Purification, Characterization and Immobilization of Lipase from *Proteus vulgaris* OR34 for Synthesis of Methyl Oleate

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A newly isolated strain, *Proteus vulgaris* OR34, from olive mill waste was found to secrete an alkaline extracellular lipase at $11 \text{ U} \cdot \text{ml}^{-1}$ when cultivated on an optimized liquid medium. This lipase was purified 94.64-fold with a total yield of 9.11% and its maximal specific activity was shown to be 3232.58 and 1777.92 $\text{U} \cdot \text{mg}^{-1}$ when evaluated using the pH-stat technique at 55 °C and pH 9 and Tributyrin TC4 or olive oil as the substrate. The molecular mass of the pure OR34 lipase was estimated to be around 31 kDa, as revealed by SDS-PAGE and its substrate specificity was investigated using a variety of triglycerides. This assay revealed that OR34 lipase preferred short and medium chain fatty acids. In addition, this lipase was stable in the presence of high concentrations of bile salt (NaDC) and calcium ions appear not to be necessary for its activity. This lipase was inhibited by THL (Orlistat) which confirmed its identity as a serine enzyme. In addition, the immobilization of OR34 lipase by adsorption onto calcium carbonate increased its stability at higher temperatures and within a larger pH range. The immobilized lipase exhibited a high tolerance to organic solvents and retained 60% of its activity after 10 months of storage at 4 °C. Finally, the OR34 lipase was applied in biodiesel synthesis via oleic acid mediated esterification of methanol when using hexane as solvent. The best conversion yield (67%) was obtained at 12 h and 40 °C using the immobilized enzyme and this enzyme could be reused for six cycles with the same efficiency.

Keywords: Lipase, *Proteus vulgaris*, purification, immobilization, biodiesel

Introduction

Lipases (triacylglycerol acylhydrolases EC 3.1.1.3) are important hydrolytic enzymes, termed as carboxyl esterases, which can perform their catalytic activity in both aqueous and non aqueous media. They catalyze the hydrolysis of triacylglycerides to glycerol and free fatty acids in aqueous media. In addition, they catalyze the reverse reactions such as esterification, interesterification and transesterification in non aqueous conditions

[1]. In contrast to esterases, lipases are activated only when adsorbed to an oil/water interface [2]. Lipases are ubiquitous in nature and can be produced by a variety of plants, animals and microorganisms [3]. However, microbial lipases are more suitable for biotechnological use due to their availability, high stability and wide substrate specificity [4]. They can be secreted by a large variety of microorganisms under defined culture conditions specific for each strain [5, 6]. Indeed, the environment where these microorganisms were isolated confers some characteristics to these enzymes [7, 8]. Due to these properties, microbial lipases are applied in several industrial applications, such as detergent formulations, biodiesel synthesis, production of pharmaceutical and

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cosmetic esters, food and treatment of wastewaters [9]. However, because of their biological origin, enzymes show some limitations like the complexity of their purification since they present the minimal portion of crude extract. Moreover, enzymes are soluble and unstable against some conditions like high temperatures, pH, organic solvents and detergents [10, 11]. For these reasons, many of the functional and biochemical properties of microbial lipases need to be improved in order to increase their industrial implementation. Indeed, the immobilization is a technique which consists to the attachment of enzymes to solid support materials in order to improve their activity, stability and reusability for a continuous process [12]. Several studies reported the different lipases immobilization techniques such as physical adsorption [13], entrapment or microencapsulation [14, 15] and covalence binding to a solid matrix [16]. Among these techniques, adsorption may have the highest interest due to its relatively low cost and simplicity [17]. Among lipase-producing bacteria, *Proteus* genus was reported in literature. In fact, Kim *et al.* [18] isolated a *Proteus vulgrais* K80 strain from soil collected near a sewage disposal plant, which was able to produce an alkaline extracellular lipase. This lipase was immobilized on amine-terminated magnetic microparticles by covalence binding technique, in order to increase their functional properties [19]. In addition, *Proteus* sp. SW1 strain was reported to be able to produce a highly organic-solvent tolerant lipase, which was effective at converting palm oil into biodiesel [20]. Moreover, *Proteus mirabilis* lipase was shown to be a promising biocatalyst for biodiesel synthesis and their properties were improved using directed evolution [21].

Therefore, bacteria belonging to the *Proteus* genus were known as extracellular-alkaline lipase producers. However, the majority of studies reported lipases of *Proteus* genus produced from coding genes expression [20, 45, 48]. Also, reports on immobilization of *Proteus* lipases [19] and their application under native form are still limited.

In this context, we report in this paper the purification and the characterization of an extracellular alkaline and organic-solvent tolerant lipase, produced by *Proteus vulgaris* OR34 bacterial strain which was isolated previously from Moroccan olive mill wastes. Further-

more, the pure lipase was immobilized by physical adsorption technique, onto carbonate calcium support for the first time, and compared to the soluble lipase. Finally, the immobilized OR34 lipase was tested for the synthesis of methyl oleate (biodiesel) by esterification reaction.

