Egyptian Journal of Chemistry

http://ejchem.journals.ekb.eg/

Isolation, purification and production of lipase from *Bacillus subtilis* isolated from food processing wastes and its application in biodiesel production

Nashwa A. H. Fetyan [a]*, Ismail M. Ismail [b]*, Reda Salem [c], Mohamed M. Afifi [a]

[a]*Department of Microbiology, Soil, Water & Environment Research Institute, ARC, , 12619Giza, Egypt*
[b]*Department of Microbial Molecular Biology, Agricultural Genetic Engineering Research Institute (AGERI), ARC, 12619, Giza, Egypt*
[c]*Department of Plant Molecular Biology, Agricultural Genetic Engineering Research Institute AGERI), ARC, 12619, Giza, Egypt*

Abstract

Massive amounts of waste oil generated by restaurants and native homes in Egypt are dumped into sewage systems, causing network damage. Utilization of waste cooking oil (WCO) in biodiesel production, can aid in solving the problem.

In the present study, a new lipase-producing bacterium (*Bacillus subtilis*) was isolated, identified using 16S rRNA sequence analysis and the isolate sequence was deposited in GenBank (accession number: MN238705). The lipase coding sequence was amplified from the abovementioned strain cloned, and expressed in *Escherichia coli*. The molecular weight of the purified enzyme tagged with glutathione-S-transferase (GST) was approximately 49 KDa on SDS-PAGE. Accordingly, the purified native lipase exhibited maximal hydrolytic activity at 37°C and pH 7.0, with a positive effect for Mg^{2+} and Ca^{2+} metals on its activity. Finally, purified native lipase was immobilized on Amberlite resin (IRC50) and successfully catalyzed the transformation of WCO into biodiesel with a yield of 87.39% as determined by gas chromatography/mass spectrometry (GC/MS) analysis. The physicochemical properties of generated biodiesel have met European standards. Prospective studies include large-scale production of *Bacillus subtilis* native lipase and testing the efficacy of recombinant lipase for the transformation of WCO into biodiesel.

Keywords :Biodiesel; Recombinant lipase; Immobilized lipase; Transesterification; Waste cooking oils.

1. Introduction

The demand for microbial industrial enzymes in a wide variety of processes has drawn considerable attention due to their novel & multifold applications .Lipases (triacylglycerol acylhydrolases, EC 3.1.1.3) demonstrate an ability to catalyze triglycerides hydrolysis to diacylglycerides, monoglycerides and fatty acids under aqueous conditions [1]. They also have esterolytic activity on distinct substrates [2]. These enzymes are ubiquitous in nature and are common in plants and animals as well as in micro-organisms such as bacteria, yeasts and fungi [3], however, bacterial lipases are more economical and stable [4], and gain importance for biotechnology, as they are easy to isolate, simple in genetic manipulation, possibility of high yield production, absence of seasonal variations and growing on low priced media [5].

Naturally, most of Bacterial lipases are glycoproteins, some of their extracellular lipases are lipoprotein. Bacterial lipases are diverse in their properties, such as, thermo-stability and specificity. Over the past two decades, interest in microbial lipase has increased steadily due to its benefits in biotechnology and industrial sectors, such as drugs, cosmetic products, agro chemicals, raw materials, detergents, textiles, biodiesel and oils, the synthesis of fine chemicals and new polymers and the

*Corresponding author e-mail: dr_nashwa167@yahoo.com (Nashwa A. H. Fetyan)
ismailm772003@yahoo.com (Ismail M. Ismail).
Receive Date: 27 November 2022, Revise Date: 05 April 2023, Accept Date: 20 April 2023, First Publish Date: 20 April 2023
DOI: 10.21608/ejchem.2023.177143.7237

production of single-cell protein as well as in wastewater treatment [6]. Some bacterial species such as: Achromobacter sp., Alcaligenes sp., Arthrobacter sp., Pseudomonas sp., Staphylococcus sp. and Chromobacterium sp. have been exploited for the production and manufacturing of lipases [7]. Due to the difference of the cell wall structure in Gram-negative and Gram-positive bacteria, the export of lipases and import of long-chain fatty acids may differ. For instance, in Gram-negative bacteria, long chain fatty acid transporter proteins have been identified for carrying the long-chain fatty acids to the cells. However, such transporters have not yet been identified for the Gram-positive bacteria but presumably exist [8].

Biodiesel is a promising renewable energy source to petroleum-based diesel fuels and consider as one of the most important biofuels due to its high biodegradability, no toxicity as well as sustainability. In addition, as its low CO, NOx, sulfur and particulate matter emission, its combustion emission profile is favorable [9]. Enzymes are better catalysts for the production of biodiesel as enzymes are more stable and their production is more convenient and safer [10]. One drawback of the lipase process is the high cost of the enzyme. Thus, the use of immobilized lipase is important to reduce production cost [11].

Immobilization is an advantageous method that improves the stability of the biocatalyst and provides for its repeated use and the easy separation of the catalyst from the reaction medium [12].

The purpose of the present study is to screen and identify potential lipase producing bacteria from potatoes manufacturing wastes, and optimize the production of lipase. Additionally, the partial purification of lipase from *Bacillus subtilis* and its using as micro-immobilized in biodiesel's production to serve as eco-friendly inexpensive biocatalyst, were investigated.

Materials and methods

Isolation and identification of lipase producing bacteria

Isolation of lipase producing bacteria was conducted from potatoes manufacturing residues obtained from ships factory at 6 October area, Giza, Egypt. The finely grinded residues (5 g) were suspended in 100 mL of peptone water. One mL of the suspension was serially diluted in peptone water and plated onto a TSA (tryptic Soy Agar) plate the colonies formed after incubation at 30°C for 24 h were examined and differentiated by Gram staining. A total of eighteen bacterial isolates were obtained and stored in nutrient agar slants at 4°C for further experiments.

Screening of the most potent Lipases Producing bacteria

The bacterial single colonies were screened for their ability to produce lipases by using solid media containing olive oil with phenol red as described by Singh et al., [13]. Visual inspection and measurement of the creation of a clearing yellow zone on the agar surface were used to determine the relative enzymatic activity

Lipolytic Enzyme Assay Using Olive Oil with Phenol Red Agar

The bacterial serially diluted samples were additionally plated with phenol red agar and cultured overnight at 37°C. The phenol red agar plates contain phenol red (0.01% w/v), olive oil (0.1% v/v), $CaCl_2$ (0.1% w/v), and agar (2% w/v) [13]. Phenol red has a pH end point of 7.3-7.4, after which a little reduction in pH causes the hue to change from pink to yellow. The color changes in red phenol were employed for the purposes of lipase activities, where lipase producing bacteria will turn the dye into yellow color [14] .

Morphological and biochemical characterization of lipase producing bacterial strains

The detailed procedure and methods of physiological and biochemical tests of the candidate were according to the Manual of Systematic and Determinative Bacteriology [15] and Bergey's Manual of Determinative Bacteriology, 9th edition [16].

Molecular identification of the most potent isolate: Amplification of the 16S-rRNA and lipase gene

The universal forward and reverse primers were used for amplification of the 16S rRNA gene fragment (8F: 5'- AGAGTTTGATCCTGGCTGAG-3' and 1492R: 5'ACGGCTACCTTGTTACGACTT-3') [17].

DNA template (20 ng), dNTPs (250 mM each), primers (25 pmol each), MgCl2 (2.5 mM), PCR buffer (5 µl of 5X), Taq DNA polymerase [1.5 U (Promega)], and the total volume was set to 25 µl using distilled water in the PCR reaction. Initial denaturation at 94°C for 3 min was followed by 35 cycles [94°C for 1 min, 55°C for 1 min, 72°C for 1.5 min], and a final extension cycle at 72°C for 7 min using an automatic thermal cycler (GeneAmp1 PCR System 9700, Perkin-Elmer). The electrophoresis apparatus was used to evaluate the PCR results on a 1% agarose gel. The Promega Wizard SV Gel and PCR Clean Up-system Kit Cat#A9282 was used to purify each PCR fragment, which was then cloned using the pGEM-T Easy cloning kit (Promega, Madison, USA). PCR was used to validate the successful insertion of white positive clones. One

verified positive was subjected to plasmid DNA isolation.

DNA sequencing

The Sequencing of cloned PCR fragment was performed using the ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems, USA) in conjunction with ABI PRISM (310 Genetic Analyzer). Approximately, 1.5 kb sequence was obtained. The NCBI GenBank database's BLASTn algorithm (www.NCBI.com/ BLASTn) was used to align sequence data.

