it resistant to fluctuations in pH.

The optimum temperature of both *L. theobromae* VBE-1 lipases was 30 °C, but Lip A showed 4% less relative activity at 40 °C. Their temperature stability varied, with Lip A remaining stable to 60 °C, while Lip B lost its stability beyond 40 °C, when kept at these temperatures for 1 h and pH 8.0. A thermostable purified lipase isolated from *Antrodia cinnamomea* was stable within the range 25–60 °C [49]. Directed evolution has been employed to produce mutants of *Proteus mirabilis* producing lipases with greatly improved thermal stability at 50 °C compared to the wild type [35], and this biomolecular approach appears to be the way forward to obtain lipases with enhanced thermostability. A purified lipase from *Geobacillus* sp. exhibited optimal lipolytic activity at pH 9.5, and at 55 °C with a half-life of 1.5 h at 55 °C [50].

Thermostability is an important property in terms of enzyme application as many industrial bioprocesses proceed at elevated temperatures, and immobilization of enzymes can confer temperature and pH stability on enzymes.

The comparison of the stability of the immobilized lipase preparation from *L. theobromae* VBE-1 with that of the crude-free enzyme towards a range of temperatures (20–80 °C), and pH values (2.5–10.0) are shown in Table 2. In each case, the stability of the lipases produced by this fungal isolate was superior for the immobilized enzyme preparation compared to the free enzyme. The free enzyme lost activity at > 40 °C, while for the immobilized preparation, this commenced at 60 °C. Both enzyme preparations were stable over the pH range of 6–8, but at pH 10, the immobilized enzyme had lost only 20% of its initial lipase activity compared to 92% for the free enzyme. Likewise, the free and immobilized preparations of lipase from *A. niger* showed similar biochemical properties and exhibited maximum activity at pH 6.0 and within a temperature range of 30–40 °C [51].

The influence of various metal cations (Na^+, Mg^{2+}, K^+, Ca^{2+}, Mn^{2+}, Fe^{2+}, Mo^{2+}, Co^{2+}, Cu^{2+}, Zn^{2+}, and Ba^{2+}) on enzyme activity differed for the two lipases, with some cations activating lipase activity, while others caused some degree of enzyme inhibition (Table 3). Lip A was activated by Ca^{2+} to the extent of 55% over it its original activity, while Mg^{2+} and Mn^{2+} ions also activated this enzyme. None of the metal cations activated Lip B activity. Na^+ and K^+ decreased lipase activity. Metal ions play a key role in enzymology as certain metals function

Table 2 The temperature and pH stability of the crude free and immobilized lipase preparations of *Lasiodiplodia theobromae* VBE-1

Physicochemical properties	Residual lipase activity (%)	
	Free lipase	Immobilized lipase
Temperature (°C)[a]		
20	79.2 ± 0.8	99.33 ± 0.63
40	93.06 ± 0.62	99.12 ± 0.81
60	35.64 ± 2.0	60.14 ± 0.20
80	14.85 ± 0.35	20.35 ± 0.47
pH[b]		
2.5	0 ± 0	10.23 ± 0.45
4	61.38 ± 0.62	70.42 ± 0.37
6	110.88 ± 0.12	120.17 ± 0.77
8	115.83 ± 0.44	135.32 ± 0.53
10	8.91 ± 0.09	80 ± 0.41

[a] The temperature stability of free and immobilized lipase was determined by incubating the enzyme preparations for 1 h at temperatures within the range of 10 to 80 °C

[b] The stability of the free and immobilized enzyme was tested by incubating the enzyme preparations for 1 h at 37 °C with various buffers over the pH range 2.5 to 10.0

as cofactors, which are crucial for enzyme configuration and metabolic functions. A purified lipase from *Mucor hiemalis f. hiemalis* was found to require Ca^{2+} for its activity [52]. Metal ions such as Fe^{2+} and Fe^{3+} have also been reported to decrease lipase activity [53]. Three distinct purified isoforms Lip A, Lip B, and Lip C from *C. rugosa* produced by SSF showed that all three enzymes were strongly inhibited by Ag^{2+} and Hg^{2+}, while Mn^{2+} showed no effect, and Ca^{2+} and Mg^{2+} enhanced lipase activity [54].

