Biocatalysis and Agricultural Biotechnology 18 (2019) 101017

Contents lists available at ScienceDirect

Biocatalysis and Agricultural Biotechnology

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

Enhanced lipolytic activity potential of mutant *Bacillus niacini* EMB-5 Grown on Palm Oil Mill Effluent (POME) and biochemical characterization of purified lipase

Solomon A. Oyedele[a], Adeyemi O. Ayodeji[a,*], Olufemi S. Bamidele[a], Joshua O. Ajele[a], Temitope B. Fabunmi[b]

[a] Enzymology and Enzyme Biotechnology Unit, Department of Biochemistry, The Federal University of Technology, Akure, 340001, Nigeria
[b] Department of Microbiology, The Federal University of Technology, Akure, 340001, Nigeria

ARTICLE INFO

Keywords:
Lipase
Biotechnology
POME
Agro-wastes
Mutation
Purification

ABSTRACT

Palm oil mill effluent (POME) is a major waste from oil-palm industries often disposed indiscriminately into surrounding water bodies, adding up to retarding soil health and environmental pollution. Here, POME was beneficially used for lipase production by newly isolated microorganisms from oil-polluted soil. The identified microorganisms, using 16S rRNA gene analysis, were screened for lipolytic activity and treated with ethidium bromide for possible mutation in nucleotide sequence and enhancement of their lipolytic potential. A mutant strain of *Bacillus niacini* as confirmed by CodonCode aligner software showed the highest lipolytic activity compared to the wild type organisms. The crude lipase produced from the mutant strain was purified to apparent homogeneity using ammonium sulphate precipitation, ion-exchange chromatography on DEAE-Sephadex A-50 and gel-filtration on Sephadex G-100. Some biochemical properties of the lipase were studied prior to its application for the hydrolysis of different oils. The 59 kDa purified lipase exhibited optimal activity at pH 9.0 and 40 °C. The enzyme is alkaline thermostable being active at pH 8.0–12 and retained about 60% of initial activity after 60 min of incubation at 90 °C. At 10 mM salt concentration, Al^{3+} and Ca^{2+} modulated the activity of the enzyme but inhibited by Cu^{2+}, Hg^{2+}, EDTA, β-mercaptoethanol, SDS and Urea. The K_m and V_{max} of the lipase with olive oil as substrate are respectively, 6.12 mg/mL and 59.64 μmol/min/mg as the enzyme was able to hydrolyze various natural oils. These remarkable properties make the purified EMB-5 lipase employable for biotechnological applications in lipid-utilizing industries.

1. Introduction

The processing of oil palm to consumable products like edible palm oil is often accompanied with the generation of many unfriendly environmental wastes such as palm oil mill effluent (POME), Oil palm fronds (OPF), Palm shells (PS) and empty fruit bunches (EFBs) that are mostly disposed improperly (Anyanwu et al., 2013). According to production statistics, Nigeria is the fifth largest producer of palm oil in the world after Indonesia, Malaysia, Thailand, and Colombia, with an annual production volume of 970,000 metric tonnes (USDA, 2018). The oil palm industry is one of the largest agro-businesses in Nigeria and it is responsible for the means of livelihood of many families within the oil palm belt where local processing and palm oil production take place while concomitantly generating not lesser than 633,250 dry tonnes of POME annually (Anyanwu et al., 2013), amongst other wastes.

The prevalent disposal method of POME involves dumping the aqueous waste into streams surrounding oil mills thereby constituting environmental problems such as water pollution and heavy stench in the areas close to the processing sites. These wastes become hazardous to both the flora and fauna in the water bodies as well as causing repulsion of humans in these areas. Industrialists and researchers alike continue to find more ecologically compatible disposal methods and possible advantageous use of the generated wastes from agro-processing industries including the multi-million dollar oil palm industry. Hence the channeling of these agro-wastes for the production of enzymes of industrial importance such as lipases, proteases and amylases (Bhange et al., 2016; Bakir and Metin, 2017), cannot be overemphasized.

* Corresponding author.
E-mail addresses: oyedelesolomon@yahoo.com (S.A. Oyedele), ade.oayodeji@gmail.com, aoayodeji@futa.edu.ng (A.O. Ayodeji), osbamidele@futa.edu.ng (O.S. Bamidele), joajele@futa.edu.ng (J.O. Ajele), tbfabunmi@gmail.com (T.B. Fabunmi).

https://doi.org/10.1016/j.bcab.2019.01.055
Received 12 September 2018; Received in revised form 22 November 2018; Accepted 28 January 2019
Available online 31 January 2019
1878-8181/

Lipases (triacylglycerol acylhydrolases, E.C. 3.1.1.3) are serine hydrolases that catalyze the breakdown of fats and oils with subsequent release of free fatty acids, diacylglycerols, monoglycerols and glycerol (Joseph et al., 2007). They are ubiquitous enzymes that display their activity in aqueous solutions containing soluble substrates such as triacylglycerols (TAGs) where they catalyze a number of useful reactions including esterification, transesterification, regioselective acylation of glycols and menthols, and synthesis of peptides (Sharma et al., 2011; Abdel-Fattah et al., 2012). Under low water activity, different transesterification reactions such as alcoholysis, acidolysis, aminolysis or interesterification are catalyzed by lipases (Borrelli and Trono, 2015).

Accounting for more than one-fifth of the global enzyme market, lipases are considered to be an attractive class of industrial biocatalysts with high future prospects (Borrelli and Trono, 2015; Daiha et al., 2015). They remain a subject of intensive study owing to their wide-ranging applications in organic chemical processing, detergent formulations, synthesis of biosurfactants, dairy, agrochemical, paper manufacture, nutrition, cosmetics, pharmaceutical and biofuel (biodiesel) industries (Yu et al., 2010; Andualema and Gessesse, 2012; Verma et al., 2012).

Though, lipases are produced by animals, plants and microorganisms, lipases of microbial origin are mostly preferred in biotechnology which has necessitated the exploration of their biodiversity for novel enzymatic variants (Sharma et al., 2016). Microbial enzymes are often more useful than enzymes derived from plants or animals because of the great variety of catalytic activities available, the high yields possible, ease of genetic manipulation, regular supply due to the absence of seasonal fluctuations and rapid growth of microorganisms on inexpensive media (Joseph et al., 2007).