Expression and purification under optimized expression conditions

PCR was used to amplify the lipase-coding sequence, which was then sub-cloned into the $P^{Gex-4t1}$ vector (Invitrogen). There are two specific primers, forward:

LG1CGC*GGATCC*ATGAAATTTGTAAAAA GAAGG-3' and the reverse: LG2 primer 5' CGC*GTCGAC*ATTAATTCGTATTCTGGCC-3') the primer sequences flanked by *BamHI* and *SalI* (italic) were designed. The $CaCl_2$ protocol was used to generate competent *E. coli* BL21 (DE3) cells, which were then transformed with the recombinant PGex-4t1+lipase vector.

The Glutathione Sepharose 4B GST-tagged protein purification resin kit, GE Healthcare, life sciences, for recombinant lipase expression and purification. An overnight culture of a clone bearing the recombinant vector PGex-4t1+lipase was inoculated into fresh LB broth medium containing ampicillin (100 mg/ml) and cultured at 37 °C until OD600 was reached 0.6.

IPTG (1mM) was used to induce the culture, which was subsequently re-incubated at 28 °C for further 3 h. The bacteria were extracted and re-suspended in lysis buffer after centrifugation at 4000 xg for 20 min (50 mM NaH2PO4, 300 mM NaCl, pH 8.0). The cells were frozen-thawed (–80 °C/ 37 °C) and then sonicated on ice for a few seconds to lyse them.

Sepharose beads were used to selectively purify clear lysates containing the GST-lipase fusion polypeptide under natural conditions. The expression and purity of lipase were determined using a 12 percent SDS-PAGE gel, and the concentration was determined using a Bradford test.

Lipase Production

Lipase Production and Isolation

The isolate with higher zone production on phenol red agar plate was inoculated in 15 ml of broth media containing the following composition: g/L (glucose 20, yeast extract10, peptone10, $CH_3COONa.3H_2O10$, MgSO40.09, MnSo40.03, $CuSO_4.5H_2O1.5$, KCL0.5 in addition to 5 ml Olive oil, 1000 ml Distilled water and pH 7.0). The inoculum flasks were then incubated overnight on a rotary shaker at 120 rpm at 37°C [18].

Production media: The lipase producing bacteria was grown in LB medium and transferred into Minimal Salt Media (per litre salt solution) NH_4 H_2PO_4 0.1gm%, NaCl 0.25gm%, $MgSO_4.7H_2O$ 0.04gm%, $CaCl_2.2H2O$ 0.04gm%, Tween 20- 2-3 drops and 2% (v/v) Olive oil, as a carbon source and the pH was maintained at 7.0.

The culture was incubated at 37^0C for 96 hours. The culture was centrifuged at 10,000 rpm for 20 min at 4oC after 96 h of incubation, and the cell free culture supernatant was employed as an extracellular enzyme source. Lipase activity was assayed by alkali titration using olive oil as substrate, as described by Jensen, with some modifications [19].

Lipase purification

In a chilled centrifuge, the crude lipase enzyme from the production medium was centrifuged at 8000 rpm for 20 min at 4 °C. Cell free supernatant was saturated with (30-990 %) ammonium sulphate with continuous stirring at 4°C followed by centrifugation at 14,000 rpm for 20 min [20]. Ammonium sulphate fraction was dialyzed against 50mM Tris-Chloride buffer (pH 7.5) for 6 hours at 4°C in a Dialysis bag .After dialysis, the concentrated enzyme was loaded onto a sephadex G-100 column. The enzyme was eluted from the column at a flow rate of 1ml/min. The fractions of the enzyme (5ml each) have been collected and the protein content measured using the Lowry method. Lipase assay was conducted with the highest protein-containing fractions.

Enzyme Assay activity

The activity of lipase was evaluated by titrimetric technique using olive oil as substrate. In a 50 mM sodium phosphate buffer pH 7.0, 10 % olive oil (v/v) was emulsified with 5 percent gum Arabic (w/v). 100 µl l of cell free culture supernatant was added to the emulsion and incubated for 15 min at 37ºC. After stopping the process, fatty acids were extracted using a 1.0 ml of acetone: ethanol solution (1:1). The amounts of fatty acids liberated were estimated by titrating with 0.05 M NaOH until pH 10.5 using phenolphthalein as an indicator [19**]**

Lipase activity (Units/ml) =

Volume of alkali consumed × Strength of alkali × 1000/Volume of sample × Time in min

One enzyme unit was defined as the amount of lipase that liberated 1 pmol of fatty acid per minute.

Immobilization of Lipase on Amberlite Resin

For immobilization the method of Kang and Rhee [21] was used: 4 g crude lipase preparation

was dispersed in 40 cm^3 sodium acetate buffer (50 mM, pH 3.5) over night with magnetic stirring at room temperature. After centrifugation at 8000g for 15 minutes at 4 °C, the undissolved material was discarded. Beforehand, the carrier (5 g of, Cation exchange resin Amberlite IRC-50 Sigma- Aldrich) were washed with 30 cm^3 of sodium acetate buffer (50 mM, pH 3.5) and stored at 10 °C overnight. After vacuum filtration, a continuous circulation of the enzyme preparation (40 cm^3) occurred at 10^0C through a column (1x 30 cm, Pharmacia) containing the pretreated supports, using a peristaltic pump. The flow rate was 3.0 cm^3 min^{-1}. To assess the lipase activity and protein concentration of the enzyme solution, samples were obtained at regular intervals. The immobilization process was monitored using the decrease in enzyme activity in the solution, which was stopped when activity reached a constant value. At different periods, the enzyme-resin complex was rinsed with buffer and dried in a vacuum desiccator over $P_2 O_5$, then stored at 4 °C [21].

Evaluation of the immobilization technique:

Protein loading (mg protein/g immobilizate), lipase activity (reported as U/g immobilizate and U/g immobilized lipase), and immobilization effectiveness were used to evaluate immobilization technique. The protein loading, P, was estimated as follows:

$$\mathbf{P = (c_0V_0 – c_fV_f) / Wg}$$

Where the concentration of protein in the lipase solution before (c_0) and after immobilization (c_f) are given in mg/mL, while the volumes of the solution, V_0 and Vf, are in mL.Wg is the weight of the wet immobilizates in g.

The efficiency of the immobilization techniques stated byWarmuth et al, [22], was estimated by comparing the lipolytic activity of the lipase solution before (E0) and after immobilization (Ef), using the relation

$$\mathbf{\eta = (E0V0 – EfVf) / V0E0 \times 100}$$

The activities are given in U/mL, and the volumes are in ml.

Characterization of Free and Immobilized Lipase

The optimum temperature for the purified lipase activity was determined by enzyme assay at different temperatures (20 – 70 °C). The effect of temperature on lipase stability was determined by incubating the enzyme solution at different temperatures (20–90°C) for 30 min. The residual activity was determined, after centrifugation, under standard assay method, [23].

The activity of purified lipase was examined within the pH range of 3.0-10.0. The lipase activity was measured as described by Jensen, [19]. The effect of pH on lipase stability was determined by incubating the lipase fraction in various buffer solutions ranging from 3.0 to 11.0 for 24 h at room temperature. After the incubation period, the residual activity was determined, after centrifugation, under standard assay method [19].

Effect of metal ions on lipase activity

For determining the effect of metal ions on lipase activity, the purified enzyme was pre incubated with 1 mM of Ca^{2+}, Mg^{2+}, Cu^{2+}, Fe^{2+} and Zn^{2+} for 1 h at 37°C and the residual activity was determined by using olive oil as substrate, according to Zouaoui et al, [24].

Biodiesel production using the transesterification process

In this experiment waste cooking oil (sunflower 75% + soybean 25%) mixture was used. One liter sample size of waste cooking oil was heated to 60 ^{0}C to remove any free water and allowed to settle for 24 h before reacting with methanol. The transesterification process was performed using oil: methanol molar ratio (1:4), catalyst NaOH (1%)/ immobilized lipase (3.0 g) / l and shaken for 3 hours at 300 rpm. Samples were left overnight for settlement of different layers (fatty acid methyl ester and sediment layer).The biodiesel layer was separated from the sediment by centrifugation and FAMEs were extracted and analyzed through GC/MS. Determination of the free fatty acid content was done titrametrically. The titrant used was Sodium hydroxide with the phenolphthalein as an indicator and free fatty acid content was calculated [25**].**

The yield of the transesterification processes was calculated as a sum of the weight of FAME (fatty acid methyl ester) produced to the weight of cooking oil used, multiplied by 100[25**].** The formula is given as:

$$\mathbf{Yield\ of\ FAME} = \frac{\mathbf{Weight\ of\ fatty\ acid\ methylester}}{\mathbf{Weight\ of\ fat\ used}} \times \mathbf{100}$$

Gas Chromatography Analysis of Waste Cooking Oil (WCO)

The GC/MS analysis of waste cooking oil (WCO) was performed using a Thermo- Scientific, Trace GC Ultra / ISQ Single Quadrupole MS, TG-5MS fused silica capillary column (30m, 0.251mm, 0.1 mm film thickness). For GC/MS detection, an electron ionization system with ionization energy of 70 eV was used, Helium gas was used as the carrier gas at a constant flow rate of 1mL/min. The injector and MS transfer line temperature was set at 280 °C. The oven temperature was programmed at an initial temperature 50 °C (hold 2 min) to150 °C at an increasing rate of 7 °C /min. then to 270 at an increasing rate 5 °C /min (hold 2min) then to 310 as a final temperature at an increasing rate of 3.5 °C /min (hold 10 min). The quantification of all the identified components was investigated using a percent relative

peak area. A tentative identification of the compounds was performed based on the comparison of their relative retention time and mass spectra with those of the NIST, WILLY library data of the GC/MS system [26].

