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Production and Purification of Lipase from *Pseudomonas* sp. AB2 with Potential Application in Biodiesel Production

Emad A. Abada[1,2]

[1]Department of Botany and Microbiology, Faculty of Science,
Helwan University, Ain Helwan, Cairo, Egypt.
[2]Department of Biology, Faculty of Science, Jazan University, Jazan, KSA.

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Lipase produced from microorganisms can be used in many industrial applications, such as detergent formulation, oil/fat degradation, pharmaceutical synthesis, biodiesel and cosmetics production. *Pseudomonas* sp. AB2 was isolated and identified by 16SrRNA from waste cooking oil (WCO). The lipase showed a molecular weight of 28.6 KDa by SDS–PAGE. The enzyme activity was highest after 24 h at 200 rpm agitation when ($4X10^4$ CFU) was used as inoculums. The maximum lipase production was observed at 35°C and pH 7. As the Tributyrine increase the enzyme activity decrease, while the yeast extract increase the activity with concentration higher than 0.5 g/l. The GC analysis of the WCO showed that the fatty acid profile of the oil was Palmetic (31.79%), Stearic (2.41 %), Oleic (65.8 %); while the total saturated acid was (34.2 %) and unsaturated acid was (65.8%). *Pseudomonas* sp. AB2 lipase, which exhibited a potential for catalyzing the biodiesel production, was further purified and immobilized on 20.4 mmol of Tetramethoxyorthosilicate (TMOS). The immobilized lipase from *Pseudomonas* sp. AB2 could be used as a biocatalyst for the application of enzyme-catalyzed biodiesel synthesis

Key words: Waste cooking oil, *Pseudomonas* sp., Lipase, TMOS, Biodiesel.

Microbial lipase (triacyl glycerol acylhydrolases, E.C.3.1.1.3) catalyzes the hydrolysis of oils/fats to form free fatty acids and glycerol, while the reverse reaction is also driven by lipase in low water content conditions[1]. Lipases are industrially useful versatile enzymes that catalyze numerous different reactions including hydrolysis of triglycerides, transesterification, and chiral synthesis of esters under natural conditions. Lipase can be obtained from various sources, such as plants, microorganisms and animals[2]. The rising interest in lipase is mainly due to the wide range of industrial applications of the enzyme, including detergent formulation, oil/fat degradation, pharmaceutical synthesis, cosmetics production, dairy and textile industries[3, 4]. The range of uses is because lipase shows activity with regard to hydrolysis, esterification, transesterification, interesterification, alcoholysis and acidolysis[4]. To date most lipase has come from microorganisms due to the low production cost. Several microorganisms, such as *Candida rugosa*, *Candida antarctica*, *Burkhoderia cepacia* and *Pseudomonas alcaligenes*, produce lipase efficiently, and this is now are commercially available[1].

Biodiesel is a fatty acid ester, which is synthesized from alcohols and variety of oils, animal fats or waste oil via a reaction catalyzed by acid, alkali or biocatalyst. The use of enzyme catalysts (lipases) in biodiesel production is being increasingly studied because of some advantages it has over chemical or thermal hydrolysis, because enzyme reactions require lower temperatures, thereby preventing the degradation of the products and reducing energy costs. Furthermore, enzymes

* To whom all correspondence should be addressed.
E-mail: emadm_abada@yahoo.co.uk

are biodegradable and are therefore less polluting than chemical catalysts. The disadvantages of enzyme catalysis are mainly its longer reaction time and the higher cost of the biocatalysts[5,6]. Biodiesel is a non-toxic, biodegradable, environmental friendly and non fossil fuel (renewable) that produces significantly lower emissions than petroleum-based diesel when it is burned, whether used in its pure form or blended with petroleum diesel. Importantly, biodiesel does not contribute to a net rise in the level of carbon dioxide in the atmosphere and leads to minimize the intensity of greenhouse effect and they are better than diesel fuel in terms of sulfur content, aromatic content, flash point and biodegradability[7-8]. An ideal lipase to be used in biodiesel production should have the following characteristics: able to utilize all mono, di, and triglycerides as well as high yield of free fatty acids, low product inhibition, high activity and yield in non-aqueous media, low reaction time, temperature and alcohol stable, reusability of immobilized enzyme, etc.[9]. For the search of lipases with potential biotechnological applications, various bacteria, yeasts, and fungi have been focused. Among bacteria, *Pseudomonas*, *Staphylococcus*, *Chromobacterium*, *Bacillus*, *Acinetobacter*, *Corynebacterium*, and *Streptomyces* have been extensively studied[10-12].