The production capacity of industrial enzyme by microorganisms has been boosted through strain improvement techniques such as molecular cloning or chemical mutation using ethyl methyl sulfonate (EMS), sodium nitrite solution, N-methyl-N-nitro-N-nitrosoguanidine (NTG) and ethidium bromide (Pathak et al., 2015; Sharma et al., 2016). Therefore, in the quest to obtain improved lipase-producing bacteria strains, this work was designed to identify and characterize organisms with increased lipolytic potential, subjected to ethidium bromide-induced chemical mutagenesis while using the oil palm waste POME as sole carbon source. This work also evaluates the physiochemical and kinetic properties of the purified lipase from a mutant strain of *B. niacini* EMB-5 for possible biotechnological application.

2. Materials and methods

2.1. Materials

Palm oil mill effluent (POME) was obtained from oil palm processing industries in Ipinsa, Akure, Southwest Nigeria (coordinates 7°10'N 5°05'E). Tributyrin, Tween-80, Ethidium bromide, *p*-nitrophenyl palmitate (*p*-NPP), Sephadex G-100, Carboxyl methyl (CM)-Cellulose, Acetic acid, Sodium acetate, Potassium phosphate monobasic, Di-potassium hydrogen phosphate, Trizma base, Urea, Glycine were acquired from Sigma-Aldrich Fine Chemicals, St. Louis, Mo, USA. All other reagents and chemicals used were of analytical grade purchased from local accredited suppliers.

2.2. Collection of soil samples

Soil samples were collected from different palm oil producing sites in Ipinsa, Akure metropolis of Ondo State, South-western Nigeria (coordinates 7°10'N 5°05'E). Samples were taken from a depth of about 5 cm and transported in sterile plastic bags to the Laboratory, Department of Microbiology, The Federal University of Technology, Akure, Nigeria for microbial analysis.

2.3. Microbial analysis

2.3.1. Isolation and identification of lipase producing microorganisms

Isolation of lipase-producing organisms was done by spreading the serial-diluted soil samples on mineral salt agar media according to the method of Kashmiri et al. (2006) with slight modification. The medium (100 mL) contained: Na_2HPO_4 (0.6%); $(NH_4)_2SO_4$ (0.5%); KH_2PO_4 (0.2%); $MgSO_4$ (0.3%); $CaCl_2$ (0.3%), Agar (1.2%) and palm oil 10 mL (emulsified in Tween-80 at 10 mL oil:1 mL Tween-80). The plates were incubated at 28 °C for 3days and checked every day for growth. Pure cultures of the isolated organisms were obtained and identification of bacteria species was done by observing their morphological, physiological and biochemical characteristics as described in Bergey's manual of systematic bacteriology (Claus and Berkeley, 1986).

2.3.2. Molecular characterization and identification of isolate

Molecular characterization of lipolytic organisms was done using 16S rRNA gene analysis. The gene sequencing was done according to standard operating procedures at the Bioscience Laboratory of the International Institute of Tropical Agriculture (IITA), Ibadan, Nigeria. Briefly, each strain's genomic DNA was extracted as previously described by Cutting and Van der Horn (1990). The 16S rRNA genes were PCR-amplified from the genomic DNA using the bacterial universal primer set of 518F: (5'-CCAGCAGCCGCGGTAATACG-3') and 800R: (5'-TACCAGGGTATCTAATCC-3'), which were also used for sequencing (Weisburg et al., 1991). The amplified PCR products were analyzed by 2% (w/v) agarose gel. The sequence of 16S rRNA was aligned by using the BLAST program to identify the most similar sequence in the database (Altschul et al., 1990). 16S rDNA sequences of different strains of *Bacillus* genera were downloaded from GenBank database (http://www.ncbi.nlm.nih.gov/entrez) and aligned to construct a neighbor-joining phylogenetic tree using Clustal W algorithm with the help of MEGA software version 7.0 (Kumar et al., 2016).

2.3.3. Random mutation of microorganisms

The mutation procedures were carried out according to the method of Khodayari et al. (2014). The 24-hr old cultured isolate was diluted with sterile deionized water and then spread on Luria-Bertini (LB) agar plates by glassy stick. The mutagen (ethidium bromide solution, 20 mg/ml) was dropped on the pre-marked points on the plate with a micropipette. The surfaces of plates were dried for 20 min and then incubated overnight at 55 °C. The colonies around the toxic zones (ethidium bromide dropped points) were picked and inoculated onto new LB agar plates containing *p*-nitrophenyl palmitate (*p*-NPP) (0.5% w/v). The plates were incubated at 55 °C for a period of 24 h to check for lipolytic activity of the mutant microbial strains. The strains were selected and classified as very good mutant when the clear halo-zone around the colonies is at least 30 mm, good mutant when the halo-zone is between 20 – 29 mm and weak mutant when less than 20 mm. Molecular characterization (16S rRNA gene analysis) of the mutant strains was done as earlier described for the wild type. The 16S rRNA sequences of both wild and mutant strains were aligned using the CodonCode Aligner software (CodonCode Corporation, Centerville, MA) for mutation detection.

2.3.4. Screening of isolates for lipolytic activity

Freshly prepared agar plates containing tributyrin (20 g/L) mixed with 20 mL of Tween-80 and phenol red (0.02 g/L) were inoculated with the isolated organism using spotting technique (Lawrence et al., 1967). The plates were incubated at 35 °C and observed for zone of clearance after 48 h. The strains were selected and classified as either good producer of lipase or not depending on the size of the clear halo-zone around the colonies. Further, quantitative screening for lipolytic activity was done by transferring a single colony about 10^7 *cfu* from each agar slant aseptically about into 50 mL basal medium containing 10 mL of palm oil (emulsified in Tween 80 at 10 mL oil:1 mL Tween);

$(NH_4)_2SO_4$ (0.25 g); Na_2HPO_4 (0.3 g); KH_2PO_4 (0.1 g); $MgSO_4$ (0.15 g); $CaCl_2$ (0.15 g). The pH of the media were adjusted to 5.0 and incubated at 37 °C under a constant shaking condition (150 rpm) for 48 h. Biochemical assays were carried out to determine the activity of lipase and the concentration of protein in liquid culture by the methods described below.

2.4. Enzyme activity assay and determination of protein concentration

Lipase activity in the supernatant after biomass separation was determined according to a standard assay procedure earlier described by Lotrakul and Dharmsthiti (1997). Briefly, 200 µL of enzyme solution was added to 1800 µL of the substrate solution containing 1.3 µM of p-NPP dissolved in 10 mL of 2-propanol, 0.4% triton-100 and 0.1% gum Arabic dissolved in 100 mL of 0.05 M Tris-HCl (pH 8.0). The reaction mixture was incubated at 37 °C for 15 min and absorbance was read at 420 nm against the blank. The results were expressed as lipase units (µmol/min/mL) of enzyme.