The influence of certain lipase inhibitors on enzyme activity was assessed on both isoforms. With the exception of SDS, all of the compounds tested exerted an inhibitory effect on the purified lipases from *L. theobromae* VBE-1 (Fig. 4). Actually, Lip B activity was more affected than Lip A by all of the compounds tested, with $KMnO_4$ and dithiothreitol exerting the most pronounced inhibitory effect. Iodine, $AgNO_3$, and mercuric chloride are regarded as

Table 3 Effect of metal cations on the activity of lipases Lip A and B from *Lasiodiplodia theobromae* VBE-1

Metal cation salts[a]	Relative lipase activity (%)	
	Lip A	Lip B
None (control)	100 ± 2.5	100 ± 1.2
$BaCl_2$	66 ± 1.6	62 ± 0.32
$CaCl_2$	155 ± 3.9	93 ± 0.43
$CoCl_2$	64 ± 1.6	56 ± 0.46
$CuSO_4$	81 ± 2.0	74 ± 0.54
$FeSO_4{\cdot}7H_2O$	88 ± 2.2	82 ± 0.72
KCl	85 ± 2.10	52 ± 0.12
$MgSO_4$	138 ± 3.4	80 ± 0.91
$MnSO_4$	124 ± 3.1	64 ± 0.24
$MoCl_2$	62 ± 1.5	47 ± 0.65
NaCl	58 ± 1.4	46 ± 0.46
$ZnSO_4$	88 ± 2.2	74 ± 0.74

[a] At 5 mM concentration

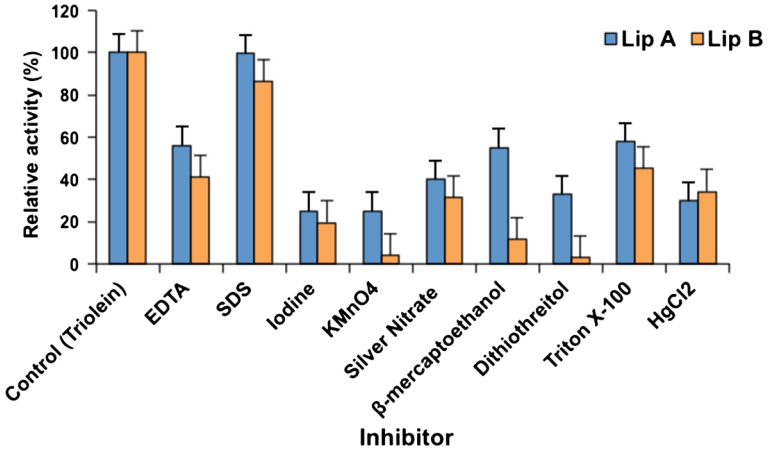

Fig. 4 Effect of inhibiting substances on the activity of lipases A and B from *Lasiodiplodia theobromae* VBE1

potent inhibitors affecting the sulfhydryl groups at the enzyme's active site, but their inhibitory action on the lipases studied here was less. The influence of the surfactant Triton X-100 was more inhibitory on lipase activity than SDS. Conversely, SDS and Triton X-100 were reported to strongly inhibit the lipase activity from a *Mucor* sp. [53]. Moreover, the lipase from *Mucor hiemalis f. hiemalis* was strongly affected by the surfactants taurocholic acid, Triton X-100, and Tween 20, but EDTA did not affect enzyme activity [52].

Substrate Specificity of Lipases A and B from *Lasiodiplodia theobromae* VBE-1

The substrate specificity of the two purified lipases from *L. theobromae* VBE-1 was studied against certain vegetable oils, and esters of *p*-nitrophenyl with different chain lengths (C:2–C:18), under standard hydrolysis conditions. The efficacy of the various vegetable oils' substrates on the activity of the two purified lipases is shown in Fig. 5. The control used was triolein. Coconut oil favored a high relative activity for Lip A (168%). Lip A also showed higher affinities for the oils derived from mustard, olive, peanut, pongamia, and sunflower, than compared to Lip B. The specificity towards the *p*-