Recently, it has been found that enzymatic catalyst (immobilized lipase) can be used in transesteriûcation reaction. No byproduct, easy product removal, reusability without any separation step and lower operating temperature are the key advantages of this method[13]. However, it is found to be very expensive[14,15]. The enzymatic reaction is insensitive to water and FFA content in waste cooking oil[15,16]. There are different types of lipases that can be used as the catalyst such as: *Rhizaopus oryzae*, *Candida rugosa*, *Psuedomonas fluorescens*, *Burkholderia*, *Cepacia*, *Aspergillus niger*, *Thermomyces lanuginose* and *Rhizomucor miehei*[17]. Meanwhile, researchers have reported that immobilized lipase can be used many times without a serious loss of activity. Yagiz *et al*.[18] used immobilized lipase on hydrotalcite and zeolite catalyst and waste oils for biodiesel production. In this study, a production, purification, characterization and application of the lipase isolated from *Pseudomonas* sp. AB2 in biodiesel production.

MATERIALS AND METHODS

Isolation of the lipase producing strains

Samples of High lipid polluted soil (LS), waste Cooking oil (WCO) and waste cake of corn oil were collected in triplicate in a screw-capped sterile a dark glass jars. The samples were homogenized well, and then transferred to the sterile jars. Isolation of lipase producing bacteria was done according to[19] with some modifications**.** Ten grams of each sample was transferred to 500 ml conical flask containing 100 ml of sterile saline solution; 9 g/l NaCl. The flask was incubated at 30^{o}C on a rotary shaker at 200 rpm. Serial dilution 10^{-1} of this sample was inoculated on the nutrient agar medium. The plates were then incubated at 30^{o}C for 24 h. The grown colonies were purified as a single colony by streaked separately on new nutrient agar medium plates. The plates were incubated at 30°C for 24 h.

Screening of lipase production

The lipase producing bacterial isolates were surveyed many times on tributyrin-basal salt agar medium (g/l^{-1}) (Tributyrine 2, Arabic gum 2, Na_2HPO_4 5.57, KH_2PO_4 2.44, NH_4Cl 2, $MgCl_2$. $6H_2O$ 0.20, $FeCl_3$.$6H_2O$ 0.001, $CaCl_2$. $2H_2O$ 0.001, Yeast extract 0.50, $MgSO_4$ 0.001, Agar 20) (pH 7). The plates were incubated for 24 h at 30°C. Isolates that showed clear zones of tributyrin hydrolysis were identified as lipase producing bacteria[20]. The bacterial isolate with highest lipase activity was chosen for further study. The activity of the lipase enzyme was estimated in terms of mean diameters of clearing zones (mm) using a standard curve covering the range of 40 µg/ml to 1000 µg/ml, of the standard lipase enzyme (*Aspergillus niger* lipase) (Sigma).

16S rRNA identification of the potential lipase isolate

Genomic DNA was extracted from the lipase potential strain using protocol of Gene Jet genomoic DNA purification kit (Thermo). Isolation of the 16SrRNA gene was carried out by means of PCR with the primer pair 16SrRNA Forward (F) (5'AGAGTTT GATCCTGGCTCAG3') and 16SrRNA Reverse(R) (3'GGTTACCTTGTTACGACT T5'). PCR was performed in a 50 µl reaction mixture containing 25µl 2X Maxima hot Start PCR master Mix, 1µl (20 µM) of each of the primers F and R, 5µl Template DNA and 18µl water nuclease-free.