Materials and Methods

Lipase production and extraction

Proteus vulgaris OR34 (NCBI accession number: MN 622879) was previously isolated from Moroccan olive mill wastes [22] and routinely maintained on Luria-Bertani agar. The production of *Proteus vulgaris* OR34 lipase is ensured on a liquid medium previously described [22], which composed of ($\text{g}\cdot\text{l}^{-1}$): 15 casein peptone, 5 yeast extract, 5 NaCl, 2.5 glucose, 3 K_2HPO_4 , 1 KH_2PO_4 , with a final pH of 7.4. After 30 h of incubation at 37°C and 180 rpm, the supernatant that contains the crude extracellular lipase, was recovered by centrifugation at 13,000 rpm for 20 min at 4°C.

Lipase activity determination

The lipase activity was measured titrimetrically at pH 9 and 55°C using pH-stat technique (Switzerland), using olive oil emulsion (10 ml) in 20 ml of 2 mM Tris-HCl buffer (150 mM NaCl, 2 mM NaDC and 3 mM CaCl_2). The olive oil emulsion was obtained by mixing 10 ml of olive oil in 90 ml of 10% (w/v) Gum Arabic. Lipase activity was also measured using tributyrin TC4 (0.3 ml) as substrate in 30 ml of 2 mM Tris-HCl buffer. Other lipid substrates (sunflower oil, soybean oil, palm oil, peanut oil) were used following the same protocol as that of olive oil [23]. Synthetic substrates (Tricaprylin TC8, Trilaurin TC12, Tripalmitin TC16 and Triolein TC18) were used following the protocol established for TC4 [24]. The fatty acids released were titrated by adding sodium hydroxide (0.1 N) to the reaction medium. The enzymatic activity was expressed in International Units (1 IU corresponds to 1 μmol of fatty acid released per min).

Determination of protein concentration

Protein concentration was determined as described by Bradford [25] using BSA as standard.

Procedure of OR34 lipase purification

The culture supernatant (1 L) containing extracellular lipase was precipitated using solid ammonium sulfate at 65% saturation. After centrifugation (9500 rpm for 30 min at 4°C), the pellet obtained was dissolved in a minimum of buffer A (50 mM Tris-HCl, pH 8, 20 mM NaCl, 2 mM benzamidine) and then centrifuged (13,000 rpm for 5 min at 4°C) to remove insoluble material. The supernatant obtained, containing the concentrated protein solution was applied to a Sephacryl S-100 column (2.5 × 150 cm) previously equilibrated with buffer A at a rate of 30 ml·h⁻¹. The fractions containing lipase activity were pooled and deposited on a Mono-Q Sepharose column anion exchanger equilibrated with buffer A. The column (2 × 30 cm) was rinsed with 300 ml of the same buffer. No lipase activity was detected in the washing flow and adsorbed material was eluted with a linear gradient of NaCl (20–500 mM) in the buffer (A) (600 ml) at rate of 45 ml·h⁻¹. The active fractions were collected and concentrated to a volume of 1 ml by ultra filtration technique using a centrifugal concentrator (Vivaspin®) containing a 10 kDa membrane. The enzyme concentrate was deposited on a Sephadex G-75 column (1.6 × 110 cm) previously equilibrated with buffer A at rate of 8 ml·h⁻¹. Then the active fractions were collected and concentrated again by the same ultra filtration technique.

Analytical methods

The purified lipase was analyzed by polyacrylamide gel (15%) electrophoresis under denaturing conditions in the presence of sodium dodecyl sulfate and β-mercaptoethanol (SDS-PAGE) according to the Laemmli method [26]. Non-denaturing or native PAGE (electrophoresis at 4°C in the absence of SDS and β-mercaptoethanol) was performed using the discontinuous gel system of Ornstein [27] and Davis [28]. After electrophoresis, native gel was rinsed three times with buffer A containing 1% Triton X-100. Next, Zymogram was accomplished on a solid revealing medium [29] containing: 30 ml of buffer A, 0.3 ml of TC4, 3 mM CaCl₂, 2 mM NaDC, 1% (w/v) agar-agar and 0.01% (w/v) of phenol red as pH indicator. Finally, the incubation was performed at 55°C for 20 min and the lipase activity was revealed by the color deviation from red to yellow.

Effect of bile salts, calcium ions and THL inhibitor on purified lipase activity

In order to study the bile salt effect on OR34 lipase activity, the hydrolysis of tributyrin by purified lipase was measured by the pH-stat technique under standard conditions (the concentration of Ca²⁺ was fixed at 3 mM), in the presence of increasing concentrations of bile salts NaDC (1 to 10 mM).

To study the calcium dependence of the OR34 lipase, the activity was measured by the pH-stat technique, using tributyrin under standard conditions (with 2 mM NaDC), in the presence of increasing calcium concentrations (from 1 to 10 mM). In the absence of calcium, the lipase activity was measured in the presence of 10 mM EDTA which is a metalloenzyme inhibitor.

In order to confirm that OR34 lipase is a serine enzyme, the purified lipase was incubated with 0.1 mM THL (Tetrahydrolipstatin C₂₉H₅₃NO₅; Orlistat), in both the presence and the absence of 4 mM NaDC [30], for one hour at room temperature. The residual lipase activity was measured after each 10 min by the pH-stat technique using tributyrin substrate under standard conditions. A control containing the enzyme in the absence of THL was incubated under the same conditions.