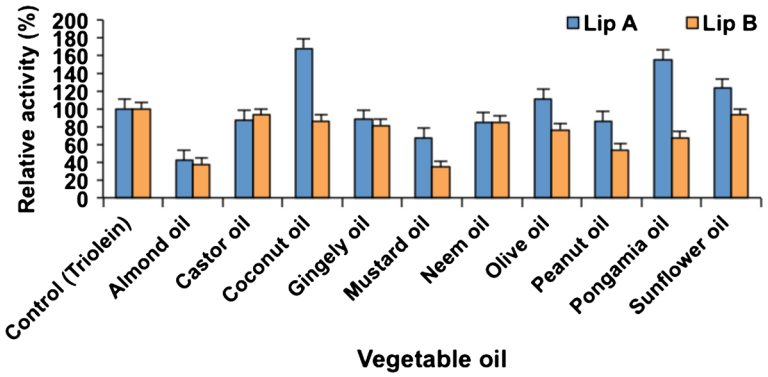

Fig. 5 Substrate specificity of lipases A and B from *Lasiodiplodia theobromae* VBE1 towards vegetable oils

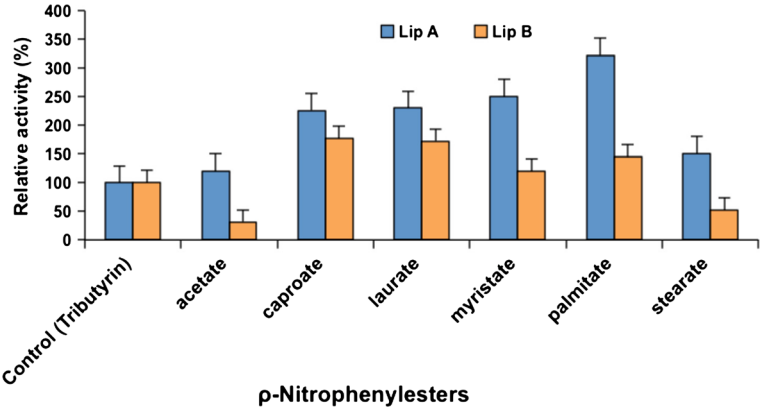

Fig. 6 Substrate specificity of lipases A and B from *Lasiodiplodia theobromae* VBE1 towards *ρ*-nitrophenyl-fatty acyl esters

nitrophenyl acyl esters by the two purified lipases was highest with palmitate (C:16), but in each case, the relative activity was higher for Lip A than Lip B on all of the substrates tested (Fig. 6). Another study [43] reported that two lipase isoforms from *Ophiostoma piliferum* exhibited higher activity towards *p*-nitrophenyl butyrate (C:4) than for the longer chained *p*-nitrophenyl stearate (C:18). This finding indicates that enzymes from different sources exhibited different substrate specificities.

Effect of Solvents on Lipase A and B Activity

The nature of the solvents is crucial in maintaining enzyme activity especially when this applies to enzymes targeted in transesterification reactions to produce fatty acyl alkyl esters from vegetable oils in making biodiesel. Different solvents were tested for their influence on the relative activity of Lip A and Lip B (Fig. 7). Lip A remained relatively stable in the alkanes, hexane, and isooctane (100%), as well as ethanol, but Lip B was less stable than Lip A in all of the solvents tested. In isooctane, 85% of the lipase activity of Lip B was retained. Of

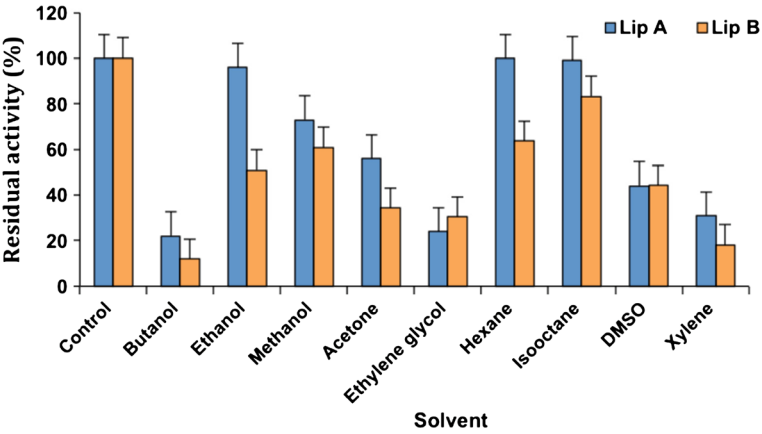

Fig. 7 Effects of organic solvents on the stability of lipases A and B from *Lasiodiplodia theobromae* VBE1. Triolein was used as the control

the alcohols tested, Lip A retained 73% of its activity in methanol, but together with Lip B, both purified enzymes were strongly inactivated in *n*-butanol. A directed evolutionary lipase from *P. mirabilis* was reported to greatly enhance the tolerance to methanol [55]. Acetone and DMSO were poor solvents, along with xylene and ethylene glycol for both purified lipases from *L. theobromae* VBE-1. This trend indicates the manner in which the chemical nature of the solvents exerted their influence on lipase activity. Similar organic solvent-tolerant lipases and their applications have been documented [56].