Amplification was performed with an initial denaturation (10 min at 94°C), followed by 35 cycles of denaturation at 94°C for 30 sec., Annealing at 65°C for 1 min, extension at 72°C for 1.5 min and a final extension at 72°C for 10 min. The amplified fragments were purified from agarose gels using GeneJET™ PCR purification Kit (Thermo). Finally we made sequencing to the PCR product on GATC Company (GATC Company, South Korea) by use ABI 3730 xl DNA sequencer by using F and R primers. Related sequences were retrieved from public databases using BLAST at the NCBI server (http://www.ncbi.nlm.nih.gov/ blast/).

Effect of the culture conditions on AB2 Lipase production

The following factors were studied to optimize the lipase production and activity by AB2 strain. The deduced optimal conditions resulted from each experiments were taken in consideration. At the end of each parameter, the lipase activity was estimated.

Incubation time, agitation speed and inoculums volume

According to[21], the *Pseudomonas* sp. strain AB2, was incubated on the tributyrin-basal salt agar medium at different incubation periods 0, 6, 12, 24, 36, 48 and 72 h, respectively. Also, the effect of agitation speed on lipase production was investigated at 0, 50, 100, 150 and 200 rpm. While, different inocula concentrations were applied at 0.1, 0.2, 0.4, 0.8 and 1.0 ml.

Incubation temperature and pH

The effect of temperature was investigated at temperatures of 15, 20, 25, to 60 °C. Also, the effect of pH on lipase production was investigated in the range of 3–9, these pH values were adjusted by using 0.2 M phosphate buffer (pH 7.0).

Substrate (tributyrine) concentrations and addition of activators

The effect of different concentrations of the substrate tributyrine was studied ranging from 1-10 g/l. Moreover, the effect of different concentrations of yeast extract and magnesium sulfate on the lipase activity has been studied. The concentrations of yeast extract were applied at 0.0, 0.5, 2.5 and 5.0 g/l, while concentrations for magnesium sulfate were applied at 0.0, 0.2, 0.5 and 1.0 g/l.

Lipase assay and protein estimation

Lipolytic activity determination was performed by spectrophotometric assay using *p*-Nitrophenyl-Laurate (pNPL) as substrate according to[22] with slight modifications.

The reaction mixture consisted of: 0.1 ml enzyme extract, 0.8 ml 0.05M phosphate buffer (pH 7.0), and 0.1 ml 0.01M pNPL in iso-propanol. The hydrolytic reaction was carried out at 60 °C for 30 min, after which 0.25 ml of 0.1M Na_2CO_3 was added. The mixture was centrifuged at 16,000×g for15 min and 25°C. The absorbance at 410 nm was determined. One unit of lipase activity was defined as the amount of enzyme that caused the release of 1 mol of *p*-nitrophenol (molar absorption coefficient 4.6 mM^{-1} cm^{-1}) from pNPL in 30 min under test conditions. The protein content estimation was carried according to[23]. The Bovine serum albumin (BSA) (Sigma) was used as a standard protein in a range of 30-400µg/ml.

Batch fermentation for maximum production of lipase by AB2 strain

The *Pseudomonas* sp. strain AB2 was cultivated in 10 L fermentor (Biotron Liflus SL) with a volume of 7 L. The basal salt-tributyrine medium was used as a culture medium and the growth of AB2 strain was done under optimum condition previously studied.

Purification of the lipase

Ammonium sulfate precipitation

Solid ammonium sulfate was added to the culture supernatant to 60% saturation. The mixture was kept at 4°C for 2 h under very slow stirring, and then the solid contents were centrifuged at 12,000 ×g at 4°C for 20 min. The supernatant was discarded, further solid ammonium sulfate was added to the precipitate up to 50% saturation, and the resulting mixture was kept at 4°C for a further 4 h. The resulting precipitate was collected by centrifugation at 12,000 ×g for 30 min. This precipitate was re-dissolved in 0.05 M sodium phosphate buffer (pH 7.0) and dialyzed overnight in the same buffer[24].