The concentration of the protein in the mixture was determined by the method of Bradford (1976) using bovine serum albumin (BSA) as the standard protein.

2.5. Inoculum preparation and time-course production of lipase

A seed inoculum of both the wild and mutant *B. niacini* EMB-5 were first prepared by transferring a colony of the organism from agar slant into 50 mL of freshly prepared nutrient broth and adjusting the Optical density (OD) to 0.1 at 600 nm. After 12 h of incubation at 37 °C under a constant shaking condition (150 rpm), 2.5 mL of the seed inoculum (5% v/v) was transferred to 50 mL of the basal medium containing; $(NH_4)_2SO_4$ (5 g/L); Na_2HPO_4 (6 g/L); KH_2PO_4 (2 g/L); $MgSO_4$ (3 g/L); $CaCl_2$ (3 g/L) and palm oil mill effluent 10 mL (emulsified in Tween 80 at 10 mL oil:1 mL Tween). The pH of the medium was adjusted to pH 5.0 and the flask was incubated in an orbital shaker set at 37 °C and 150 rpm for 72 h. The culture medium was sampled at 6 h interval to monitor the microbial growth rate and enzyme production in liquid culture. The Absorbance of fractions collected was read at 600 nm to evaluate the progressive growth of the organism. The fractions were later centrifuged at 10,000 *g* for 20 min in a refrigerated centrifuge (Harrier 18/80) before carrying out lipase activity and protein concentration assays.

2.6. Production of lipase from mutant B. niacini EMB-5

Seed culture of mutant *B. niacini* EMB-5 was first prepared by transferring 5% (v/v) of inoculum containing about 10^7 cells/mL prepared earlier into 100 mL of growth medium containing: $(NH_4)_2SO_4$ (5 g/L); Na_2HPO_4 (6 g/L); KH_2PO_4 (2 g/L); $MgSO_4$ (3 g/L); $CaCl_2$ (3 g/L) and palm oil mill effluent 10 mL (emulsified in Tween 80 at 10 mL oil:1 mL Tween), initial pH of 5.0 and incubated for 12 h at 37 °C (150 rpm). The seed culture (50 mL) was transferred to 950 mL of the basal medium containing $(NH_4)_2SO_4$ (5 g); Na_2HPO_4 (6 g); KH_2PO_4 (2 g); $MgSO_4$ (3 g); $CaCl_2$ (3 g) and palm oil mill effluent 10 mL (emulsified in Tween 80 at 10 mL oil:1 mL Tween), with pH adjustment to 5.0. The medium was incubated at 37 °C (150 rpm) in an incubator orbital shaker (Stuart, S1600) for 36 h. After incubation period, the culture medium was centrifuged at 10,000 *g* for 20 min in a refrigerated centrifuge (Harrier 18/80) at 4 °C to remove the cells. Lipase activity in the cell-free supernatant was determined using the earlier described method of Lotrakul and Dharmsthiti (1997).

2.7. Purification of enzyme

Firstly, solid ammonium sulphate was added stepwise to culture supernatant until it attained 60% saturation. The precipitate was collected by centrifugation at 10,000 rpm for 10 min in a refrigerated centrifuge and dialyzed against 50 mM Tris-HCl buffer (pH 8.0) at 4 °C periodically for 24 h. The salt-free dialysate was loaded on a DEAE-Sephadex A-50 column (2.5 × 40 cm) previously equilibrated with 50 mM Tris-HCl buffer (pH 8.0) at a flow rate of 2 mL/min. Elution of proteins was done using the same buffer while the bound proteins were eluted from the column by linear salt gradient (0.1–1.0 M NaCl) in the elution buffer. The presence of protein in the eluted fractions was monitored by measuring their absorbance at 280 nm using UV spectrophotometer (Shimadzu, UV 1800) and lipase activity of the fractions were determined. The active fractions were pooled and concentrated using an ultra-filtration system before loading unto a Sephadex G–100 column (2.5 × 75 cm, flow rate of 20 mL/hr) already equilibrated with 50 mM Tris-HCl buffer (pH 8.0) also used as elution buffer. Absorbance of eluted fractions was read at 280 nm and lipase activity assay was carried out. The active fractions were pooled and concentrated (Ajele and Afolayan, 1992; Ayodeji et al., 2017). The purified enzyme was thereafter used for biochemical characterization studies.

Table 1
Mutation of isolated bacteria isolates.

Isolate code	Clear zone diameter (mm)	Remark	Name of Isolate
EB-A7	25	Good	*Bacillus subtilis*
EB-A8	32	Very Good	*Bacillus niacini*
EB-A4	23	Good	*Bacillus polymyxa*
EB-A1	20	Good	*Bacillus cereus*

2.8. Determination of molecular mass

SDS-PAGE was performed on a 10% polyacrylamide gel using Tris-Glycine-SDS buffer system according to the method of Laemmli (1970) to determine the purity/homogeneity and the sub-unit molecular mass of the purified lipase. Electrophoresis was performed at 80 V for 4 h using the Bio-Rad electrophoresis system (Bio-Rad, UK). The gel was stained with Coomassie brilliant blue and the protein bands were observed after destaining the gel.

2.9. Biochemical characterization of purified lipase

2.9.1. Effects of temperature on the activity and stability of purified lipase

Diluted purified enzyme (200 µL) was pipetted into a test tube containing 1800 µL of the substrate solution as previously described and the mixture was incubated at 30 °C for 15 min. The assay procedure was repeated for other temperatures between 40 - 90 °C. The thermal stability of the purified lipase was determined by incubating the diluted enzyme solution at varying temperatures (30–90 °C) for 120 min. Aliquot of 200 µL was withdrawn at 20 min interval to determine the residual activity of the purified lipase.

2.9.2. Effects of pH on the activity and stability of purified lipase

To determine the optimum pH, lipase activity was measured under standard assay conditions at different pH values using the following buffers; 50 mM glycine-HCl buffer (pH 3.0), 50 mM sodium acetate buffer (pH 4.0–5.0), 50 mM phosphate buffer (pH 6.0–7.0), 50 mM Tris-HCl buffer (pH 8.0–9.0) and 50 mM glycine-NaOH (pH 10.0–12.0). The effect of pH on the stability of the purified lipase was determined by incubating the purified enzyme with relevant buffer solutions (pH 3.0–12.0) for 120 min with periodic withdrawal of aliquot enzyme every 20 min to measure the residual activity of the purified lipase.