Immobilization of OR34 lipase

The purified lipase was immobilized by adsorption on CaCO₃ support according to the protocol described by Ghamgui *et al.* [31]. First, a volume of 1 ml of pure enzyme (1002.1 IU) was added to 1 g of calcium carbonate, under stirring at 4°C. The lipase adsorbed onto the support was washed with 20 ml of acetone priority chilled at -20°C, and filtered on wattman paper. Finally, the immobilized enzyme was dried and stored at 4°C until use. The immobilization yield (Ri) was measured at different incubation times ranging from 30 min to 3 h, as the ratio of the adsorbed activity recovered at the end of the immobilization period divided by the total soluble lipase activity initially added to 1 g of the support.

Characterization of free and immobilized OR34 lipase

Effect of storage. The free and the immobilized lipases were stored at 4°C for 10 months. Samples were taken throughout the storage period and the residual activities

were measured by the pH-stat technique, using tributyrin, under standard conditions.

Effect of pH. The effect of pH on lipase activity was studied in the pH range varying from 4 to 11 using tributyrin at 55°C. The effect of pH on lipase stability was determined by incubating the free and the immobilized lipases to different pH values varying from 4 to 11 for 1 h at 4°C, using different buffers: Citrate buffer (50 mM, pH: 4–5), phosphate buffer (50 mM, pH: 6–7), Tris-HCl buffer (50 mM, pH: 8–9) and Glycine-NaOH buffer (50 mM, pH: 10–11). Then the residual lipase activities were measured under standard conditions.

Effect of temperature. Effect of temperature on the free and the immobilized OR34 lipase activity was determined at pH 9 in temperature range varying from 20 to 60°C, using tributyrin as substrate. Relative activities were calculated as the ratio of the lipase activity measured at different temperatures to the maximal activity of the enzyme measured as described above.

The thermal stability assays were performed by incubation for 1 h of the immobilized or the free OR34 lipase at various temperatures (20–80°C). After that, the residual activities were measured under standard conditions (pH 9, 55°C) as the ratio of the activity measured after incubation to the maximal activity of the enzyme.

Effect of organic solvents. The immobilized OR34 lipase was incubated with different organic solvents at 50% (v/v) concentration (DMSO, methanol, ethanol, iso-propanol, acetone, hexane and chloroform) for 24 h at 40°C. Then the solvent was removed by centrifugation and the immobilized lipase was dried under vacuum. The residual activities were measured, using tributyrin under standard conditions, as the ratio of the activity of treated immobilized lipase to the activity of the untreated immobilized lipase.

Esterification assay and reusability of OR34 immobilized lipase

Esterification of oleic acid with methanol, catalyzed by the free and the immobilized lipase was tested according to some literature protocols [32–34]. The reaction was performed in screw-capped flask at 40°C and 150 rpm for 24 h. The reaction mixture was composed of: 500 U of

lipase (free or immobilized), 3 ml of hexane and a molar ratio of 3:1 methanol/oleic acid. An enzyme-free control was carried out under the same conditions. Aliquots of 0.2 ml were withdrawn every 4 h from the reaction mixture to determine the degree of conversion. Firstly, the enzyme was recovered by centrifugation at 13,000 rpm for 5 min at 4°C. Secondly, the supernatant was dissolved in 2 ml of ethanol and the residual fatty acids were assayed by titration with 0.1 N NaOH solution using phenolphthalein as indicator colored. The degree of conversion (%) was calculated as follows:

$$\text{Conversion (\%)} = \frac{IA1 - IA2}{IA1} \times 100$$

With: IA1: Acid Index before esterification

IA2: Acid Index of the sample after esterification

The enzyme recovered by centrifugation at 13,000 rpm for 5 min, after esterification reaction, was washed once with hexane in order to remove any substrate or product retained in the support. Then it was dried at room temperature for 1 h and reused again for another reaction cycle using fresh substrates. A total of 10 continuous esterification cycles using the same initial immobilized lipase were established and the degrees of conversion were calculated after each cycle.

Results

Production of OR34 lipase

The maximal lipase production was obtained after 30 h of culture incubation at 37°C under 180 rpm shaking. The supernatant obtained after centrifugation of the culture broth at 13,000 rpm for 20 min at 4°C, showed a lipolytic activity of 11 U·ml⁻¹ on tributyrin and 5.7 U·ml⁻¹ on olive oil emulsion as substrates, using pH-stat technique under standard conditions.