Fractionation on Sephadex G-100 gel chromatography

The dialyzed crude enzyme preparation was applied onto column packed with sephadex G-100 (Sigma, Germany) previously equilibrated with (0.2 M) phosphate buffer (pH 7.0), then eluted with

the same buffer. A column (2.5 X 80 cm) (Pharmacia) has been used. Five grams of sephadex G-100 were suspended into 500 ml (0.2 M) phosphate buffer at (pH 7.0) and allowed to swell overnight in the refrigerator. Sodium azide (0.02%) was added to prevent any microbial growth. The column was packed carefully by pouring the previously degased slurry of gel into vertical column partially filled with the same buffer. The addition of the gel was continued until a bed height of 70 cm in the column was attained. The column was connected to the buffer reservoir and the flow of the buffer was maintained at a rate of approximately 20 ml/h for two h to allow the settlement of the bed. The gel height was adjusted at 70 cm by the precise addition or removal of the extra gel. One ml of the crude enzyme was applied carefully to the top of the gel. It was allowed to pass into the gel by running the column. Buffer was then added without disturbing the gel surface and then the column was connected to the reservoir. Fourty fractions were collected (5 ml) and tested for their lipase activity and protein content to calculate the enzyme specific activity. The active fractions were pooled and concentrated by dialysis against sucrose crystals to achieve constant volume.

Gel Electrophoresis

The molecular mass of the purified enzyme was determined by sodium dodecyl sulfatepolyacrylamide gel electrophoresis (SDS-PAGE) as described by[25] using a 5 % (w/v) stacking and 12%(w/v) polyarcylamide resolving gels. Following electrophoresis, the gel was stained with Coomassie Brilliant Blue (CBB) R-250. A low range protein marker kit (Fermentas, USA) was used for the calibration.

Potential application of *Pseudomonas* sp. AB2 in biodiesel production

Immobilization of the (purified and partially purified) lipase produced by *Pseudomonas* sp. AB2

At room temperature, 20.4 mmol of Tetramethoxyorthosilicate (TMOS), 0.7 ml of distilled water, and 30 µl of 40 mM HCl were mixed in a test tube to form a homogeneous solution. This mixture was cooled to 4°C, then 4.5 ml of 100 mM phosphate buffer (pH 7.5) was added. Three handereed mg/ml of enzyme solution (pH 7.5) were mixed with the buffered solution. The resultant mixture was spread over the inner surface of petri dish before it gelled, and left for one day at room temperature. The hydrogels formed were dried *in vacuo* for one day, crushed in a mortar, and equilibrated for one day in a desiccator containing a saturated aqueous KCl solution (water activity, a_w = 0.84) at 30°C. This procedure yielded approximately 1.6 g of the immobilized lipases on a dry weight basis[26].

Determination of the physico-chemical properties and GC analysis of the WCO

The Saponification value, Total acid number, Iodine number, Density value of the WCO was determined according to the method described by[27]. Also, Fatty acid composition was determined by Gas Chromatography analysis (GC) in the Regional Center for Mycology and Biotechnology, Alazhar University. The oil sample was methylated before GC injection. The instrumental and chromatographic conditions of GC was: GC/MS system: SHIMADZU GC/MS-QP5050A, Column: DB1, 30 m; 0.53 mm, Carrier gas: Helium, Temprature program: 115°C (1 min) 200°C (1 min) at 10°C/min-240°C (2 min) at 5°C/min- 260°C(3 min) at 3.5°C/min, Injector temprature: 300°C and Detector temprature: 230°C. Identification of fatty acids contained in WCO was performed by comparison of retention time with fatty acids standard.