2.9.3. Effect of metallic ions on the activity of purified lipase

The effect of different metal ions (Ca^{2+}, Cu^{2+}, Mg^{2+}, Mn^{2+}, Fe^{2+}, Hg^{2+}, Na^{+}, Zn^{2+}, K^{+} and Al^{3+}) on the activity of the purified lipase was determined by pre-incubating the enzyme with the metal ions at a final concentration of 10 mM using their chloride salt prepared in

Table 2
Qualitative and Quantitative screening of isolated soil bacterial species for lipolytic potential.

Isolate code	Halo-zone diameter (mm)	Lipolytic potential	Protein Concentration (mg/mL)	Specific Activity (U/mg)	Name of Isolate Identified
EB-A7	27	+ +	0.87	18.32	*Bacillus subtilis*
EB-A8	30	+ + +	1.15	29.88	*Bacillus niacini*
EB-A4	24	+ +	0.91	26.51	*Bacillus polymyxa*
EB-A1	22	+ +	1.03	22.29	*Bacillus cereus*
EMS-2	29	+ +	1.22	24.16	mutant *B. subtilis*
EMB-5	34	+ + +	1.68	36.87	mutant *B.niacini*
EMP-3	25	+ +	1.14	30.23	mutant *B. polymyxa*
EMC-8	22	+ +	1.37	28.53	mutant *B. cereus*

Remark: + + + very good producer.
+ + good producer.
+ weak producer.

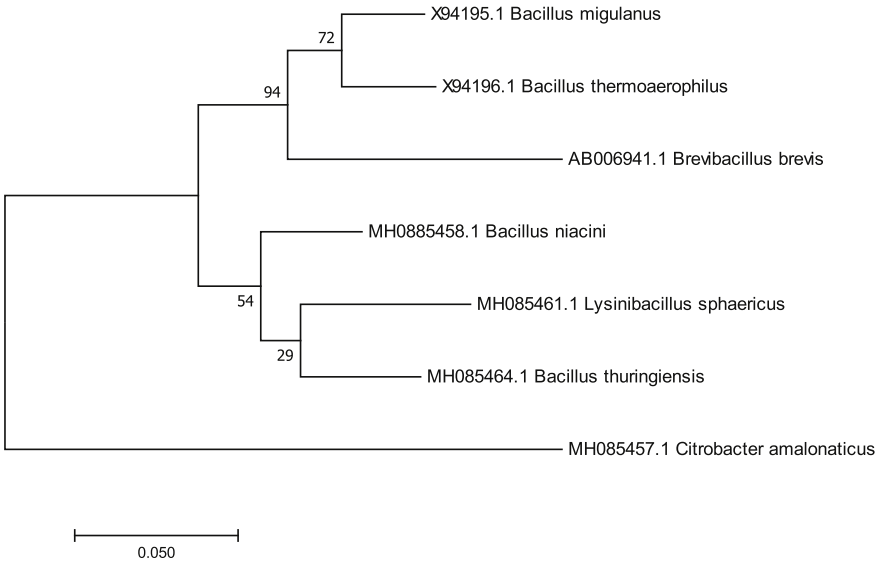

Fig. 1. Molecular Phylogenetic analysis by Maximum Likelihood method.
The tree with the highest log likelihood (−5037.15) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 7 nucleotide sequences. There were a total of 1540 positions in the final dataset.

Table 3
Codon aligner of 16S rRNA of wild and mutant *B. niacini*.

Feature	Source	Found In	Parent Contig	Start	End	Content
heterozygoteAG	Aligner	Mutant 16SForward	Contig1	121	121	Heterozygous 121A > G Arg41Gly
heterozygoteCG	Aligner	Mutant 16SForward	Contig1	209	209	Heterozygous 209G > C Arg70Pro
heterozygoteCG	Aligner	Mutant 16SForward	Contig1	253	253	Heterozygous 253C > G Leu85Val
heterozygoteCG	Aligner	Mutant 16SForward	Contig1	257	257	Heterozygous 257C > G Thr86Arg
heterozygoteAG	Aligner	Mutant 16SForward	Contig1	344	344	Heterozygous 344G > A Arg115Lys
heterozygoteAG	Aligner	Mutant 16SForward	Contig1	462	462	Heterozygous 462A > G Gly154Gly
heterozygoteGT	Aligner	Mutant 16SReverse	Contig1	1116	1116	Heterozygous 1116G > T Val372Val
heterozygoteGT	Aligner	Mutant 16SReverse	Contig1	1117	1117	Heterozygous 1117G > T Gly373Trp
heterozygoteCT	Aligner	Mutant 16SReverse	Contig1	1121	1121	Heterozygous 1121T > C MET374Thr
heterozygoteCT	Aligner	Mutant 16SReverse	Contig1	1142	1142	Heterozygous 1142C > T Pro381Leu
heterozygoteCG	Aligner	Mutant 16SReverse	Contig1	1222	1222	Heterozygous 1222G > C Val408Leu
heterozygoteCT	Aligner	Mutant 16SReverse	Contig1	1223	1223	Heterozygous 1223T > C Val408Ala
heterozygoteCT	Aligner	Mutant 16SReverse	Contig1	1225	1225	Heterozygous 1225T > C Cys409Arg
heterozygoteCT	Aligner	Mutant 16SReverse	Contig1	1240	1240	Heterozygous 1240T > C Trp414Arg
heterozygoteCT	Aligner	Mutant 16SReverse	Contig1	1262	1262	Heterozygous 1262T > C MET421Thr
heterozygoteAC	Aligner	Mutant 16SReverse	Contig1	1278	1278	Heterozygous 1278A > C Leu426Leu
heterozygoteCT	Aligner	Mutant 16SReverse	Contig1	1298	1298	Heterozygous 1298C > T Ala433Val
heterozygoteCT	Aligner	Mutant 16SReverse	Contig1	1299	1299	Heterozygous 1299T > C Ala433Ala

50 mM Tris-HCl buffer, pH 8.0. After 30 min of incubation at 30 °C, lipase activity relative to the control without metal ions was determined according to the assay procedure previously described.