Fatty Acid Methyl Esters (FAMEs) Analysis (Biodiesel Products)

Five hundred milliliters of the reaction mixture were mixed with 1.0 mL isooctane for two min. Following centrifugal separation, the upper organic layer was collected and washed twice with distilled water and dried over anhydrous Na_2SO_4. The solvent was dried under N_2 steam and dissolved in 0.25 mL of CH_2Cl_2 [27**].** The previous GC condition of WCO analysis was applied. The prepared FAMEs were then analyzed using particular fatty acid methyl ester standards (methyl palmitate, methyl stearate, methyl oleate, methyl linoleate, and methyl linolenate; Sigma-Aldrich).

Physicochemical features of produced biodiesel

Biodiesel Properties according to fatty acid Profile Analysis of Waste cocking oil and derived biodiesel was carried out using the BiodieselAnalyzer© software (version 2.2, BRTeam, Karaj, Iran), according to the methodology described in the works of **[**28 and 29].

Statistical analysis

Statistical analysis was done by ANOVA test using Microsoft Excel program. The difference in values was indicated in the form of probability ($p < 0.05$) values.

Results and Discussion

Isolation of Lipase Producing Bacteria

A total of sixteen strains with lipase activity were isolated. A wide range of lipolytic activity was observed, ranging from a large clear zone (high lipolytic activity) to a small zone (low lipolytic activity). Different zone size in mm was given in **Table.1.** The microscopic examinations of the selective lypolytic bacterial isolates were shown in **Table. 2.**

Because of the change in pH of the medium as a result of the liberation of fatty acids, the isolate NI13 produced the most yellow zone on the Phenol red agar plate, indicating lipase production (**Figure 1**). Previously, Patel and Desai [5] have demonstrated the isolation of 41bacterial isolates obtained oil contaminated soil by primary screening on a Nutrient agar plate and out of them 20 isolates grown in the selective medium like Tributyrin agar plate, Phenol red agar plate, and Tween-80 agar plate were found to produce lipase.

Table 1 Screening of lipase-producing bacteria by measuring the yellow zone Produced in the phenol red medium

Bacterial Isolate Code	Zone size (in mm)
NI 1	14
NI 2	11
NI 3	16
NI 4	12
NI 5	9
NI 6	26
NI 7	19
NI 8	24
NI 9	15
NI 10	21
NI 11	24
NI 12	14
NI 13	30
NI 14	22
NI 15	17
NI 16	19

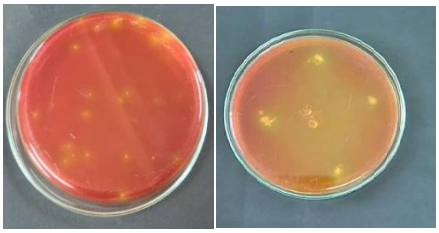

Fig. 1 Phenol red agar plates supplemented with 1%olive oil (substrate) showing lipase production as the pH indicator

Characterization and Identification of the most lipase producing isolate:

Molecular identification

The isolate NI13 was characterized both morphologically and biochemically. Light microscopic observation revealed that the isolate was a rod shaped Gram positive bacteria. Biochemical tests are shown in **Table 3.** According to The Bergey's manual of systematic bacteriology and considering the physiological and biochemical tests performed, The isolate NI13 was initially identified as *Bacillus sp*."

Identification of dominant bacteria

Because the strains were comparable to Bacillaceae in terms of morphological, physiological, and biochemical features (Tables1 and 2), a molecular approach was used to further characterize them. The results revealed that utilizing PCR amplification, the 16S rRNA gene of these isolates was amplified to a length of 1500 bp (Figure 2). Sequence data of the isolate deposited in the GenBank database accession number MN368195. The results showed that our isolate had the highest identity with *Bacillus subtilis* 16S rRNA gene sequence homology.

Table 2 Microscopic examination of selective lypolytic bacterial isolates

Bacterial Isolate Code	Cell shape	Motility	Gram reaction	ore formation
NI 1	Rod	motile	positive	+
NI 2	Short- rod	motile	negative	+
NI 3	cocci and form in grape-like cluster	non-motile	Positive	-
NI 4	Rod	motile	positive	+
NI 5	Short- rod	motile	negative	+
NI 6	Rod	motile	positive	+
NI 7	Rod	motile	positive	+
NI 8	Rod	motile	positive	+
NI 9	Rod	motile	positive	+
NI 10	Rod	motile	positive	+
NI 11	Rod	motile	positive	+
NI 12	Short- rod	motile	negative	+
NI 13	Rod	motile	positive	+
NI 14	Rod	motile	positive	+
NI 15	Rod	motile	positive	+
NI 16	Rod	motile	positive	+

Table 3 Biochemical tests of most efficient lipase producing bacterium (NI13).

Biochemical tests	Isolate No. NI13
Gram staining	+
Voges-Proskauer	-
Motility	+
Catalase	+
Oxidase	-
Citrate	+
Nitrate	+
Starch hydrolysis	+
Casein	+
Gelatin	+
Indole	-
Glucose	+
Mannitol	+
Lactose	+
Sucrose	+
Fructose	+

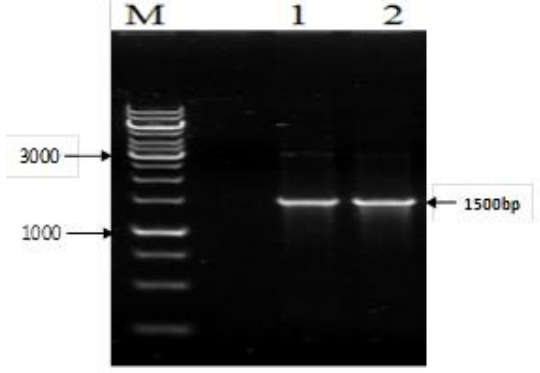

Fig. 2 PCR amplification of 16s rRNA. M: 1Kb DNA ladder; 1, 2: PCR product of 16s of *B. subtilis* NI13.

The identified bacteria are Gram-positive bacteria that excrete extracellular enzymes and they are beneficial for the bacterial population. The presence of any signaling molecules such as protein or lipid serves as the substrate or inducer for the increased secretion of both the lipase and the protease by the *Bacillus spp*. [14].*Bacillus subtilis*, *B. amyloliquefaciens*, and *B. licheniformisare* widely exploited for the purpose of protein production due to the immense fermentation nature, very high product production, and the very low level of toxic by-products [14].

Cloning and Expression of lipase in *E. coli* BL21

Using PCR, a lipase encoding gene was amplified from *B. subtilis* NI13; the lipase gene sequence was *identified* and submitted to Gen Bank (accession no. MN238705). The putative lipase gene sequence has a 639-bp open reading frame (ORF). The deduced protein has 212 amino acid residues and a molecular weight of 22.819 kDa (**Figure 3).**

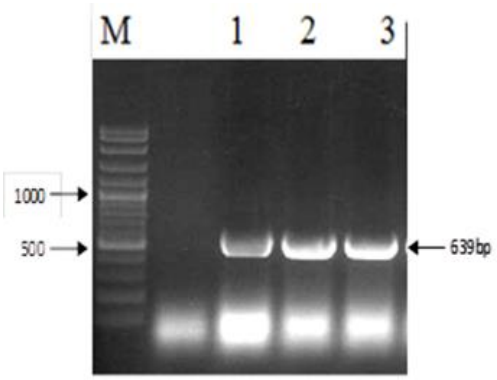

Fig. 3 PCR amplification of lipase gene. M: 100bp DNA ladder; 1, 2, and 3: PCR product of lipase gene of *B. subtilis* NI13.

The amplified PCR product (lipase gene 639 bp) was purified by QIAquick Gel Extraction Kit (Cat. # 28704), Qiagen. Subsequently, the purified lipase gene amplicon was mixed with the PGEM-T easy vector, Promega in the presence of ligase, after incubation for 1 h at room temperature, 10 µL of the ligation reaction was transformed into Top10 competent cells.