Production of biodiesel from waste (WCO) using trans-esterification process

According to[28], the transesterification reactions between WCO and alcohol were conducted in closed 15-mL batch reactors, with constant mechanical stirring, coupled to condensers in order to avoid alcohol loss by volatilization. This experiment was carried out to compare the biodiesel obtained by using different types of lipase (partially purified, purified and standard immobilized lipase) and KOH. In case of different types of biocatalysts (lipase), the trasesterification reaction condition were methanol/WCO molar ratio 3/1, enzyme concentration 5 %, reaction temperature 40°C, reaction time 6 h, and reaction shaking speed 400 rpm. While in case of KOH the trasesterification reaction condition were methanol/WCO molar ratio 3/1, KOH concentration 5 % of oil weight, reaction temperature 60°C, reaction time 3 h, and reaction shaking speed 250 rpm.

After the reaction is completed, glycerol was separated using separating funnel and the

reaction product was washed by equal volume of hot water for 3 or 4 times to remove the excess of methanol, glycerol and residue of WCO. The yield was dried for 24 h on the oven at 70°C to get constant weight. The transesterification percentage was calculated by determining the comparison of the weight of fatty acid and the weight of the resulted methylester. In all cases, samples (1 ml) were taken from the reaction mixture at specified times and centrifuged at 13000 rpm for 5 min to separate lipase powder and obtain supernatant which is diluted with equal volume of n-hexan for GC analysis.

RESULTS AND DISCUSSION

Screening and identification of strain producing lipase

Twenty five, 18 and 23 bacterial isolates were isolated on the nutrient agar medium from different sources including waste cooking oil, high oil polluted soil and waste cake of corn seed oil. The total 66 (coded as AR1 to AR66) bacterial isolates were subjected to 2 rounds for their lipase enzyme production by using tributyrin-basal salt agar medium. The enzyme activity was estimated in terms of mean diameters of clearing zones (mm). After 1st round, 7 of 66 bacterial isolates showed the highest lipase activity, while the isolate no 10 showed the most highest lipase activity after the 2nd round of study. Isolate no 10 was selected as a potential isolate for lipase enzyme production with activity of 21 U/ml (Fig. 1). In order to identify the isolate no 10, the genomic DNA of the isolate was extracted, purified and the 16SrRNA was sequenced in order to achieve the phylogeny. Search for similar sequences in the databases using BLAST at the NCBI server (http:// www.ncbi.nlm.nih.gov/ blast/) and subsequent alignment of the retrieved sequences indicated that the DNA sequence shows 97% homology with the 16SrRNA gene sequences of *Pseudomonas aeruginosa* PAO1, the strain was identified as *Pseudomonas* sp. strain AB2. A phylogenetic tree is presented in (Fig. 2).

Effect of physical conditions on *Pseudomonas* sp. AB2 lipase activity

Incubation time, agitation speed and inoculums volume

The lipase activity was increased with the increase in incubation time starting from the first 6 h. The maximum activity of lipase was observed after 24 h (24 U/ml), but after 48 h, it was decreased (Fig. 3). The enzyme activity was increased by increasing the agitations speed with maximum production at 200 rpm but further increase in agitation speed decreased the enzyme activity (Fig. 4). While, the maximum enzyme activity was achieved (26 U/ml) when 0.1 OD_{600} (4×10^4 CFU) was used as an inoculums (Fig. 5). It has been proposed that lipase activity can be improved by increasing agitation or air sparging[29, 30]. The same suggestion was also made by[31], who reported that the lipase yield could be improved and the