Purification of OR34 lipase

The extracellular lipase produced by *Proteus vulgaris* OR34 was purified to homogeneity from the culture medium. The enzyme was precipitated with 65% saturation of ammonium sulfate and the precipitate was solubilized in buffer A and loaded to Sephacryl S-100 gel filtration column. The elution was performed with the same buffer (Fig. 1A). The active fractions were applied

Fig. 1. Purification steps of OR34 lipase. (A) Chromatography on Sephacryl S-100. The column (2.5×150 cm) was equilibrated with buffer A (50 mM Tris-HCl, pH 8, 20 mM NaCl, 2 mM benzamidine). Elution of proteins was performed with the same buffer at a rate of $30 \text{ ml} \cdot \text{h}^{-1}$. (B) Chromatography on Mono-Q Sepharose. The column (2×30 cm) was equilibrated with buffer A and elution was performed with a linear gradient of NaCl (20–500 mM) at a rate of $45 \text{ ml} \cdot \text{h}^{-1}$. (C) Chromatography on Sephadex G-75. The column (1.6×110 cm) was equilibrated with buffer A. The elution was performed with the same buffer at rate of $8 \text{ ml} \cdot \text{h}^{-1}$.

to a Mono-Q Sepharose chromatography. Unbound proteins were washed out with buffer A. Then the elution of adsorbed proteins was performed with a linear gradient of NaCl (20–500 mM). As shown in Fig. 1B, OR34 lipase

was eluted between 100 and 220 mM. After that, the active fractions were concentrated using ultrafiltration technique (molecular weight cutoff of 10 kDa) and the concentrate was applied on a second gel filtration

Table 1. Flow sheet of the purification procedure of OR34 lipase.

Purification step	Lipase activity (U·ml ⁻¹)	Volume (ml)	Total activity (Units) ^a	Proteins (mg) ^b	Specific activity (U·mg ⁻¹)	Activity recovery (%)	Purification factor
Culture supernatant	11	1 000	11 000	322	34.16	100	1
(NH ₄) ₂ SO ₄ precipitation (65%)	715	10	7150	84	85.12	65	2.49
Sephacryl S-100 chromatography	73.33	60	4400	9.8	448.98	40	13.14
Mono-Q Sepharose chromatography	60.5	40	2420	1.9	1273.68	22	37.28
Ultrafiltration	2095.5	1	2095.5	0.72	2910.42	19.05	85.22
Sephadex G-75 chromatography	40.45	30	1213.3	0.4	3033.25	11.03	88.82
Ultrafiltration	1002.1	1	1002.1	0.31	3232.58	9.11	94.64

^aunit: 1 μ mol of fatty acid released per minute using TC4 as substrate under standard conditions.

^bproteins were estimated by Bradford method.

Sephadex G-75. Proteins elution was performed with buffer A and the peak of lipase activity was detected in fraction 48 (Fig. 1C). A summary of the purification data is presented in Table 1. The specific activity of the pure lipase reached 3232.58 and 1777.92 U·mg⁻¹ using TC4 and olive oil as substrates, respectively. The OR34 lipase was purified 94.64-fold with an overall recovery yield of 9.11% using TC4 as substrate.

SDS-PAGE and Zymographic analysis

The active fractions of the last step were pooled, concentrated and analyzed on 15% SDS-PAGE using proteins standard. As shown in Fig. 2A, a single band

with an apparent molecular weight of 31 kDa was observed. The lipase activity of this homogeneous band was confirmed by Zymogram assay under non-denaturing conditions. As shown in Fig. 2B, a positive lipolysis was revealed by the deviation of red color of phenol to yellow (pH indicator), due to hydrolysis of TC4 and liberation of free fatty acids.

Substrates specificity of OR34 lipase

The substrate specificity and chain length selectivity were determined by pH-stat technique using a variety of triglycerides. The OR34 lipase was able to hydrolyze all natural lipid substrates tested (Fig. 3A). The highest activity (3119.16 U·mg⁻¹), which was taken as 100%, was shown against palm oil which has the highest saturated fatty acid content of the oils assayed. The enzyme can also hydrolyze peanut oil (2183.41 U·mg⁻¹) and soybean oil (2058.65 U·mg⁻¹) with relative activities of 70 and 66%, respectively. The hydrolysis of olive oil (1777.92 U·mg⁻¹) and sunflower oil (1372.43 U·mg⁻¹) achieved 57 and 44%, respectively. With respect to the fatty acid specificity, OR34 lipase preferred triglycerides containing short and medium chain fatty acids (Fig. 3B). It showed the highest activity (3232.58 U·mg⁻¹), which was taken as 100%, towards Tributyrin TC4 followed by Tricaprylin TC8 (2844.67 U·mg⁻¹/88%) and Trilaurin TC12 (2359.78 U·mg⁻¹/73%). However the triglycerides with long acyl chain lengths like Tripalmitin TC16 (1131.4 U·mg⁻¹) and Triolein TC18 (937.45 U·mg⁻¹) were less hydrolyzed with relative activities of 35 and 29%, respectively.

Fig. 2. SDS-PAGE and Zymography of the purified OR34 lipase. (A) SDS-PAGE (15%) analysis of OR34 lipase. Lane 1: molecular mass markers (Pharmacia). Lane 2: purified OR34 lipase obtained after Mono-Q sepharose chromatography and ultrafiltration. (B) Zymogram demonstrating the OR34 lipase activity toward TC4. Native PAGE gel was incubated for 30 min at 55 °C. A positive lipolysis is showed by a deviation of red color to yellow using phenol red as pH indicator.

Fig. 3. Hydrolytic activities of OR34 lipase towards different substrates. (A) Substrate specificity of the lipase toward various emulsion oils. Lipase activities are expressed as the percentage of that of palm oil, which was taken as 100%. (B) Substrate specificity of the lipase toward various triglycerides. Lipase activities are expressed as the percentage of that of Tributyrin, which was taken as 100%. The results are the mean $\pm$ S.D. of three separate experiments.