Clones were selected on ampicillin LB plates and recombinant plasmid was isolated from positive clones using GeneJET Plasmid Miniprep Kit (Cat. # K0502).

Recombinant plasmid was confirmed through digestion with restriction enzymes *BamH1* and *Sal1* (**Figure 4**), the lipase gene was sub-cloned into PGex-4T1 expression vector in frame with the GST-tag

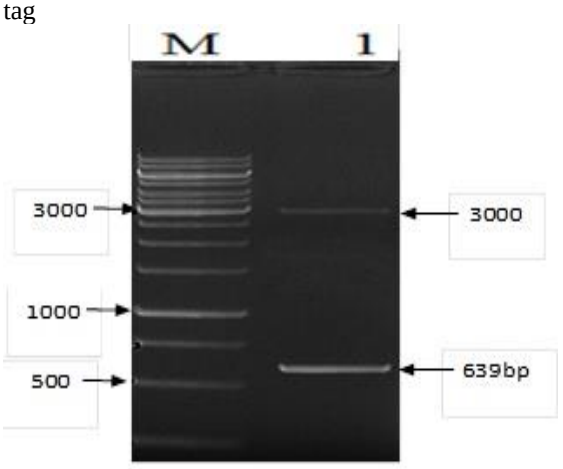

Fig.4 1% agarose gel confirms the Plasmid recombination through restriction enzymes digestion by *BamH1* and *Sal1*restrictionenzymes.

The positive recombinant clones were re-transformed into BL21 (DE3) cells for expression of the cloned lipase gene. Protein apparent bands were clearly observed after separating the total extracted proteins on SDS-page and staining with Commassie brilliant blue. SDS-page analysis visualized a major protein band at ~ 49KDa (~ 23KDa lipase + ~ 26KDa GST) (**5**).

Production and Purification of native Lipase from *Bacillus subtilis* MN238705

The crude enzyme (500 ml) produced after mass production showed a lipase activity of 20.66U/ml with a specific activity of 2.46U/mg (Table 3.2). *B. subtilis* MN238705 lipase was purified from the culture supernatant at 4°C and the purification results are summarized in Table 4. Ammonium sulphate precipitation up to 80% exhibited maximum activity beyond which showed a marked decrease in lipase activity. The enzyme obtained after dialysis showed a specific activity of 6.0 U/mg with 2.43-fold purification and 73.82 % enzyme recovery. The enzyme was further purified on a gel exclusion sephadex G-100 column. The elution pattern from sephadex G-100 column showed a single peak with lipase activity of 12.52 U/ml (Table 4 and Figure 7). The pooled active fractions of gel filtration showed a significant increase in specific activity from 6.00 U/mg to 23.19 U/mg which indicates that most of the unwanted proteins have been removed (Figure 8). The enzyme was purified to 9.45 fold with an overall lipase recovery of 60.5 %. [30] Has reported two-step purification protocol for *Staphylococcus sp*. CJ3 lipase and resulted in higher specific activity and 32% lipase recovery.

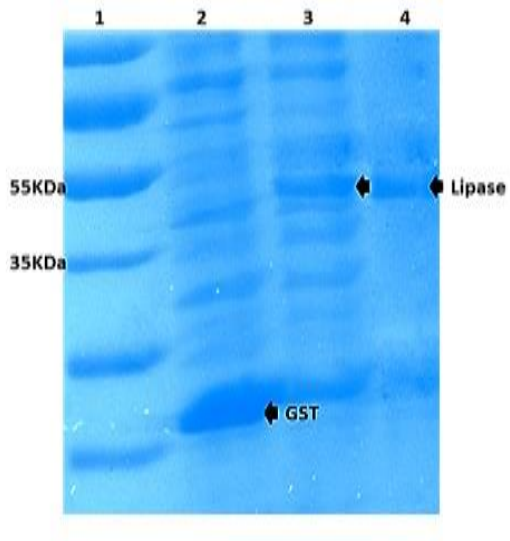

Fig. 5 12% SDS-page shows the expression of lipase in clear lysates of transformed BL21. 1: protein ladder; 2: clear lysate of BL21 transformed with non-recombinant PGex-4T1 expression vector; 3: clear lysate of BL21 transformed with recombinant PGex-4T1-lipase, 4: partially purified recombinant lipase fused with GST.

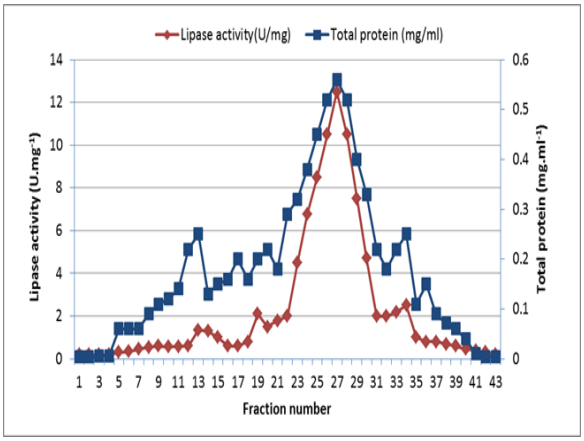

Fig. 6 Protein and enzyme activity profile of fractionsof Sephadex G-100 column chromatography of the dialyzed lipase of *B. subtilis* MN238705

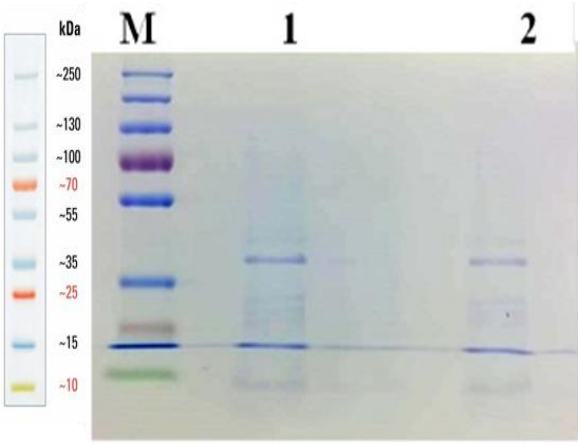

Fig. 7 SDS-PAGE of *Bacillus subtilis* isolate native lipase at different stages of purification. Lane M: Thermo Scientific™ PageRuler™ Plus Prestained Protein Ladder; Lane1:contains Ammonium sulphate precipitation; Lane 2: Sephadex G-100.

Activities of immobilized lipase

Immobilization of Microbial Lipase: Choosing an appropriate enzyme immobilization technique is critical. The bounding ability of the carrier is one of the most important indicators of enzyme immobilization, which is measured by the residue activity of the enzyme solution **[**22**].** As shown in Table 5, Protein loading capacity and immobilization yield, respectively, were 13.2 mg/g support and 81.77 %. Lipase enzyme should be immobilized to increase its stability, recovery, and reusability [31]. Support binding can be physical or chemical, involving weak or covalent bonds. The purified lipase was immobilized by the physical absorption method as it is simple and economical. The use of Amberlite resin IRC50 to immobilize an enzyme increased its biocatalytic properties, such as stability and reusability, as well as providing a greater surface area for high enzyme loading **[**32, 33**].** For free and immobilized enzymes, the optimal conditions for maximum enzyme activity differ depending on the type of support, method of activation, and method of immobilization **[**34**].** Activities of immobilized lipase were measured at various pH and temperatures values.

Characterization of Lipase from *Bacillus subtilis* MN238705

As indicated in Figure 8 , the maximum enzyme activity of free enzyme was obtained at 37 °C , while the immobilized lipase's optimal temperature was 45°C, which was much higher than that of the free enzyme. Moreover, the immobilization of the enzyme improved its stability against high temperatures recording an increase in relative activity % by 1.2 and 2.4 folds at 50 and 60°C than the free enzyme, respectively. Never the less, the activity of the immobilized enzyme recorded as twice as that of the free form at extreme temperatures of 20 and 60°C, as shown in Figures (8 a and 8b). The immobilized enzyme had greater thermal stability at all temperatures investigated as compared to the free enzyme, which could be attributed to the interaction of lipase with the support that stabilizes the enzyme's structure and improves the protein's resistance to thermal denaturation [35**,** 36 and 37**]**. For the effect of pH values on lipase activity , the immobilized lipase's optimal pH was 8.0, while the free lipase was 7.0. Immobilized lipase also had higher activity than free lipase, particularly at pH higher than 7.0. The optimal pH value of free lipase shifted one unit to the alkaline region after binding on the support. On the other hand the immobilized enzyme recorded higher relative activity % by 1.2 to 1.3 folds than the free form along variable acidic and alkaline pH values in the stability test, while recorded increase by 1.3 to 1.4 fold in relative activity % under activity test at extreme pH values of pH 3 and 10, as shown in Figures **(8c and 8d).**

Table 4 Summary of the purification procedure of lipase from *Bacillus subtilis*MN238705

Purification step	Volume (ml)	Lipase Activity (U/ml)	*Total activity (U)	**Total protein content (mg/ml)	*** Specific Activity (U/mg)	**** Fold purification	**** Yield (%)
Crude enzyme	500	20.63±1.12	10330±55.59	8.41± 1.04	2.46±.19	1±0.00	100
Ammonium sulphate precipitate	60	15.23±0.65	913.8±38.70	2.54±0.11	6.20±0.15	2.43±0.28	73.72±7.24
Sephedex G -100	60	12.52±0.55	750.0±32.8	0.560±0.02	23.185±0.8	9.45±1.09	60.5±1.78

*Total activity: Enzyme activity in given volume (IU)**Total protein: mg/ml., ***Specific activity: Enzyme activity per unit protein concentration (IU/mg), ****Purification fold: increase in specific activity., *****Percent recovery is remaining protein concentration as % of the initial protein concentration.