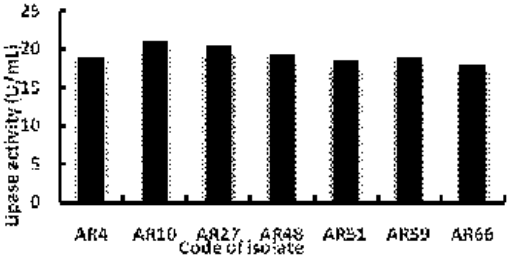

Fig. 1. Production of lipase by the most potent isolated bacterial strains

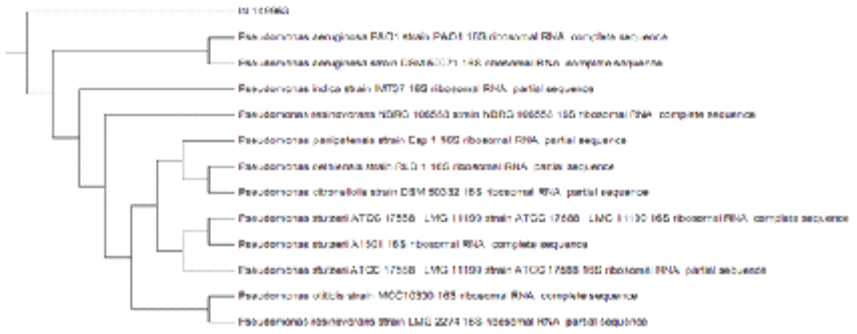

Fig. 2. Phylogenetic tree of the 16SrRNA sequence of *Pseudomonas sp.* AB2

cultivation time could be reduced when bacteria were cultured with good aeration or agitation. Enzymes are very sensitive to mechanical force. Shear stress at higher stirring rates might disturb the intricate shape of a complex molecule to such a degree that enzyme denaturation occurs. The decrease in enzyme activity observed at the highest stirring rate tested in the present study may have been due to perturbation of the protein structure during the synthesis of lipase[32].

Incubation temperature and pH

In the present study, the lipase activity was decreased as the temperature on a range of 25 to 35 °C. The maximum lipase activty was observed at 35°C (25 U/ml) (Fig.6. The *Pseudomonas* sp. AB2 strain was found to produce lipase over wide range of pH from 3-9 with optimum at pH 7.0 (25U/ml) (Fig.7). It has been reported that the lipase of *Pseudomonas aeruginosa* LST-3 (Lip9) showed optimum temperature of 37°C and pH of 6 [33, 34]. Moreover, the lipase of *Pseudomonas aeruginosa* LST-3 (Lip3) reached optimum activity at 35°C and pH 6-7 [35]. Also, the lipase of *Pseudomonas aeruginosa* LST-3 (Lip8) reached optimum activity at 30°C and pH 7 [35]. The optimum temperature and pH for the lipase activity were in the range of other lipase published data for *Pseudomonas* genus.

Substrate (Tributyrine) concentrations and addition of activators

Tributyrine was added to the culture medium with the concentration ranging from 1 to 10 g/l. The maximum activity of the lipase reached at 4 g/l (28 U/ml); while as the substrate concentration increase there was a decrease in the activity at concentration of 6 g/l (Fig.8). In addition, the lipase activity decreased with the increase of yeast extract concentration of which was higher than 0.5 g/l. Since there is minor increase in promoting the yield of lipase by additional yeast extract, it is economical to use 0.5% (w/v)[36]. the lipase activity was the maximum when Mg SO_4 used at concentration of 0.2 g/l with activity of (29 U/ml) (data not shown). Ions like, magnesium were reported to greatly enhance the lipase production from different type of microorganisms. For instance, improvement of lipase production was done by *P. fluorescens* NS2W in the medium containing[37] Mg^{2+} and Ca^{2+}; stimulation of lipase activity using

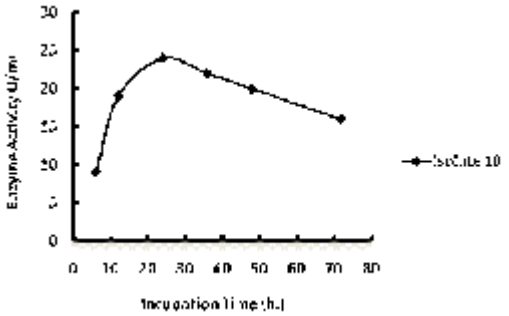

Fig. 3. Relation between incubation period and lipase activity by *Pseudomonas* sp. AB2 (isolate 10)

Fig. 4. Relation between Agitation speed and lipase activity by *Pseudomonas* sp. AB2 (isolate 10)