2.9.4. Effect of inhibitors and surfactants on the activity purified lipase

The diluted purified enzyme solution (200 µL) was pre-incubated differently in 100 µL of 1, 5 and 10 mM β-mercaptoethanol (β-Me), ethylenediaminetetraacetic acid (EDTA), sodium dodecyl sulphate (SDS) and Urea at 30 °C for 30 min. The mixtures were used under standard assay conditions to determine the effect of the surfactant and inhibitors on the purified lipase activity.

2.9.5. Determination of kinetic parameters

Kinetic parameters (K_m and V_{max}) were determined at optimum conditions of temperature and pH by measuring the initial reaction rate in the presence of *p*-nitrophenyl palmitate (*p*-NPP) as substrate at

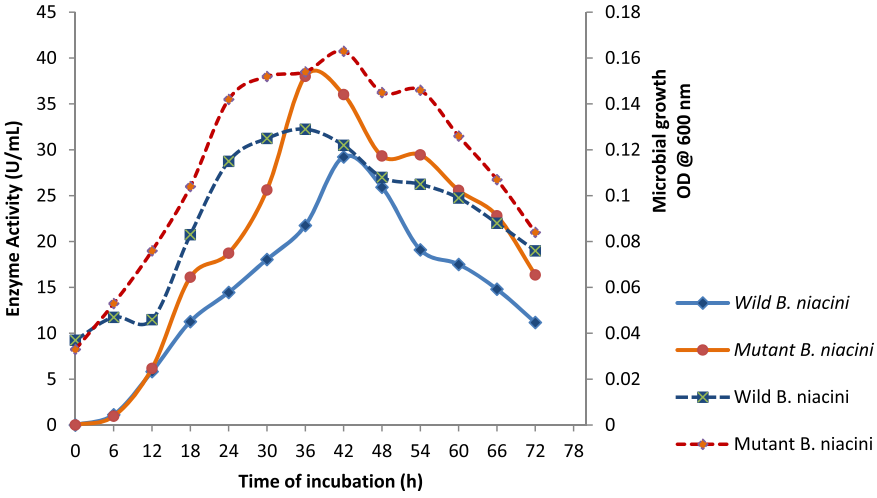

Fig. 2. Time-course production of lipase from wild and mutant *B. niacini* EMB-5 using POME as carbon source (….. dotted lines show the microbial growth curve; —— block lines represent enzyme activity curve).

Table 4
Summary of Purification of Lipase from mutant *B. niacini* EMB-5.

Purification Step	Volume (mL)	Total Activity (U)	Total Protein (mg)	Specific Activity (U/mg)	Yield (%)	Purification Fold
Crude Enzyme	700	21700.2	644.51	33.67	100	1
Ammonium sulfate precipitation (60%)	40	3036.32	33.20	91.46	13.99	2.72
Ion-exchange Chromatography on DEAE-Sephadex A-50	18	1929.6	6.30	306.29	8.89	9.10
Gel filtration on Sephadex G-100	20	1558.08	2.43	641.42	7.18	19.05

All Purification steps were carried out at 4 °C.

various concentrations (1.5–4.0 μM). The values of the apparent kinetic parameters were obtained from the double reciprocal plot by Lineweaver and Burk.

2.9.6. Hydrolysis of different oils

Six different oils including palm oil, soybean oil, vegetable oil, corn oil, carnola oil and olive oil, each mixed in 2-propanol were used for the application study of hydrolysis of oils. This was done by monitoring the enzymatic hydrolysis of the oils spectrophotometrically. Enzyme activity assay was carried out according to the standard method already described.

3. Results and discussion

3.1. Isolation, mutation and screening of microorganisms for lipase production

Microbial lipases have found broad application potential in many day-to-day industrial activities. Here, four bacteria species isolated from an oil-contaminated soil environment and their respective mutant strains obtained from subjection to ethidium bromide treatment were identified (Table 1). The mutants were observed to have increased production potentials for lipolytic enzyme compared to the wild type organisms as seen in the specific activity result. Notably, a strain (EB-A8) together with its mutant showed the highest lipolytic potential from both qualitative and quantitative screening (Table 2). This EB-A8 strain identified as *Bacillus niacini* through its 16S rRNA analysis has 94% sequence similarity with *Bacillus* species (Fig. 1) and was registered with GenBank database of which an ascension number MH0885458 was obtained. The evolutionary history was inferred by using the Maximum Likelihood method based on the Jukes-Cantor model (Jukes and Cantor, 1969).

Both wild and mutant strains of the *B. niacini* coded EMB-5 in our Laboratory with mutation points identified through sequence analysis using the CodonCode Aligner software (Table 3) were thereafter employed for a time-course lipase production. The wild strain reached its maximum lipase production capacity at the 42nd hour of incubation with enzyme activity of 29.23 U/mL whereas the mutant strain reached maximum enzyme production at the 36th hour with an activity value of 38.01 U/mL (Fig. 2) under optimized conditions. The production of lipases at a maximum rate has been between 24 – 48 h using wild type microorganisms of *Bacillus sp* (Amjad, 2011; Bakir and Metin, 2017). Here, there was a boost in the lipolytic potential of *B. niacini* through chemical mutagenesis with ethidium bromide which is comparable to the higher production capacity of lipases from different mutants reported by Bisht et al. (2013) and Sharma et al. (2016). The perceived molecular changes in the genetic make-up of mutant *B. niacini* compared to the wild strain possibly add up to the increased oil hydrolysis rate by the mutant strain (EMB-5) lipase which gave rise to an increased specific activity recorded (see Table 2).

3.2. Production and purification of lipase

The crude enzyme solution with total lipase activity of 21700 U and 644 mg total protein was purified to apparent homogeneity. The activity recovery of the purified lipase was 7.18% and 19.05–fold increase in specific activity. As seen in the summary of purification reported in Table 4, each purification step enhanced the specific activity of the enzyme which is comparable to the 16% yield of a purified lipase from *B. subtilis* NS 8 by Akanbi et al. (2001) and Bhosale et al. (2016). The purification of enzymes obtained from microbial fermentation is necessary to have the protein of interest in its simple active form, separated from other concomitantly produced proteins in solution which allows for the characteristics of the enzyme of interest to be well studied.