Fig. 4. Characterization of the purified OR34 lipase. (A) Effect of increasing concentration of bile salt (NaDC) on OR34 lipase activity in presence of 3 mM CaCl_2 using Tributyrin TC4 as substrate under standard conditions. (B) Effect of increasing concentration of CaCl_2 in presence of 2 mM NaDC. The star indicates the lipase activity measured in the absence of CaCl_2 and in the presence of 10 mM EDTA. (C) Effect of Orlistat on the OR34 lipase activity. The pure enzyme was incubated at room temperature with 0.1 mM THL (either with or without NaDC) and the residual activities were measured using Tributyrin TC4 under standard conditions. The results are the mean $\pm$ S.D. of three separate experiments.

Effect of bile salts, calcium ions and THL inhibitor on purified lipase activity

In order to check if the purified OR34 lipase is able to hydrolyze triacylglycerols in the presence of surface-active agents like bile salts, the hydrolysis rate of TC4 was measured in the presence of increasing concentrations of NaDC, under standard conditions. Fig. 4A showed that the specific activity of OR34 lipase reached the maximum ($3232.58 \text{ U} \cdot \text{mg}^{-1}$) at 2 mM NaDC. This natural detergent has no inhibitory effect on the OR34 lipase ($1789 \text{ U} \cdot \text{mg}^{-1}$) even at a large concentration (10 mM).

Concerning the effect of Ca^{2+} ions, Fig. 4B shows that calcium ions seems to be not necessary to trigger the OR34 lipase activity which can be detected ($2100 \text{ U} \cdot \text{mg}^{-1}$) in the absence of Ca^{2+} and the presence of 10 mM EDTA as chelator. The specific activity of OR34 lipase reached its maximal ($3232.58 \text{ U} \cdot \text{mg}^{-1}$) at 3 mM of CaCl_2 and in the absence of chelator.

In order to check if the purified OR34 lipase is a serine enzyme, it was incubated with THL, either in the presence or in the absence of NaDC, and the residual activities were measured under standard conditions using TC4 as substrate. Fig. 4C shows that after 30 min of incubation in the absence of NaDC, the OR34 lipase was totally inhibited. The addition of 4 mM of NaDC in the medium accelerated the inactivation of the OR34 lipase which was reached at 20 min of incubation.

Immobilization of OR34 lipase

The immobilization yield (R_i) was measured at differ-

Fig. 5. Residual activity of the free and the immobilized OR34 lipase after storage period of 300 days at 4 °C. The results are the mean $\pm$ S.D. of three separate experiments.

ent incubation times of enzyme (1002.1 IU) with 1 g of support (CaCO_3) at 4°C under stirring. An incubation time of 60 min was ideal to reach the maximal adsorption of the OR34 lipase onto the support (901.89 IU), with the highest immobilization yield of 90% (data not shown).

Characterization of free and immobilized OR34 lipase

Effect of storage. The ability to be stored for certain period of time was investigated (Fig. 5). The immobilized OR34 lipase retained 100% of its initial activity ($3232.58 \text{ U} \cdot \text{mg}^{-1}$) after a storage period of 60 days at 4°C, whereas the activity of the free lipase decreased to 90% ($2909.32 \text{ U} \cdot \text{mg}^{-1}$) during the same period. Moreover the decrease of lipase activity over time was more accentuated in the case of the free lipase which retained only 10% ($323.252 \text{ U} \cdot \text{mg}^{-1}$) of its initial activity after 10 months, while the immobilized lipase maintained 60% ($1939.548 \text{ U} \cdot \text{mg}^{-1}$) after the same period.

Fig. 6. Effect of pH on OR34 lipase activity (A) and stability (B) for both the free and the immobilized enzyme. Enzymes were incubated 1 h with different buffers at 4 °C for stability study. The results are the mean $\pm$ S.D. of three separate experiments.

Effect of pH. As shown in Fig. 6A, the immobilization has no effect on the optimal pH of activity. In fact, the highest activity ($3232.58 \text{ U} \cdot \text{mg}^{-1}$) for both free and immobilized OR34 lipase were obtained at pH 9. Moreover, the immobilized lipase presented between 10 and 20% of activity ($323\text{--}646 \text{ U} \cdot \text{mg}^{-1}$) at acid pH from 4 to 6, while the free lipase was not active at these pH values. Concerning the pH stability, Fig. 6B shows that the immobilized OR34 lipase remained stable and kept 100% of its activity ($3232.58 \text{ U} \cdot \text{mg}^{-1}$) within a large range of pH varying from 7 to 11, while the free OR34 lipase retained about 94% of its activity ($3038.62 \text{ U} \cdot \text{mg}^{-1}$) at pH 11. Moreover, the immobilized lipase was more stable than the free one at acid pH. It retained between 60 and 90% of its activity ($1939\text{--}2909 \text{ U} \cdot \text{mg}^{-1}$) at pH varying from 4 to 6, while the free OR34 lipase maintained between 50 and 72% of its activity ($1616\text{--}2327 \text{ U} \cdot \text{mg}^{-1}$) at the same pH range.