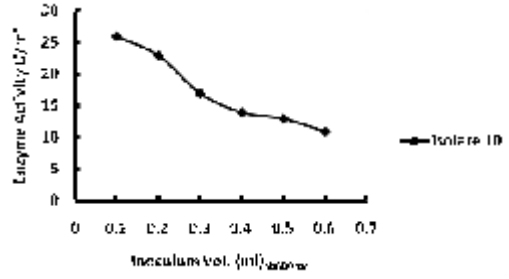

Fig. 5. Relation between inoculums volume and lipase activity by *Pseudomonas* sp. AB2 (isolate 10)

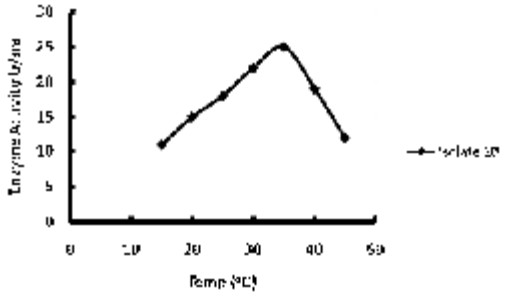

Fig. 6. Effect of incubation Temperatures and lipase activity by *Pseudomonas* sp. AB2 (isolate 10)

Burkholderia sp. GXU56 by addition of Mg^{2+} was observed[38].

Purification of the lipase enzyme

The extracellular lipase was purified by 60% ammonium sulphate precipitation, and **Sephadex G-100** chromatography. The purification was 3.3-fold and has specific activity of 212 U mg/ml protein (Fig. 9). The purified lipase showed a single protein band on SDS-PAGE with an estimated molecular mass of 28.6 kDa (Fig.10). Therefore, the present lipase related to the lower molecular mass lipases. However, the recovery was comparable with [39] (20.4%) and [40] (22%) and was even better than that reported by[41] (5%) and[42] (12.5%). Enzyme purification factor, although low, was comparable with the lipase recently reported by[43]. RL74 is close to many known Pseudomonas lipases with 29–33 kDa molecular mass[44].

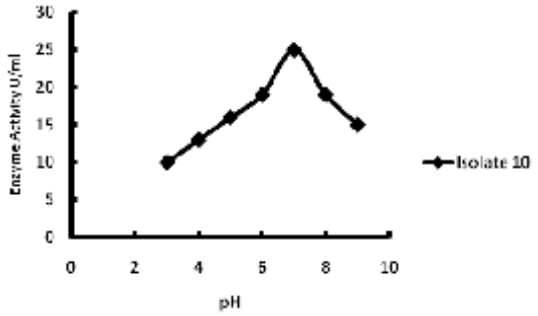

Fig. 7. Effect of pH on the lipase activity by *Pseudomonas* sp. AB2 (isolate 10)

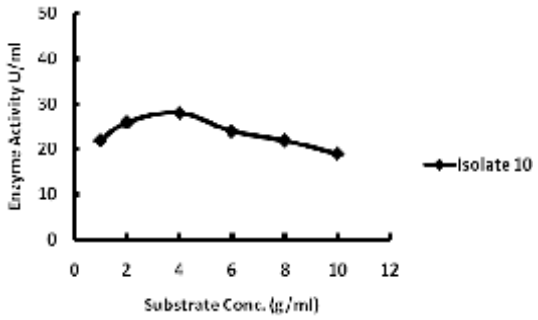

Fig. 8. Effect of tributyrin concentrations on the lipase activity by *Pseudomonas* sp. AB2 (isolate 10)

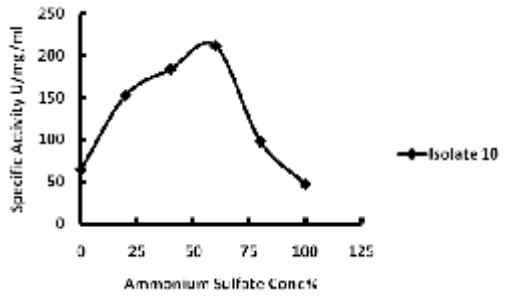

Fig. 9. Ammonium sulphate saturation levels and the corresponding specific lipase activities

Determination of the physico-chemical properties and GC analysis of the WCO

The WCO obtained was used in our experiments to determine their physical and chemical properties. Some chemical properties of waste cooking oil and its compositions was identified by GC analysis. The data showed that the WCO fatty acid profile was Palmetic (31.79%), Stearic (2.41 %), Oleic (65.8 %); while the total saturated acid was (34.2 %) and unsaturated acid was (65.8%) (data not shown). Moreover, Acid value (1.899 mg) KOH/g oil, Iodine number (122 g) I_2/100g oil, Saponification value (200 mg) KOH/g oil and Density at 40°C was 62 g/cm^3.