The correlation coefficient calculated from results showed strong negative effect of temperature variability on relative activity % for both free and immobilized enzyme forms under stability test, as shown **in Figure (9)**. Lipase's application range in severe conditions was expanded by immobilization, to accelerate hydrolysis and esterification [38, 39]. Bacterial lipases generally have a neutral or alkaline pH **[40, 41]**. However, in many cases, maximal activity was recorded at a higher pH (>7.0) [42, 43, 18, 44]. The optimum physical conditions for *B. megaterium*AKG-1 extracellular lipase were 55°C and pH 7.0 [44**]**. While for Lipase produced by the *Bacillus sp*. MPTK 912 the optimum pH was 8.0 and the optimum temperature was 35°C [45]. Maximum lipolytic activity of *Pseudomonas aeruginosa* KM110 extracellular lipase was exhibited at 45°C where enzyme stably was also maintained [46]. It could be deduced that the immobilization of the enzyme protected it from deterioration specifically under extreme temperatures (20 and 60°C) and pH (3 and 10) confirmed by stability and activity results.

Table 5 Specific activities of Enzyme-Resin complexes and immobilization yields

Immobilization Yield (%)	Protein loading mg/g support	Complex hydrolysis Activity, C (U./g)	Theoretical activity immobilized [a], A – B (U./g)	Resin
81.77±1.22	13.20± 0.8	3614.234±62.7	4420± 6.54	IRC50

Note: A, specific activity of the initial solution; B, specific activity of the residual solution; C, Specific activity of the enzyme-resin complex.

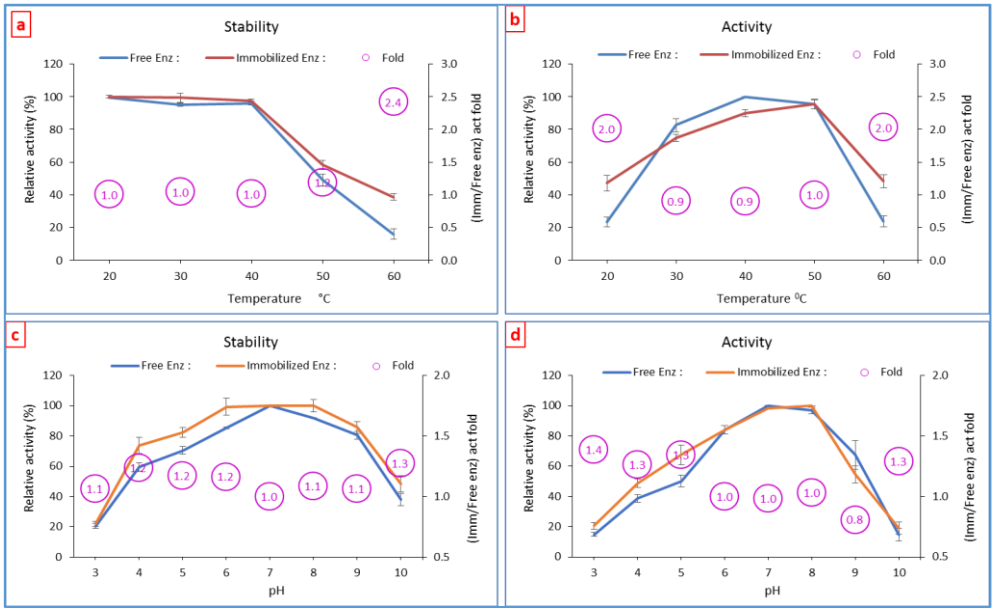

Fig. 8 Effects of temperature and pH on stability and activity of the of free and immobilized Enzyme.

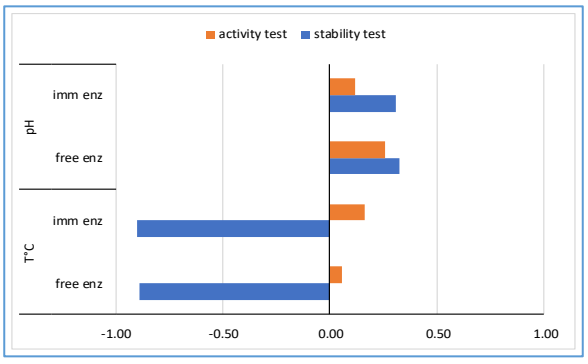

Figure (9): Correlation coefficient for Temperature (T° C) and pH values against activity and stability test relative activities %.

Effect of metal ions on immobilized lipase activity ; The enzyme activity increased by 127.42 % and 105.2 %, in presence of metal ions Ca2+ and Mg2+ respectively, suggesting the requirement for metalloprotein **(Figure 10).** The importance of these ions as lipase cofactors has been reported [21]. Metal ions play a key link in the binding between the enzyme and the substrate, contacting with both and holding the substrate and the enzyme's active site together. Moreover, the role of calcium ions, in the binding process to alter the active site's position specificity has been reported by several authors [47]. However, a decrease in relative activity was observed using Cu2+, Fe2+, Zn^{2+} with about 41.44,

28.36%, and 18.60%. This metal ion inhibitory effect may be due to a change in the solubility and the behavior of the ionized fatty acids at interfaces, or the change in the catalytic properties of the enzyme itself [48]. A similar inhibitory effect of Cu2+, Fe2+, Zn^{2+} metal ions on lipase from *B. methylotrophicus* PS3 was reported by [49].

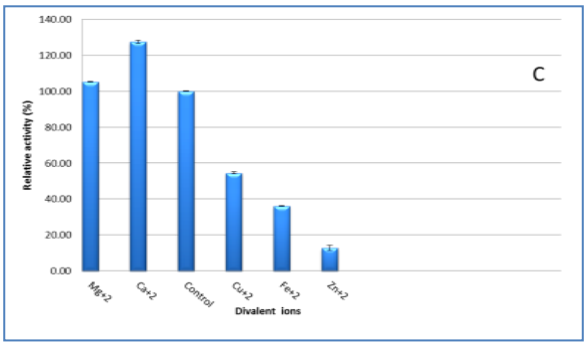

Fig. 10. Effect of divalent ions on the activity of *B. subtilis* MN238705 lipase.The control has no divalent ions.

GC-MS analysis of cooking oil (WCO) and its biodiesel product.

The application of lipase in methanolysis was investigated. The immobilized lipase was used in the transformation of waste cooking oil (WCO) from food waste. Application of lipase in methanolysis was investigated. The immobilized lipase was used in the transformation of waste cooking oil (WCO) from food waste.

In this study, biodiesel was produced under the following transesterification conditions: Reaction temperature: 45°C; catalyst concentration: 5.0 wt. %; reaction time: 6 h. The molar ratio of methanol to oil is 4:1, yielding 90.5 percent biodiesel.

In biodiesel production, Gas-chromatography-mass spectrometry studies are required to clarify the compounds contained in pure and used frying oils, as well as biodiesel produced, and are useful in determining biodiesel quality [50-52]. The conversion of WCO to biodiesel was determined using gas chromatography (GC). As standards, the standard methyl esters of stearic, oleic, and linoleic acids were used. The three fatty acid methyl ester mass spectra are shown in **Figure 11, 12,** and **13**.

The area percentage of WCO to biodiesel was calculated and interpreted with the help of peak integration software GC solution (Shimadzu cooperation).

The principal components of the WCO and its biodiesel product were determined by GC-MS (**Figure 14 and 15)**, and their chemical composition are described in **Table 6**.