3.3. Determination of molecular mass

The molecular mass of the purified *B. niacini* EMB-5 lipase, as revealed by SDS-PAGE was approximately 59 kDa (Fig. 3), a value comparable to the 59.4 kDa obtained for lipase from *Pseudomonas aeruginosa* (Singh and Banerjee, 2007) but higher than the 30 kDa of *P.*

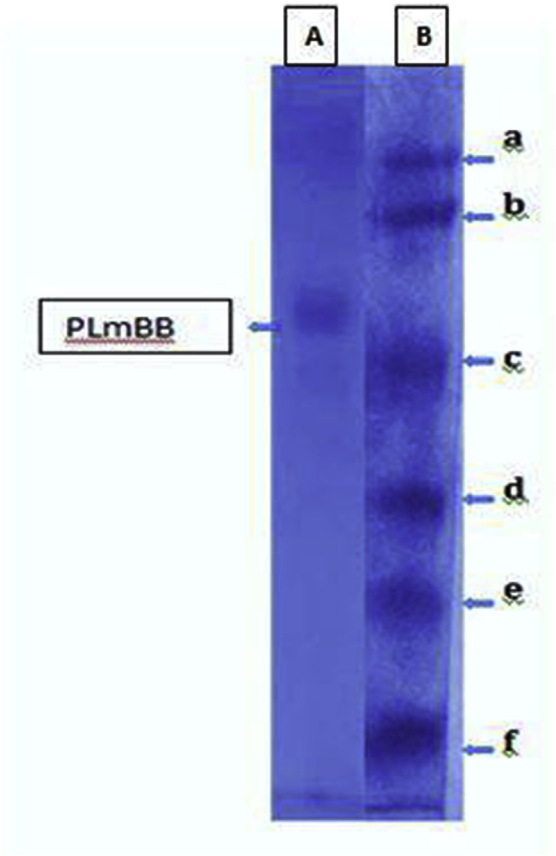

Fig. 3. The polyacrylamide gel electrophoresis of the lipase from mutant *B. niacini* EMB-5 under denaturing conditions (SDS-PAGE).
Lane A: purified lipase from mutant *B. niacini* EMB-5 (PLmBB).
Lane B*: Standard molecular weight markers*.
a = Phosphorylase b (113 kDa) b = Bovine Serum Albumin (81 kDa).
c = Ovalbumin (47 kDa) d = Carbonic Anhydrase (34 kDa).
e = Soyabean Trypsin Inhibitor (27 kDa) f = Lysozyme (17 kDa).

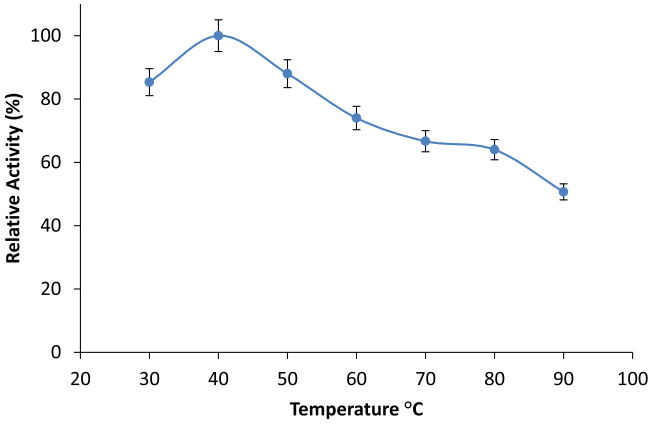

Fig. 4. Effect of temperature on the activity of lipase from mutant *B. niacini* EMB-5.

aeruginosa ATCC 27853 lipase (Izrael-Zivkovic et al., 2009). The presence of a single band lipase from *P. aeruginosa* mutant was also confirmed by Bisht et al. (2013) on SDS-PAGE and its molecular weight was approximately 67 kDa. The variation in molecular weights could be attributed to isozymic properties as well as types of amino acid resides present in the *B. niacini* EMB-5 lipase. Further studies on the sequence and structure of the purified lipase needs to be carried out to confirm these.

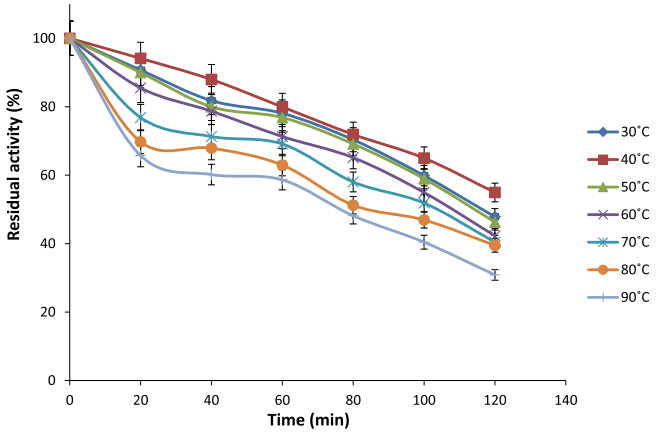

Fig. 5. Effect of temperature on the stability of lipase from mutant *B. niacini* EMB-5.

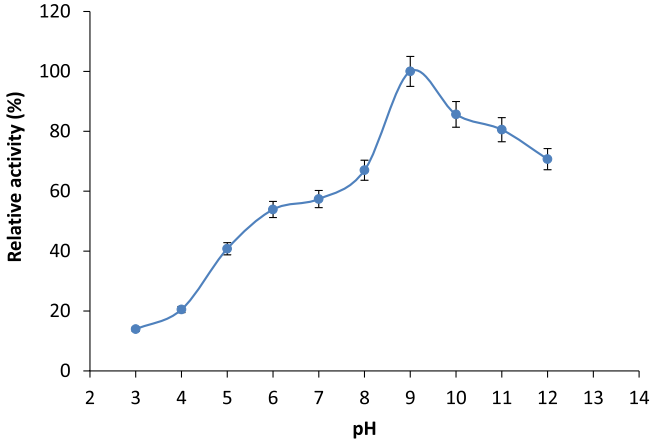

Fig. 6. Effect of pH on the activity of lipase from mutant *B. niacini* EMB-5.

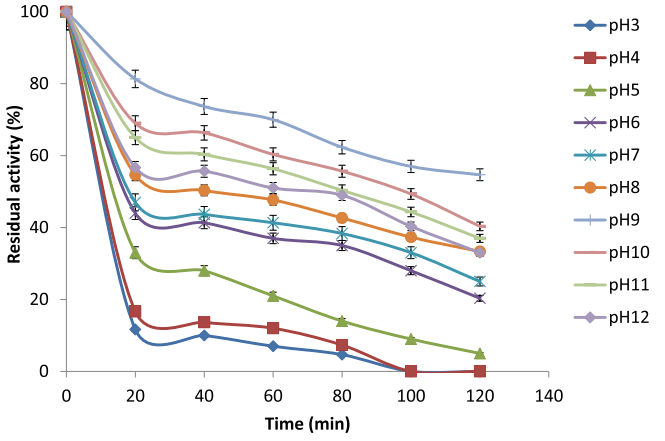

Fig. 7. Effect of pH on the stability of lipase from mutant *B. niacini* EMB-5.