Application of the lipase for biodiesel production

The GC analysis on transesterification conversion products, indicated that the main components in the WCO-derived biodiesel were methyl palmitate, methyl oleate and methyl stearate respectively. The methyester content (Biodiesel content) were 77% by using Immobilized partially purified lipase-catalyzed transesterification under the optimum processing conditions, 89 % in case

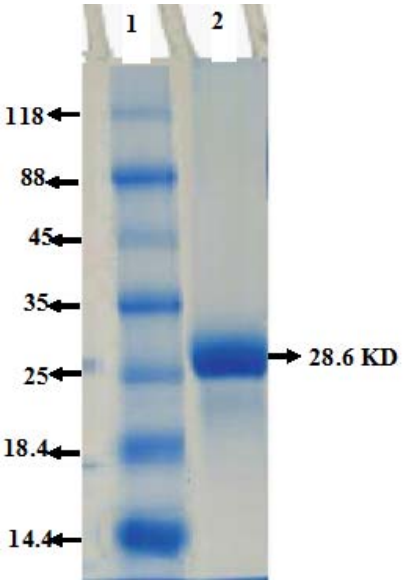

Fig. 10. SDS-PAGE analysis of the purified lipase. Lane 1 molecular mass markers, lane 2 purified lipase

of Immobilized purified enzyme, while it achieve 91% when we used standard immobilized enzyme. In case of using KOH as a transesterification catalyst; the biodiesel content was 94%. The immobilization of the lipase forms a hard external back bone of the enzyme molecule and increases the temperature, so faster reaction rate will occur. The best method for immobilization is physical adsorption. This method is recommended by a majority of scientists for such reasons as it is non-toxic and inexpensive, able to retain activity and able to be regenerated[45]. The immobilized lipase by physical adsorption was used successfully in biodiesel production)[46,47,48].

The immobilized lipase was found to catalyze biodiesel synthesis with higher yield, and this might be due to its larger surface area[49]. Free lipases were known to have mass transfer problem since they form aggregates in low water media[50]. It has been shown that the lipase from strain W33 had relatively higher efficiency. Similarly, biodiesel production by the immobilized lipase from *B. cepacia* reached more than 90% yield[51,52]. The biodiesel sample produced in this study is following within the European Biodiesel Standard (DIN EN 14214), Egyptian diesel oil and the fuel properties of ASTM Biodiesel D-6751 (Table 1).

Table 1. Comparison of physico-chemical properties of Biodiesel sample of this work with standard data

Test	Biodiesel sample of this work	Egyptian Diesel oil	European Biodiesel standard DIN EN 14214	ASTM Biodiesel D-6751
Flash point °C	202	> 55	> 101	> 130
Density g/cm3 @ 15.56 °C	0.8734	0.82-0.87	0.86-0.9	-
Kinematic Viscosity cSt @ 40 °C	6.95	1.6-7	3.5-5	1.9-6
Total acid number (mg KOH/g)	0.177	Nil	< 0.5	< 0.8
Cloud point °C	-3	-	- 4	-
Pour point °C	-9	4.5-15	-	-
Cetane number	61.60	Min. 55	> 51	> 47
Calorific value MJ/Kg	33.39	Min. 44.3	32.9	-
Total S wt%	0.057	Max. 1.2	< 0.01	< 0.05
Water content wt%	0.08	Max. 0.15	< 0.05	0.05
Saponification value mg KOH/g oil	207			
Iodine number mg I2/100g	106	-	120	-

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