As illustrated in **Table 6,** Stearic acid, methyl ester (23.57± 1.954%), oleic acid, methyl ester (15.73±1.372%), and palmitic acid, methyl ester (14.27±0.775%) are the main components of waste cooking oil (WCO), While the components of the used WCO are converted to biodiesel components during the methyl esterification process. Oleic acid, methyl ester (41.574± 3.3%) is the most abundant component in the WCO biodiesel sample, followed by linoleic acid, methyl ester (19.510±1.19%) and Palmitoleic acid (14.204±0.95). Total fatty acid methyl ester in the WCO biodiesel sample was 87.39%.

The fatty acid profile has been considered as a suitable biodiesel quality indicator **[**53**].** The contents of the C16 and C18 of biodiesel (as percentages of total FAMEs) are used to assess the oil/biodiesel productivity; this correlates to an occasional degree of unsaturation and is preferred for biodiesel production **(**54,55). In recent years, waste oil is becoming increasingly popular for biodiesel synthesis due to its high content of free fatty acids (FFAs) and low production costs **[**53].

Finally, the physicochemical features of WCO and produced biodiesel were analysed as cetane number (CN) 55.47, Cold Filter Plugging Point (CFPP) (◦C) - 2.568, kinematic viscosity (υ) (mm2 •s -1) 5.10, and density (g•cm−3) 0.87 (**Table 7**).

The cetane number (CN) is one of the first indicators of fuel quality and is calculated according to ASTM D 613.It is a well-known fact that higher cetane numbers are associated with shorter ignition delay times and vice versa [56]. The density of biodiesel is also another factor that influences atomization efficiency; it is depends on the alkyl ester content and the amount of alcohol remaining.

The lowest temperature at which crystal formation in biodiesel is visible as a cloudy suspension is known as the Cold Filter Plugging Point (CFPP) **[**57**]**. It depends mainly on the saturated fatty acid percentage; high levels of those acids enhance the freezing temperature and provide a high CFPP [58**],** causing the biofuel to solidify and making it more suitable for use in warmer climates.

The physicochemical properties of WCO-derived biodiesel met the European biodiesel standards of kinematic viscosity, cetane number, density, and iodine value.

Our findings are consistent with those of [59], who studied biodiesel production from waste vegetable oils and reported that a mixture (sunflower 75 percent + soybean 25 percent) waste oils was superior in biodiesel production, with a fatty acid, methyl ester content of 91.03 percent, the second was cotton waste oil at 89.56 percent, followed by sunflower waste oil at 86.92 percent. The significance of this endeavor is to reap the benefits of local environmental wastes as renewable energy sources.

Table 6 GC/MS chromatogram of Fatty acids methyl ester of waste cooking oil (WCO) and its biodiesel product

Fatty acid (% total FA)	WCO	Biodiesel
Oceanic acid ,2-butyl ester	5. 52± 2.185	00.00
1,4dimethyladamantane	3.47± 0.185	0.538±0.064
Lauric acid (C12:0)	4.09± 1.047	5.511±0.856
Myristic acid (C14:0)	9.55± 1.064	10.046± 1.075
Palmitic acid (C16:0)	14.27± 0.775	14.204±0.950
Palmitoleic acid (C16:1)	0.72± 0.151	3.00±0.155
Stearic acid (C18:0)	23.57± 1.954	4.387±0.998
Oleic acid (C18:1)	15.73± 1.372	41.574± 3.340
Linoleic acid (C18:2)	8.91± 0.733	19.510±1.196
Linolenic acid (C18:3)	1.48± 1.331	0.432±0.120
Arachidonic acid (C20:4)	3.64 ± 0.450	0.770±0.147
Erucic acid (C22:1)	8.57± 0.253	0.597±0.056
Σ C16	14.99± 0.927	17.204±1.107
Σ C18	49.69± 5.392	65.874±5.534
Σ C20	3.64 ± 0.450	0.77±0.1470
Σ MUSFA	25.02± 1.778	44.574±3.550
Σ PUSFA	24.03± 2.515	20.88±1.641
TUSFA	39.17 ±4.346	65.385±4.930
TSFA	61.83± 4.841	34.6043±3.664

**MUF, monounsaturated FA; PUFA, polyunsaturated; TUSFA, total unsaturated FA and TSFA, total saturated FA

Table 7 Some fuel properties of WCO-derived biodiesel

Parameter	**DU**	**SV**	**IV**	**CN**	**LCSF**	**CEPP**	**KV**	**Density at 25°C**
Biodiesel from WCO	58.53±2.04	211.91±2.48	73.74±1.48	55.47±0.78	4.43±1.15	-2.568±0.65	5.10±0.34	0.865±0.28

DU: degree of unsaturation; SV: saponification value; IV: iodine value; CN: cetane number; LCSF: long-chain saturated factor; CFPP: cold filter plugging point; Vis: Viscosity KV: Kinematic Viscosity ($mm^2 \cdot s^{-1}$).

As a result, the lipase Amberlite IRC-50 complex delayed the denaturing effect of increased pH and/or temperature, as indicated by a shift in the optimal pH/temperature for activity. As indicated by its highest percentage content of fatty acid, methyl ester (87.39%), the biodiesel produced from the investigated waste oils suggests that the waste oil has a strong potential for biodiesel production.

Thus, immobilized lipase proves to be a versatile biocatalyst, as it accepts all of the various fatty acids found in the WCO.

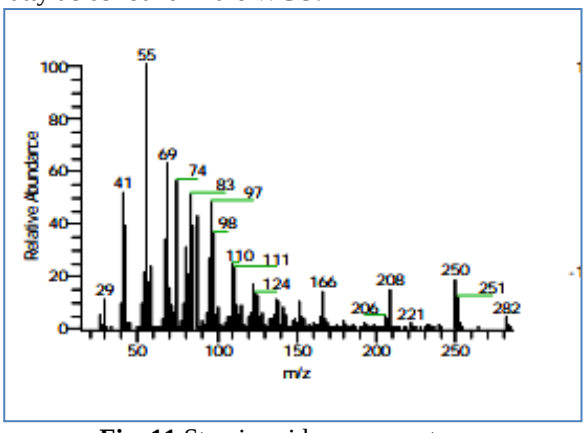

Fig. 11 Stearic acid, mass spectrum

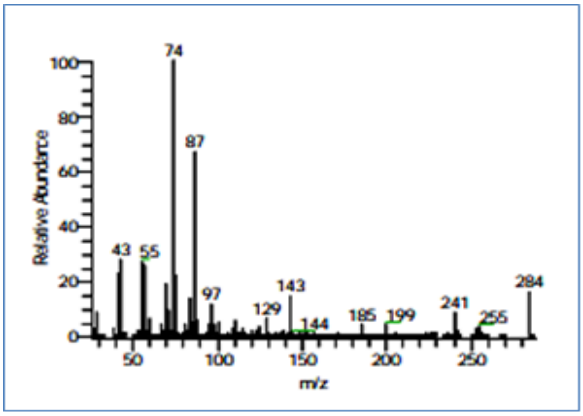

Fig. 12 Oleic acid, mass spectrum

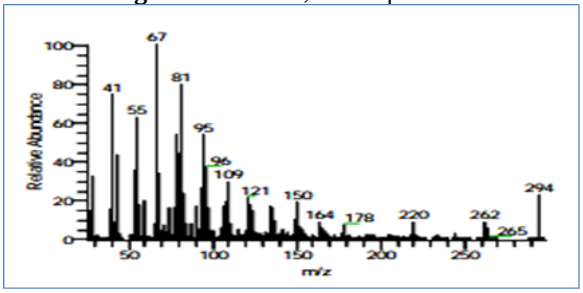

Fig. 13 Linoleic acid, methyl ester mass spectrum

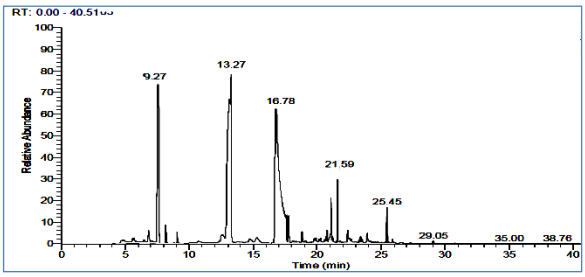

Fig. 14 The GC-MS chromatogram of (WCO).

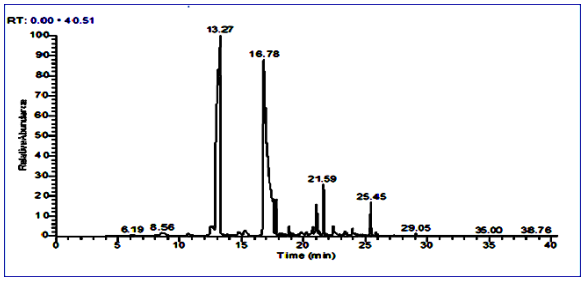

Fig. 15 The GC-MS chromatogram of biodiesel obtained from (WCO).