3.4. Biochemical characterization

3.4.1. Effect of temperature on lipase activity and stability

The enzyme showed high lipolytic activity between 30 °C and 60 °C. Further increase in temperature up to 90 °C resulted in a decrease in enzyme activity (Fig. 4). Thus, 40 °C was found to be the optimum temperature for the activity of this lipase from mutant *B. niacini* EMB-5 which is comparable to lipases reported to be active in the temperature range of 35–55 °C (Sharma et al., 2002; Bisht et al., 2013). The thermal stability studies of this lipase, as shown in Fig. 5, revealed that the

Table 5
Effect of metal ions and some chemical inhibitors on the activity of Lipase from Mutant *B. niacini* EMB-5.

	Relative Activity (%)		
Metal ion/Inhibitor/denaturant	1 mM	5 mM	10 mM
Control	100 ± 0.0	100 ± 0.0	100 ± 0.0
Ca^{2+}	118 ± 2.3	115 ± 1.8	106 ± 1.2
Al^{3+}	107 ± 1.4	104 ± 0.5	102 ± 1.1
K^{+}	112 ± 1.2	105 ± 1.3	100 ± 2.1
Cu^{2+}	65 ± 1.7	62 ± 1.3	53 ± 2.4
Hg^{2+}	58 ± 0.5	57 ± 1.1	55 ± 1.0
Mg^{2+}	78 ± 1.2	70 ± 1.4	67 ± 1.3
Na^{+}	115 ± 1.6	111 ± 1.8	97 ± 1.2
Fe^{2+}	96 ± 0.2	93 ± 0.8	88 ± 1.1
Mn^{2+}	108 ± 2.2	102 ± 1.8	100 ± 1.0
Zn^{2+}	97 ± 1.9	91 ± 1.4	81 ± 0.8
EDTA	70 ± 1.1	64 ± 1.7	59 ± 1.8
SDS	93 ± 1.2	89 ± 1.3	88 ± 1.3
Urea	81 ± 1.3	77 ± 1.5	73 ± 1.4
β-mercaptoethanol	48 ± 2.1	44 ± 1.3	41 ± 1.2

(data values represent mean ± standard deviation of three repetitions).
All reactions were carried out at standard assay conditions after pre-incubation of the lipase at 30 °C for 30 min in the absence (control) and presence of chemical agents at final concentrations of 1 mM, 5 mM and 10 mM. The lipase activities are expressed as the percentage of control, which was set as 100%.

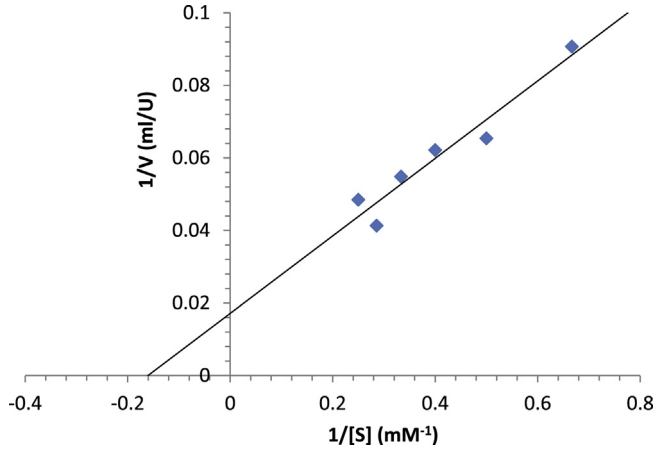

Fig. 8. Double reciprocal plot of reaction velocity against substrate concentration of purified lipase from mutant *B. niacini* EMB-5 using olive oil as substrate.

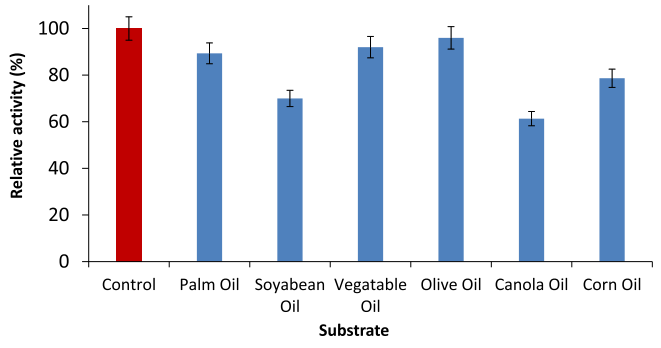

Fig. 9. Hydrolytic activity of lipase from mutant *B. niacini* EMB-5 on different oils.

enzyme retained more than 50% of its initial activity after 80 min of pre-incubation at all temperatures from 30 to 90 °C. Interestingly, the enzyme was very much stable and active after 1 h of incubation at high temperatures of 80 °C and 90 °C with respective residual activities of 63% and 59% which could be an indication that the enzyme maintained its structural integrity at these very high temperatures (Zhu et al., 2001). At an extended incubation time of 120 min, the activity of this lipase was still relatively significant for enzymatic reaction to proceed at the temperature range of 30–50 °C. The activity only reduced to 71% of initial at 60 °C which is more active and stable compared to the thermophilic *B. thermoleovorans* ID-1 lipase reported by Lee et al. (1999) which only retained 50% of its original activity after 1h incubation at 60 °C. The relative stability of this *B. niacini* EMB-5 lipase at high temperatures makes it employable for biotechnological processes as enzymes which can withstand high temperatures for a longer period of time is of interest to industries (Yildirim et al., 2017).