Fig. 7. Effect of temperature on OR34 lipase activity (A) and stability (B) for both the free and the immobilized enzyme. Enzymes were incubated 1 h at different temperatures for stability study. The results are the mean $\pm$ S.D. of three separate experiments.

Effect of temperature. The activity profiles of the free and the immobilized OR34 lipase at different temperatures are presented in Fig. 7A. The optimal temperature for both free and immobilized lipase ($3232.58 \text{ U} \cdot \text{mg}^{-1}$) was found at 55°C . However, the relative activity of the free lipase decreased to 81% ($2618.39 \text{ U} \cdot \text{mg}^{-1}$) at 60°C , while the immobilized lipase was more active and showed 96% of activity ($3103.28 \text{ U} \cdot \text{mg}^{-1}$) at the same temperature. In addition, the immobilized lipase was more active at low temperatures; it can show more than 50% of its initial activity ($1680 \text{ U} \cdot \text{mg}^{-1}$) at 20°C contrary to the free lipase which presents only 27% ($872.8 \text{ U} \cdot \text{mg}^{-1}$) at this temperature. Concerning the thermostability, as shown in Fig. 7B the immobilized OR34 lipase was more stable at large range of temperatures. It retained 61 and 40% of its initial activity, which correspond to 1971.87 and $1293 \text{ U} \cdot \text{mg}^{-1}$ at 70 and 80°C , respectively, while the free lipase was totally inhibited at these temperatures.

Effect of organic solvents. The tolerance of immobilized OR34 lipase to organic solvents (polar and non-polar) was studied and the results are presented in Fig. 8. As it can be shown, the immobilized OR34 lipase was highly stable in the presence of 50% (v/v) hexane; it retained more than 90% of its initial activity till 24 h of incubation at 40°C . Moreover, it maintained more than 80% after 24 h of incubation at 40°C , either with ethanol or

Fig. 8. Effect of different organic solvents at 50% (v/v) concentration on the stability of immobilized OR34 lipase. The residual activities were calculated after 24 h of incubation at 40°C .

Fig. 9. Esterification reaction and reusability of the immobilized OR34 lipase. (A) Synthesis of methanol oleate by the free and the immobilized OR34 lipase. Esterification reaction was conducted at 40 °C and 150 rpm for 24 h using 500 U of enzyme in 3 ml of hexane and methanol/oleic acid with molar ratio of 3:1. (B) Effect of reusability of immobilized OR34 lipase on the conversion yield. The incubation time was 12 h for each cycle. The results are the mean $\pm$ S.D. of three separate experiments.

iso-propanol at 50% (v/v). In the presence of methanol or DMSO the OR34 lipase conserved more than 70% of its activity, while it lost about 46 or 38% of its activity when incubated with chloroform and acetone, respectively.

Esterification reaction catalyzed by OR34 lipase

After immobilization of OR34 lipase, its ability to catalyze esters synthesis in organic media was studied. The synthesis of methyl oleate (biodiesel) by direct esterification of methanol and oleic acid in hexane was taken as a reaction model and the conversion yield (%) is calculated.

Kinetic synthesis. As seen in Fig. 9A, the highest conversion yield (67%) was obtained with the immobi-

lized OR34 lipase after 12 h of incubation at 40 °C, whereas the free lipase allowed only 30% of conversion at the same conditions.

Reusability. As shown in Fig. 9B, the immobilized OR34 lipase was reused 6 times with the same efficiency (conversion yield of 67%). After 6 cycles the conversion starts to decrease and it reduced to approximately 49% after 10 successive cycles.

Discussion

Diverse lipases from many bacterial genera have been isolated and studied, such as *Pseudomonas* [35, 36], *Serratia* [37, 38], *Staphylococcus* [39, 40], *Bacillus* [41, 42] and *Halomonas* [43, 44]. Few lipases from *Proteus* genus were also reported in the literature, like those produced by *Proteus vulgaris* K80 [18], *Proteus* sp. SW1 [20], *Proteus* sp. K107 [45] and *Proteus mirabilis* [46]. Bacterial lipases are classified into 19 families based on amino acid sequences and biochemical characteristics [47]. The sequences of *Proteus* lipases reported share a high similarity with lipases from *Pseudomonas fragi* which were classified together as a separate sub-family in group I.1. They differ from other group I.1 lipases in their mechanism of secretion: they lack leader peptides, absence of two conserved Cys residues believed to be involved in disulfide bond formation, and they don't require the participation of a chaperone [18, 20, 45]. The crystal structure of lipase from *Proteus mirabilis* was solved and provides the first and unique structure of a lipase from the *Proteus* I.1 sub-family. It closely resembles the structure of the family I.1 (*Pseudomonas aeruginosa*), as well as family I.2 lipases (*Burkholderia gluma* and *Burkholderia cepacia*) with some unique features [46]. In this study, the purification of *Proteus vulgaris* OR34 lipase process resulted in 94.64-fold purification factor and a final recovery of 9.11% of the enzyme with specific activity of 3103.28 U $\cdot$ mg⁻¹. Kim *et al.* [18], purified a native lipase from *Proteus vulgaris* K80 strain, 300-fold with a yield of 25% and a final specific activity of 2000 U $\cdot$ mg⁻¹. In addition, the pure OR34 lipase showed a single band by SDS-PAGE with a molecular mass estimated to be 31 kDa which is similar to lipases from *Proteus* genus [18, 20, 45]. The OR34 lipase was able to hydrolyze a large range of oil substrates, with a prefer-