Conclusions

Lipase of *Bacillus subtilis* MN238705 is an extracellular lipase- having molecular weight of 22.819 kDa, whose optimum temperature of 37°C and pH of 7.0, was found to be a metallo enzyme with $Ca2^{+}$ and Mg^{2+} activator. Purified lipase was successfully immobilized on Amberlite IRC-50 support. Extracellular lipase produced *Bacillus subtilis* MN238705 immobilization retained its activity at extreme temperatures and pH values, facilitating its application in cooking oil catalyzed transesterification for biodiesel production, whose physicochemical properties of kinematic viscosity, cetane number, density and iodine values were found to follow European biodiesel standards. The laboratory scale is an excellent starting point for large-scale production**.**

References

1. Payá-Tormo L., Rodríguez-Salarichs J., Prieto A., Martínez M.J. and Barriuso J. Improvement of the Activity of a Fungal Versatile-Lipase Toward Triglycerides: An in silico Mechanistic Description. Front. Bioeng. Biotechnol. 7:71.(2019). doi: 10.3389/fbioe.2019.00071.
2. Reihaneh B., Ben, A. , Burhan S., Martin P., Thomas P. C., Irina D. O. Looking for lipases and lipolytic organisms in low-temperature anaerobic reactors treating domestic wastewater,Water Research 212,(2022). . https://doi.org/10.1016/j.watres.2022.118115
3. Kumar S., Kikon K., Upadhyay A., Kanwar S., and Gupta R **.**Production, Purification, and Character ization of Lipase from Thermophilic and Alkaliphilic *Bacillus coagulans* BTS3, Protein.Expr.Purif., vol. 41, no. 1, pp. 38–44(2005) . DOI: 10.1016/j.pep.2004.12.010
4. Chandra P., Enespa Singh R. Microbial lipases and their industrial applications: a comprehensive review. Microb Cell Fact 19, 169 **(2020)**. https://doi.org/10.1186/s12934-020-01428-8.
5. Patel P. and Desai B. Isolation, identification and production of lipase producing bacteria from oil contaminated soil. BMR Microbiology, 4(1). 1-7(2018).
6. Nadeem U., Muhammad D., Hidayatullah, M. S., Ayşegül TaylanÖzkan, Sami ullah, M. Q. Screening identification and characterization of lipase producing soil bacteria from upper dir and Mardan Khybe Pakhtunkhwa, Pakistan, International Journal of Biosciences, Vol. 6, No. 2, p. 49-55, (2015)
7. Sardessai Y.N. and Bhosle S. Industrial potential of organic solvent tolerant bacteria.BiotechProg; 20(3):655–6 **(2004)**.
8. Salvador Lopez J.M., Van Bogaert I.N .Microbial fatty acid transport proteins and their biotechnological potential. Biotechnology and Bioengineering.;118(6):2184-201(2021) .
9. Umaiyambika N. A., Arunodhaya N., Baskar G., Vijay M., Renganathan S., Chapter 15 - Challenges and opportunities in large-scale production of biodiesel, Editor(s): Baskar Gurunathan, Renganathan Sahadevan,Biofuels and Bioenergy,Elsevier,2022,Pages 385-407, https://doi.org/10.1016/B978-0-323-90040-9.00033-3
10. Ali, S., Khan, S. A., Hamayun, M., & Lee, J .The Recent Advances in the Utility of Microbial Lipases: A Review. Microorganisms. 11(2) (**2023**)., . https://doi.org/10.3390/microorganisms11020510
11. Kim K.H, Lee Y. Environmentally-benign dimethyl carbonate-mediated production of chemicals and biofuels from renewable bio-oil. Energies, 10, 1790 (2017) . **https://doi.org/10.3390/en10111790**
12. Mulinari J., Oliveira J.V., Hotza D .Lipase immobilization on ceramic supports: An overview on techniques and materials. Biotechnol Adv. Sep - Oct; 42:107581 **(**2020**)** . doi: 10.1016/j.biotechadv.2020.107581.
13. Singh R., Gupta N. , Goswami V. K. , Gupta R. "A simpleactivity staining protocol for lipases and esterases," AppliedMicrobiology and Biotechnology. 70,. 6, pp. 679–682**,(2006**) .
14. Lee L. P., Karbul H. M., Citartan M., Subash C .B .Gopinath, Lakshmipriya T. and Tang T. Lipase-Secreting *Bacillus Species* in an Oil-Contaminated Habitat: Promising Strains to Alleviate Oil Pollution, BioMed Research

International(2015). https://doi.org/10.1155/2015/820575

15. Dong X .Z., Cai M. Y. Manual of Systematic and Determinative Bacteriology. Beijing, China: Science Press**(2001)**.
16. Buchanan R. E., Gibbons N. E. Bergey's Manual of Determinative Bacteriology. 9th. Baltimore, Md, USA: Williams & Wilkins; 1994. [Google Scholar]
17. Eden P.A., Schmidt T.M., Blakemore R.P., Pace N.R. Phylogenetic analysis of *Aquaspirillum magnetotacticum* using polymerase chain reaction-amplified 16S rRNA-specific DNA. Int. J. Syst. Bacteriol.;41(2):324–325**(1991)**. DOI:10.1099/00207713-41-2-324.
18. Patel M., Misery J., Desai S., Patel S. and Desai S. Isolation and Characterization of Lipase producing Bacteria from Vegetable Oil Spillage Site, International Journal of Current Microbiology and Applied Science, Volume 5, Number 8, : 214-232**(2016)**. . http://dx.doi.org/10.20546/ijcmas.2016.508.023
19. Jensen R.G. **(1983) "**Detection and determination of lipase (acylglycerol hydrolase) activity from various sources," Lipids, Vol. 18. Cited from; Tahany M El- Ashary. (1985). Studies on production, purification, properties and application of lipase produced by *Rhizopus delemar* [Unpublished PhD thesis]. Faculty of Agricultural, Cairo University.
20. Borkar P.S., Bodade R.G., Rao S.R., Khobragade C.N. Purification and characterization of extracellular lipase from a new strain: Pseudomonas aeruginosa SRT 9. Braz J Microbiol. 2009 Apr;40(2):358-66 (**2009**). doi: 10.1590/S1517-838220090002000028.
21. Kang S.T., and Rhee J .S. Effect of solvents on hydrolysis of olive oil by immobilized lipase in reverse phase system. Bitoechnol.Lett. 11; 37-42(1989).
22. Warmuth W., WenzigE., Mersmann A**.** Selection of a support for immobilization of a microbial lipase for the hydrolysis of triglycerides Bioprocess Eng. 12 (1995) 87 (1995).
23. Mohan T., Selva, A. ,Palavesam, and G. Immanvel. "Isolation and characterization of lipase-producing Bacillus strains from oil mill waste." African journal of Biotechnology 7.15 (2008).
24. Zouaoui. B., Bouziane A. and Bachir R. Isolation, purification and properties of lipase from *Pseudomonas aeruginosa* African Journal of Biotechnology.: 11(60: 12415-12421a.5, (2) ,48-55(2012).
25. El-Batal A. I, Farrag A. A., Elsayed M. A .and El-Khawaga A.M. Biodiesel Production by Aspergillus niger Lipase Immobilized on Barium Ferrite Magnetic Nanoparticles. Bioengineering, 3, 14;(2016) doi:10.3390/bioengineering3020014.
26. Abdel Kader E., Abo El – Enin S.A., El-Ibiari N.N., El-Diwani G. and Hawash S.I. Hydrothermal Liquefaction of Microalgae (*Spirulina Platensis*) under Subcritical Water Conditions for Bio-fuel Production. International Journal of Engineering and Innovative Technology (IJEIT).5:2**(2015)**. http:www.ijeit.com/Vol%205/Issue%202/IJEIT1412201508_07.
27. Daoud L., Kamoun J., Ali M.B., Jallouli R., Bradai R., Mechichi T., Gargouri Y., Ali Y.B., Aloulou A. Purification and biochemical characterization of a halotolerant *Staphylococcus sp*. extracellular lipase. Int J Biol Macromol. Jun;57:232-7**(2013)** . doi: 10.1016/j.ijbiomac.2013.03.018.
28. Ramírez-Verduzco L.F,Rodríguez-Rodríguez J.E., del Rayo Jaramillo-Jacob A. Predicting cetane number, kinematic viscosity, density and higher heating value of biodiesel from its fatty acid methyl ester composition. Fuel 2012, 91, 102–111**(2012)**.
29. Talebi A.F., Mohtashami S.K., Tabatabaei M.,Tohidfar M., Bagheri, A.; Zeinalabedini, M,Mirzaei, H. MMirzajanzadeh M, Shafaroudi S.M, Bakhtiari S. Fatty acids profiling: A selective criterion for screening microalgae strains for biodiesel production. Algal Res. 2013, 2, 258–267**(2013)**.
30. Kiran K.K., Chandra T.S. Production of surfactant and detergent-stable, halophilic, and alkali tolerant alpha-amylase by a moderately halophilic *Bacillus sp*. strain TSCVKK. Appl. Microbiol. Biotechnol. 77:1023–1031**(2008)**. DOI: 10.1007/s00253-007-1250-z
31. Schmitt-Rozieres M. , Vanot G., Deyris V., and Comeau L. Borago officinalis Oil: Fatty Acid Fractionation by Immobilized Candida rugosa Lipase. JAOCS, 76, (5):227-562(1999).
32. MINOVSKA V., WINKELHAUSEN E., and KUZMANOVA S. Lipase immobilized by different techniques on various support materials applied in oil hydrolysis.J. Serb. Chem. Soc. 70 (4) 609–624(2005).
33. Wang K., Zhao L., Li T.; Wang Q., Ding Z., Dong W. Selective Immobilization of His-Tagged Enzyme on Ni-Chelated Ion Exchange Resin and Its Application in Protein Purification. Int. J. Mol. Sci. . 24, 3864(2023). https://doi.org/10.3390/ijms24043864.
34. Souza P.M.P., Carballares D., Gonçalves L.R.B., Fernandez-Lafuente R., Rodrigues S. Immobilization of Lipase B from Candida antarctica in Octyl-Vinyl Sulfone Agarose: Effect of the Enzyme-Support Interactions on Enzyme Activity, Specificity, Structure and