3.4.2. Effect of pH on the activity and stability of lipase

The activity of the lipase from mutant *B. niacini* EMB-5 was found to be 5.51 U/mL at pH 3.0 and increased to 7.82 U/mL at pH 4.0. The lipolytic activity then increased steadily from 15.73 U/mL (pH 5.0) to 40.88 U/mL (pH 9.0), after which further increase in the pH of the solution showed a gradual reduction in the activity of the lipase (Fig. 6). The optimum pH for the lipase activity was observed to be pH 9.0 which is similar to the pH of *B. thermoleovorans* CCR11 lipase (Castro-Ochoa et al., 2005). The result is also comparable to reports on lipases from other species such as *B. coagulans* BTS-3 (Kumar et al., 2005) and mutant *P. aeruginosa* (Bisht et al., 2013), where the lipase was active over a range of pH 7–10 with maximum activity observed at pH 8.5 and pH 8.0, respectively. The results from the stability studies showed that this purified mutant *B. niacini* EMB-5 lipase was actively stable over a pH range in the alkaline medium (pH 8.0–11). As presented in Fig. 7, the activity of the lipase, after 60 min pre-incubation period, reduced to about 48% of the initial activity at pH 8.0, 70% at pH 9.0, 60% at pH 10.0 and 56% at pH 11.0. Whereas after 120 min of pre-incubation at pH 9.0, the enzyme was still stable and active with a residual activity of about 55% of its initial comparable to the lipase from a *Bacillus* sp. L2 (Shariff et al., 2011) and better than *Penicillium cyclopium* lipase II which was only stable between pH values of 4.5 and 7.0 (Chahinian et al., 2000).

3.4.3. Effect of metallic ions on lipolytic activity

As summarized in Table 5, the monovalent ions (Na^{+} and K^{+}) and divalent ions (Ca^{2+} and Mn^{2+}), at both 1 mM and 5 mM salt concentration, all stimulated the activity of this lipase from mutant *B. niacini* EMB-5, which is a clear deviation from the reported inhibitory effects of these ions at 5 mM salt concentration on *Anoxybacillus* sp. lipase produced from oil mill wastewater (Bakir and Metin, 2017). Metallic ions have inhibitory or stimulating effect on the activity of enzymes depending on the concentration of metallic salt solution and also on the binding affinity of the ion to side groups of the amino acid residues present in the enzyme catalytic or binding sites. In the presence of Hg^{2+}, Cu^{2+} and Mg^{2+} at different salt concentration tested (1, 5 and 10 mM), the lipolytic activity of the mutant *B. niacini* EMB-5 lipase was greatly reduced. The inhibitory effects of Hg^{2+} and Cu^{2+} as major inhibitors were also reported for lipases from *Geobacillus thermodenitificans* IBRL-nra (Balan et al., 2012), *Anoxybacillus* sp. HBB16 (Bakir and Metin, 2017) and also for some *Bacillus* species (Castro-Ochoa et al., 2005; Nawani and Kaur, 2007). Whereas, at a final concentration of 10 mM, Zn^{2+} and Fe^{2+} did not show any appreciable inhibitory effects on the EMB-5 lipase activity, Al^{3+}, a trivalent ion stimulated the activity of the mutant *B. niacini* EMB-5 lipase at these salt concentrations.

3.4.4. Effect of some inhibitors on lipolytic activity

As presented in Table 5, the results clearly showed that β-mercaptoethanol largely inhibited the activity of the lipase to about 41% of the initial lipase activity at a final inhibitor concentration of 10 mM, suggesting the presence of sulfhydryl (SH–) and disulfide (-S-S-) groups in the active site of the enzyme and are likely to participate in the catalytic

processes of the hydrolytic enzyme. This indicates a possible shift in the required ionization of the side group species participating in the catalytic activity. Whereas, at all concentrations of inhibitor tested, SDS and Urea showed moderate inhibitory effect on the enzyme activity indicating that the enzyme was able to maintain its structural integrity at these concentrations. The chelating agent, EDTA, at a concentration of 10 mM has an appreciable inhibitory effect on the activity of the enzyme suggesting that metallic ions might be required for the enzyme activity and also an indication that the enzyme is likely to be a metalloenzyme. This is similar to the report of Shariff et al. (2011) that 5 mM EDTA caused a complete inhibition of activity of a lipase from *Bacillus* sp.

3.4.5. Determination of kinetic parameters (K_m and V_{max})

The Michaelis constant (K_m) and the maximum velocity (V_{max}) of this purified lipase from mutant *B. niacini* EMB-5 were determined to be 6.1 mg/mL and 59 μmol/min/mg of protein, respectively from the double reciprocal plot (Fig. 8). This result is higher than that obtained for mutant *P. aeruginosa* alkaline lipase reported by Bisht et al. (2013) with respective K_m and V_{max} values of 2.0 mg/mL and 50 μmol/min/mg. The kinetic constant (K_m) can provide a lot of biochemical and physiological information about an enzyme (Ranaldi et al., 1999). As nature provides an amazing diversity of enzymes, identifying enzyme for a specific problem can be extremely difficult. So, the kinetic properties of any enzyme obtained from a particular source becomes crucial to appreciate its maximal catalytic performance, which in turn is indispensable for the best industrial exploitation of that enzyme (Sangeetha et al., 2011).

3.4.6. Lipolytic activity on different substrates

The lipolytic potential of this purified mutant *B. niacini* EMB-5 lipase on different natural oils with reference to *p*-NPP as standard is shown in Fig. 9. The EMB-5 Lipase has relatively the highest lipolytic activity on olive oil (96%), followed by vegetable oil (92%), palm oil (89%) and then corn oil (78%). Lower lipolytic activity of the lipase was observed on soybean and canola oils with relative activity of 70% and 61%, respectively. This is similar to report by Balan et al. (2012) that lipase produce from *Geobacillus thermodenitificans* IBRL-nra hydrolyzed olive oil, palm oil, corn oil and sunflower oil at 100%, 96%, 90% and 86% respectively while the lipase exhibited lower activity on soybean oil and canola oil. The lipolytic activity could be dependent on the composition and complexity of the oil.

4. Conclusion

The study established that both wild and mutant strains of *B. niacini* EMB-5 have lipase-producing ability. Ethidium bromide induced mutation of *B. niacini* enhanced its lipase production capacity using POME as sole carbon source. The appreciable physicochemical and kinetic properties of the purified mutant *B. niacini* EMB-5 lipase such as its alkaline thermostability during hydrolysis of oil, metallic ion enhanced activity and broad range substrate specificity suggestively affirms its applicability in relevant industries dependent on lipase-based technologies for the synthesis as well as biotransformation of novel compounds.

Author contributions

All authors contributed to the conception and design of experiments; Solomon A. Oyedele and Adeyemi O. Ayodeji performed the experiments; Bukola T. Fabunmi performed the molecular microbial analysis. Adeyemi O. Ayodeji and Olufemi S. Bamidele analyzed the data; Olufemi S. Bamidele and Joshua O. Ajele contributed to writing the manuscript;

Conflicts of interest

The authors declare no conflict of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bcab.2019.01.055.

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