ence to palm oil. Among the triglycerides tested (C4–C18), it showed high activity against short and medium chain fatty acids esters. Indeed, the lipase from *Proteus vulgaris* K80 showed the highest activity against Tricaprylin (TC8) among the triglycerides tested (C4–C16) [18], while lipase from *Proteus* sp. SW1 was more active against *p*-nitrophenyl laurate (pNL, C12) among the synthetic esters tested (C2–C18) [20]. It has been reported that calcium ions are needed for lipolytic activity of some bacterial lipases. In fact, Liu *et al.* [50] demonstrated that lipase from *Pseudomonas fluorescens* Pf0-1, which belongs to sub-family I.1, was stimulated (160%) in the presence of 10 mM CaCl₂. Moreover, the activity of lipase from *Staphylococcus hyicus* (sub-family I.6) increased about 40-fold in the presence of Ca²⁺ ions and was strongly inhibited in the presence of chelators [51]. Therefore, calcium ions might carry out three distinct roles in lipase action: removal of fatty acids as insoluble Ca²⁺ salts [52, 53], direct activation resulting from concentration of the enzyme at the lipid/water interface [54] and structure stabilizing effect of the enzyme [55]. In this study, calcium seems to be not necessary to trigger the OR34 lipase activity that was detected (2100 U·mg⁻¹) even in the presence of 10 mM EDTA. However, in the presence of 3 mM CaCl₂ the OR34 lipase activity increased to 3232.58 U·mg⁻¹ suggesting that the enzyme is partially calcium dependent. In fact, lipases belonging to sub-family I.1 have a conserved Ca²⁺ binding located near the end of a α 8 proposed to play a role in catalysis and stability [55, 57]. Since surfactants are important and frequently used in the preparation of emulsions for lipases assays, the effect of NaDC on OR34 lipase was studied in this work. It is reported that bile salts at concentrations above 2 mM reversibly inhibited the hydrolysis of Triolein by pancreatic lipase [58]. Several different mechanisms have been suggested to explain the bile salt inhibition: 1) the building of a detergent monolayer on the substrate surface that is negatively charged and that electrostatic repulsion is of importance. 2) A competition between lipase and bile salt for the interface and/or a lowering of the interfacial tension hindering lipase adsorption to the lipid/water interface. 3) The bile salts react cooperatively with lipase to form a complex with a negligible activity for the substrate [59]. In our case, NaDC has no inhibitory effect on OR34 lipase activity even at large concen-

tration (10 mM). Comparable results were obtained with lipase from *Pseudomonas aeruginosa* SRT 9, which retained 97.5% of its activity after incubation of 1 h with NaDC at 10% (w/v) [60]. Our result shows that OR34 lipase has a penetrating power allowing it to access the lipid/water interfaces even in the presence of high concentrations of bile salts. Moreover, OR34 lipase reached its maximal activity in the presence of 2 mM NaDC. This can be explained by the fact that bile salts at low concentrations ($\leq$ CMC_{NaDC}, critical micelle concentration of NaDC), due to their hydrophobicity, stimulate lipase activity by inducing the opening of the lid and the fixation of enzyme at interface [61]. Tetrahydrolipstatin (THL, Orlistat) is a hydrogenated analogue of lipstatin produced by *Streptomyces toxytricini*, and is a potent inhibitor of gastrointestinal lipases. It has been suggested that a stoichiometric enzyme-inhibitor complex of a long lived acyl-enzyme type is formed between the β -lactone ring of THL and the hydroxyl group of the catalytic serine (ser 152) of pancreatic lipase [62, 63]. In this work, the inhibition of purified OR34 lipase by THL indicates that this lipase, like all the known lipases from different origins [67–69], is a serine enzyme. The THL inhibitor effect on OR34 lipase activity was enhanced in the presence of NaDC, which is in accordance of the results founded by Salah *et al.* [68] and Zouari *et al.* [69]. In fact, it has been demonstrated that bile salts micelles are important in forming and stabilizing the THL-lipase complex, which accelerates the enzymatic inhibition [70]. After purification and characterization of OR34 lipase, the immobilization of the pure enzyme was performed by adsorption onto carbonate calcium support. The maximal adsorption (90%) was reached after 60 min, which correspond to the point of dynamic balance between adsorption and desorption. This is in accordance with the results showed for lipase from *Rhizopus oryzae* (ROL), which was immobilized by physical adsorption onto CaCO₃ and presented a yield more than 95% during 30 min [33]. Minovska *et al.* [71] reported that the same support displayed a similar immobilization yield (92%) after 60 min of incubation, using the commercial *Candida rugosa* type VII lipase. However, the adsorption of *Pseudomonas* sp. KWI 56 lipase on the same support showed an immobilization yield of only 57.8% after 60 min of incubation [72]. In fact, CaCO₃ appeared as a powder with small crystalline particles to