Enzyme-Catalyzed Transesterification/Methanolysis of Coconut Oil by Immobilized Lipase from *Lasiodiplodia theobromae* VBE-1

The industrial-scale production of biodiesel using lipases is still considered uneconomical, due largely to the high costs of the enzyme. Finding alternate methods for lipase production such as SSF and utilizing waste feedstocks (e.g., plant oil-extracted seed cakes), microbial strain improvement to enhance lipase titers, and the affect of nutritional factors by statistical design [13] that influence microbial lipase titers will ultimately reduce enzyme costs. Lipase applications for biodiesel from various plant-derived oil sources continues to be increasingly studied because of the advantages over chemically catalyzed transesterification/alcoholysis processes [1, 2, 4]. These include lower energy consumption, adaptability towards different raw (oil) substrates, process efficacy, easier purification of the FAME product and removal of glycerol as co-product, and the biodegradability of the catalyst among others, all regarded as environmentally friendly (*green*) processes [57]. Rapid changes in the price of liquid fuels from fossil oils and market fluctuations favor alternative diesel fuels based upon plant-derived oil feedstocks and waste cooking oils.

In this study, we were interested to evaluate the lipase from *L. theobromae* VBE-1 for its ability to produce biodiesel. It is impractical to use pure lipases, and the crude-free lipase preparations often suffer from temperature and pH instability, an important feature in any industrial process. As the immobilized lipase preparation was far more stable to temperature and pH (see Table 2), this preparation was chosen for transesterification/methanolysis of coconut oil, and was compared to the chemical transesterification reaction using alkali. Commercial diesel from petroleum was used for comparison purposes.

The composition of coconut oil showed a similar trend to that reported in the literature [58], except that in the coconut oil used in our study, the concentration of laurate was somewhat higher than that of palmitate, oleate, and linoleate, lower (see Table 4), and may reflect the geographical location of the different sources of coconut oil, Thailand [58] versus India (this study).

The FAMEs arising from the transesterification/methanolysis of coconut oil catalyzed by NaOH and immobilized lipase were identified and quantified by GC (Table 4). The maximum percentage (%) of FAMEs generated by catalysis with immobilized lipase was of the order laurate (46.1%), myristate (22.3%), palmitate (9.9%), and oleate (7.2%). The FAMEs of caprylate, caprate, and stearate were also detected, but at lower concentrations. With NaOH as catalyst, the FAMEs generated from coconut oil resulted in similar yields with highest amounts of esters of laurate (46.7%) and myristate (21.75%) being present.

Among the FAMEs in commercial diesel, esters of linoleate (44.4%) and oleate (34.9%) were present in highest amounts followed by palmitate (5.1%). In petrochemical-based diesel, FAMEs corresponding to carbons C:18–24 (arachidate, behanate, and lignocerate) were identified in relatively low amounts as shown in Table 4. These higher carbon-chained FAMEs

Table 4 Gas chromatography analysis of fatty acyl methyl esters (biodiesel) produced from coconut oil using immobilized lipase and NaOH as transesterification catalysts, and compared to petrochemical-based commercial diesel

Fatty acid methyl ester	Coconut oil fatty acid content[a] (%)	NaOH		Immobilized lipase		Diesel	
		Retention time (min)	FAMEs (%)	Retention time (min)	FAMEs (%)	Retention time (min)	FAMEs (%)
Caprylic (C:8)[b]	4.43	1.34	4.43	1.34	4.30	ND	ND
Capric (C:10)	4.82	2.0	4.82	2.01	4.48	ND	ND
Lauric (C:12)	46.7	3.23	46.7	3.29	46.1	1.49	1.41
Myristic (C:14)	21.7	4.99	21.7	5.06	22.34	2.02	1.84
Palmitic (C:16)	9.6	7.12	9.6	7.18	9.98	2.96	5.10
Stearic (C:18)	3.26	9.36	3.26	9.40	3.35	4.20	3.0
Oleic (C:18:1)	7.27	9.71	7.27	9.78	7.26	4.52	34.9
Linoleic (C:18:2)	1.99	10.39	1.99	10.41	1.97	5.01	44.4
Arachidic (C:20)	ND	ND	ND	ND	ND	5.54	0.24
Behanic (C:22)	ND	ND	ND	ND	ND	7.36	0.49
Lignoceric (C:24)	ND	ND	ND	ND	ND	9.11	0.04