Inactivation Pathway. Int. J. Mol. Sci. . 23, 14268 (2022). https://doi.org/10.3390/ijms232214268

35. Sani F, Mokhtar NF, Mohamad Ali MS, Raja Abd Rahman RNZ. Enhanced Performance of Immobilized Rhizopus oryzae Lipase on Coated Porous Polypropylene Support with Additives. Catalysts. .11(3):303 (2021). https://doi.org/10.3390/catal11030303
36. Bussamara R., Dall'agnol L., Schrank A., Fernandes K.F., Vainstein M.H. Optimal Conditions for Continuous Immobilization of Pseudozymahubeiensis (Strain HB85A) Lipase by Adsorption in a Packed-Bed Reactor by Response Surface Methodology. Enzyme Res. 2012:329178(2012). doi:10.1155/2012/329178.
37. Sun, J., Chen,Y., Sheng,J. and Sun,M. Immobilization of Yarrowia lipolytica Lipase on Macroporous Resin Using Different Methods: Characterization of the Biocatalysts in Hydrolysis Reaction. BioMed Research International, Article ID 139179, 7 pages(2015). http://dx.doi.org/10.1155/2015/139179.
38. Rodrigues R.C., Virgen-Ortíz J.J., Dos Santos J.C.S., Berenguer-Murcia Á., Alcantara A.R., Barbosa O., Ortiz C., Fernandez-Lafuente R. Immobilization of lipases on hydrophobic supports: immobilization mechanism, advantages, problems, and solutions. Biotechnol Adv. Sep-Oct;37(5):746-770(2019). doi: 10.1016/j.biotechadv.2019.04.003.
39. Costantini A., Califano V. Lipase Immobilization in Mesoporous Silica Nanoparticles for Biofuel Production. Catalysts . 11, 629. **(2021**). https://doi.org/10.3390/catal11050629
40. IQBAL, S. A. and Rehman, A. Characterization of Lipase from *Bacillus subtilisI*-4 and Its Potential Use in Oil Contaminated Wastewater. Braz. arch.biol.technol., vol.58, n.5: pp.789-797 **(2015)** . https://doi.org/10.1590/S1516-89132015050318
41. Lesuisse E., Schanck K., Colson C. Purification and preliminary characterization of the extracellular lipase of *Bacillus subtilis* 168, an extremely basic pH-tolerant enzyme. Eur J Biochem 216:155–160**(1993)**. DOI: 10.1111/j.1432-1033.1993.tb18127.x
42. Khyami-Horani H. Thermotolerant strain of *Bacillus licheniformis* producing lipase. World J MicrobiolBiotechnol; 12: 399-401**(1996)**.
43. Sharma R., Soni S.K0, Vohra R.M., Jolly R.S., Gupta L.K., Gupta J.K. Production of extracellular alkaline lipase from a *Bacillus* sp. RSJ1 and its application in ester hydrolysis. Ind J Microbiol.; 42: 49-54**(2002)**.
44. Sekhon A., Dahiya N., Tiwari R.P., Hoondal G.S. Properties of a thermostable extracellular lipase from *Bacillus megaterium* AKG-1. J Basic Microbiol.; 45(2): 147-154**(2005)**.
45. Mukeshkumar D.J., Rejitha R., Devika S., Balakumaran M.D., Immaculate N.R.A., Kalaichelvan P.T**.** Production, optimization and purification of lipase from *Bacillus sp*. MPTK 912 isolated from oil mill effluent. Adv. Appl. Sci. Res.; 3(2): 930-938**(2012)**.
46. Mobarak-Qamsari E., Kasra-Kermanshahi R., Moosavi-Nejad Z. Isolation and identification of a novel, lipase-producing bacterium, *Pseudomnas aeruginosa KM110*. Iran J Microbiol.;3(2):92-98**(2011**).
47. Matsumae H., Shibatani T. Purification and characterization of lipase from *Serratia marcescens* Sr41 8000 responsible for asymmetric hydrolysis of 3-phenylglycidic esters. J. Ferment. Bioeng. 77:152-158(**1994**).
48. Tripathia R., Singha J., kumar R., Band Thakur I .S. Isolation, Purification and characterization of lipase from *Microbacterium sp.* and its application in biodiesel production. Energy Procedia, 54, 518 – 529**(2014)**. https://doi.org/10.1016/j.egypro.2014.07.293
49. Sharma P., Sharma N., Pathania S., Handa S. Purification and characterization of lipase by *Bacillus methylotrophicus* PS3 under submerged fermentation and its application in detergent industry. J Genet Eng Biotechnol.;15(2):369-377(2017). doi: 10.1016/j.jgeb.2017.06.007.
50. European Standard EN 14103. (**2003a)**. European Committee for Standardization, Brussels, Belgium. https://www.cen.eu/Pages/default.aspx.
51. European Standard EN14105. (**2003b)**. European Committee for Standardization, Brussels, Belgium. https://www.cen.eu/Pages/default.aspx.
52. Nascimento F.F., Lazar A., Menezes A.N., Durans A.D.M., Moreira J.C., Salazar-Bravo J. The Role of Historical Barriers in the Diversification Processes in Open Vegetation Formations during the Miocene/Pliocene Using an Ancient Rodent Lineage as a Model. PLoS ONE 8(4): e61924**(2013)**. https://doi.org/10.1371/journal.pone.0061924.
53. Banani R., Youssef S., Bezzarga M., Abderrabba M. Waste frying oil with high levels of free fatty acids as one of the prominent sources of biodiesel production. Journal of Material and Environmental Sciences. 6(4):1178-1185**(2015)**.
54. El-Sayed AE-KB, Fetyan NA, Moghanm FS, Elbagory M, Ibrahim FM, Sadik MW, Shokr MS. Biomass Fatty Acid Profile and Fuel Property Prediction of Bagasse Waste Grown Nannochloropsis *oculata*. *Agriculture*.

12(8):1201(**2022**). https://doi.org/10.3390/agriculture12081201

55. Bhatia, S. , Gurav, R., Choi, Tae-Rim , Kim, H. j., Yang S., SongH., Park J., Park .,Han Y., Choi, Yong-Keun K., Sang-Hyoun Y.J.J., Yang Y. Conversion of waste cooking oil into biodiesel using heterogenous catalyst derived from cork biochar .Bioresource Technology. 302**(2020)**.. https://doi.org/10.1016/j.biortech.2020.122872.

56. Dagne H., Karthikeyan R. , Feleke S. "Waste to Energy: Response Surface Methodology for Optimization of Biodiesel Production from Leather Fleshing Waste", Journal of Energy, vol. 2019, Article ID 7329269, 19 pages**(2019)**. https://doi.org/10.1155/2019/7329269.

57. CEN ECfS-. EN 590:2009 - Automotive fuels - Diesel - Requirements and test methods. Organisation BS, **(2009).**

58. Knothe G., Krahl J., Van Gerpe J. The Biodiesel Handbook American Oil Chemist's society (AOCS) Press, Champagn, IL**(2005)**.

59. Zayed M.A., Abd El-Kareem M.S., Zaky N.H.S. Gas Chromatography-Mass Spectrometry Studies of Waste Vegetable Mixed and Pure Used Oils and Its Biodiesel Products. J. Pharm. Appl. Chem., 3, No. 2, 109–116**(2017)**. doi:10.18576/jpac/030204.