produce a large adsorption surface area [33, 72]. In addition to its low cost, the CaCO_3 has several advantages for use as a matrix including its lack of both toxicity and chemical reactivity with the enzyme [31, 33]. In this work, we showed that the immobilized OR34 lipase was more stable than the soluble lipase when stored at 4 °C. The immobilization of lipases to a support often limits their drastic conformational changes, and hence results in increased stability towards denaturation [33, 73, 74]. The maximal lipase activity for both the immobilized and the soluble enzyme was obtained at pH 9 that consistent with other lipases from *Proteus* genus [18, 20, 45], while the immobilized enzyme showed an activity in a broader pH range. This result corroborate with that obtained by Kharrat *et al.* [73] who reported that the immobilization of ROL onto silica aerogels significantly improved the pH range of activity. The OR34 lipase activity detected at acidic pH after immobilization of the enzyme is in agreement of the result reported by Ghamgui *et al.* [33], who showed that immobilization of the ROL on CaCO_3 support induce the activity at acidic pH values. We noted also an improvement of pH stability of OR34 lipase after immobilization, which can be explained by a multipoint covalent attachment of enzymes on the support [33]. This attachment maintained unaltered under mild denaturing environment (extreme pH values...), which induced the rigidity of the protein structure leading to its stability [75, 76]. Concerning the effect of temperature on OR34 lipase activity, both the immobilized and soluble enzymes were extremely active at 55 °C. A similar result was presented by other lipases from *Proteus* genus [18, 20], whereas Gao *et al.* [45] reported that 35 °C was the optimal temperature for *Proteus* sp. K107 lipase activity. In this work, the immobilization of OR34 lipase increased the activity in a broader temperature range and improved the stability at high temperature values. It is often found that immobilized enzyme has a higher thermal stability than free enzyme due to restriction of its conformational flexibility attributed to its multiple attachment points on the support which limit the conformational alterations and movements under various temperatures [33, 73]. In this study the biodiesel synthesis via interesterification reaction was investigated. In fact, the immobilized lipases as biocatalysts have recently drawn great attention due to their environmental benefit, economic perspective and

simpler operation over conventional chemical catalytic processes [77, 78]. The enzymatic reaction can be performed in the presence of organic solvents in order to increase the solubility of the substrates, reduce the viscosity of the reaction mixture and enhance the reaction rate. However, most organic solvents are toxic and denature the enzyme activity [79]. For this reason the stability of OR34 lipase in the presence of different organic solvents was studied. The results showed that the immobilized OR34 lipase was stable and retained more than 50% of its activity after 24 h of incubation in the presence of 50% (v/v) of various organic solvents (hexane, ethanol, iso-propanol, DMSO, chloroform and acetone). These results are in accordance with those founded by Whangsuk *et al.* [20] who reported that *Proteus* sp. SW1 Lipase was highly stable in organic solvents (acetone, ethanol, hexane and isopropanol). In addition, Kharrat *et al.* [73] showed that silica aerogels-immobilized ROL was stable in the presence of various organic solvent especially the hydrophobic ones. Generally, it was found that the biodiesel production rate increases with the increase in the hydrophobicity of the solvent used [80]. Using hydrophilic solvents resulted in much lower rates due to their tendency to strip-off the bound water from the enzyme surface, resulting in the loss of the favorable structure and a decrease in the activity [81]. The activity of enzyme was correlated against solvent polarity and the stripping of water was found to occur when the hydrophobicity (log P) of the organic solvent was less than 2, whereas the effect was not observed if the value was between 2 and 4 [79, 80]. That is why n-hexane, which has log P = 3.5, has been commonly used, as a reaction medium, and its positive effect was proven in most biodiesel production studies [32, 82, 83]. In this work, the synthesis of methyl oleate was also performed in the presence of hexane and the maximal conversion yield obtained was about 67%. Lipase from *Proteus* sp. W1 expressed in *E. coli* was used to catalyze the transesterification reaction of palm oil with ethanol to synthesis biodiesel and the conversion yield obtained was 43% [20]. However, the transesterification of rapeseed oil with methanol, catalyzed by lipase from *Proteus* sp. K107 (recombinant *E. coli* whole-cell) showed a conversion yield of 97% [45]. Furthermore, direct evolution was used to improve the organic solvents-tolerance and the thermal stability of lipase from *Proteus mirabilis* for bio-

diesel production [21]. Finally, the immobilized OR34 lipase prepared in this study was reused for six cycles with the same efficiency. Natalia *et al.* [19] reported the immobilization of lipase from *Proteus vulgaris* K80 expressed in *E. coli* cells, onto amine-terminated magnetic microparticles (Mag-MPs). They found that the immobilized lipase was more active and stable at different pH and temperature values, had broader substrate specificity compared with the soluble lipase, and showed about 75% residual relative activity after six recovery trials.

It is the first time we report the immobilization of lipase from *Proteus* genus onto CaCO₃ support and its application under native and immobilized form in biodiesel synthesis. These findings justify further studies to optimize the parameters of biodiesel esterification reaction.

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Conflict of Interest

The authors have no financial conflicts of interest to declare.

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