FAMEs fatty acyl methyl esters, *ND* not detectable

[a] Coconut oil was hydrolyzed with NaOH and methyl esters analyzed by GC

[b] Carbon chain length

were absent in biodiesel produced from coconut oil using alkali and immobilized lipase. However, lower carbon chains of C:8–10 (caprylate and caprate), although present in biodiesel of coconut oil, were absent in commercial diesel.

Comparative studies on the kinetics of lipase conversion of coconut oil to biodiesel in the presence of ethanol at 50 °C had the advantage that the enzymatic transesterification/ethanolysis reaction minimized side reactions such as saponification, which was more prominent with alkali-catalyzed transesterification, and esp., in the presence of high free fatty acid concentrations [59].

The geographical distribution of plant oils plays a major role in FAME composition, for example, Thailand coconut oil biodiesel has been reported to contain esters of lauric (64.4%), myristic (20.5%), palmitic (7.7%), palmitoleic acid (0.09%), stearic (1.7%), oleic (4.6%), linoleic (0.96%), and arachidic (0.04%) as a potential biodiesel source [60]. In another study [61], the composition of biodiesel from coconut showed predominantly methyl esters of laurate within the range 47–64%, myristate (18.5–20.5%), and palmitate (7.7–8.9%), which were within the range of FAMEs found in the present study.

Studies on fuel specification of two biodiesel feedstocks, coconut and castor oil, reported that coconut oil biodiesel is characterized by low viscosity and high cetane number, whereas castor oil biodiesel potentially exhibits excellent oxidation stability with very good cold-flow properties, but fails to fulfill specifications of viscosity, density, and cetane number [62].

In order to obtain high purity biodiesel to operate in motor engines, there is the propensity to determine any impurities such as unreacted free fatty acids and triacylglycerols, residual glycerol, and the presence of other derivatives that are not detected by analysis using GC-flame ionization detection. Other techniques such as GC-MS, FT-IR, and NMR (^{1}H and ^{13}C) can be used to analyze the impurities of coconut FAMEs, and details of this will be the subject of a further paper from our laboratory on lipases from *L. theobromae* VBE-1.

Conclusions

The agro-industrial coconut kernel *cake* residue produced lipases by *L. theobromae* VBE1 on SSF that were isolated and purified resulting in two lipases: Lip A and Lip B. The purified lipases showed elevated activity and stability over a broad range of pH, temperature, solvent, and substrate specificities applicable in biodiesel production. An immobilized lipase preparation was assessed to produce biodiesel from coconut oil. The end products (FAMEs) of transesterification/methanolysis were analyzed and identified by GC. The immobilized lipase generated methyl esters of laurate, myristate, palmitate, and oleate in major amounts along with minor amounts of methyl esters of caprylate, caprate, and stearate. The FAME profile compared to the chemical transesterification reaction of coconut oil with NaOH, but differed significantly from those identified in commercial diesel. The findings of our study conclude that the lipase system from the oilseed-associated fungus, *L. theobromae* VBE1, constitutes a potential and alternative source for industrially important exocellular lipases that can find application for biodiesel production, in this case from coconut oil.

Acknowledgements The authors acknowledge the Department of Chemistry, University of Madras and Indian Institute of Technology (IIT) Madras, and Central Leather Research Institute (CLRI), Adyar, Chennai, Tamil Nadu, India, for analysis of the biodiesel samples from coconut oil and commercial diesel. Professor R.F.H. Dekker gratefully acknowledges Universidade Tecnológica Federal do Paraná, Londrina-PR (Brazil) for support during the writing of the paper.

Funding Information The University Grants Commission—Major Research Project at the University of Madras, INDIA, is acknowledged for financial support.

Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

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