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Expression, purification of a novel alkaline *Staphylococcus xylosus* lipase acting at high temperature

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ABSTRACT

A new *Staphylococcus xylosus* strain was isolated. The extracellular lipase of *S. xylosus* (wt-SXL2) was purified to homogeneity from the culture medium. The specific activity of the purified enzyme, measured at pH 8.5 and 55 °C using tributyrin or olive oil emulsion, reached, respectively, 6300 U/mg or 2850 U/mg. The sequenced 18 N-terminal amino acid showed a high degree of identity with known staphylococcal lipase sequences.

The gene encoding the mature lipase was cloned and sequenced. The deduced amino acid sequence showed a significant similarity with various staphylococcal lipases. The highest overall identity (98.74%) was found with *S. xylosus* lipase (SXL1). The mature part of the lipase was expressed in *Escherichia coli*. The recombinant lipase was purified by affinity chromatography. The specific activity of the recombinant lipase was 4100 or 1500 U/mg using tributyrin or olive oil emulsion as substrate, respectively, at pH 8.5 and 55 °C.

The wild type and recombinant lipases presented a quite interesting thermal stability, after an incubation of 60 min at 55 °C and they are found to be highly stable at a pH ranging from 4 to 11. Due to its stability at high temperature and in organic solvent, the wt-SXL2 was used as biocatalyst to synthesise a high added value molecules.

1. Introduction

Lipases (triacylglycerol acylhydrolases, EC 3.1.1.3) are one of the most important classes of hydrolytic enzymes that catalyse both the hydrolysis and the synthesis of esters. Lipase has a number of unique characteristics, including substrate specificity, stereospecificity, regioselectivity and the ability to catalyse heterogeneous reaction at the interface soluble and insoluble water systems [1]. They are ubiquitous enzymes produced by all biological systems animals, plants and microorganisms. In contrast to animal and plant lipases, extracellular microbial lipases can be produced relatively inexpensively by fermentation and in large quantities [2]. In addition to their hydrolytic activity, lipases can also catalyse other reactions such as esterification or interesterification. A particular interest relies on lipases capacity of catalysing such reactions and, consequently, the synthesis of fine compounds used for manufacturing products of high aggregate value, such as the engineering of structured lipids for the biotransformation of oils and fats [3–6]. Each application requires unique properties with respect to specificity, stability, optimal temperature, and pH-dependence [7]. In order to use lipases for hydrolysis, esterification, or other applications, it is essential to produce the purified enzyme at high concentrations and to determine its biochemical properties. Only about 2% of world's microorganisms have been tested as enzyme sources until 2006, and lipases from different sources have large variations in specific activity, fatty acid specificity, optimal temperature, and pH [8]. More recently several new lipases have been isolated [9–13]. A number of staphylococci producing lipases have been purified and characterized. Some studies, based on sequence comparison or direct mutagenesis, have attempted to explain the biochemical differences between staphylococcal lipases.

The practical application of staphylococcal lipases may be limited due to relatively lower stabilities and catalytic activities under conditions that characterize industrial processes: high temperature and extreme pH values. However, recently we have isolated a novel lipase produced by *Staphylococcus aureus* [14]. This enzyme which is stable at high temperature and alkaline conditions, was used to

Abbreviations: Wt-SXL2, *Staphylococcus xylosus* lipase 2; SSL, *Staphylococcus simulans* lipase; SXL1, *Staphylococcus xylosus* lipase 1; SAL2, *Staphylococcus aureus* lipase 3; SHL, *Staphylococcus hyicus* lipase; BSA, bovine serum albumin; EDTA, ethylene diamine tetraacetic acid; EGTA, ethylene glycol-bis (β-aminoethyl Ether) N,N,N′,N′-tetraacetic acid; NaDC, sodium deoxycholate; NaTDC, sodium taurodeoxycholate; PC, phosphatidylcholine; PCR, polymerase chain reaction; SDS/PAGE, sodium dodecyl sulphate/polyacrylamide gel electrophoresis; TG, triacylglycerol; TC_4, tributyrin.

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catalyse several esterification reactions such as biopolymers and antioxidants [15,16]. As the industrial application may require specific properties of the biocatalysts, there is still an interest in finding new lipases that could create novel applications. Specially thermostable and alkaline lipases show higher resistance to chemical process [15,16].

Here, we conducted an extensive screening to isolate a new thermostable and alkaline lipase produced by a newly isolated *Staphylococcus xylosus* strain. The lipase was purified to homogeneity and the N-terminal sequence was determined and compared to the known staphylococcal lipases. The part of the gene encoding the mature lipase was also cloned, and sequenced. The gene was sub-cloned for over expression in *Escherichia coli* BL 21 (DE3) and the produced lipase was purified to homogeneity in one step using immobilized metal affinity chromatography. The biochemical properties of the recombinant lipase were compared to those of the wild type one. Due to its stability at high temperature and in organic solvent, the wt-SXL2 could be used as biocatalyst to synthesise some high value molecules such antioxidants [17], flavour esters, biopolymer and estolide esters.

2. Experimental

2.1. Chemicals

Isopropyl thio-β-D-galactopyranoside (IPTG) was purchased from Boehringer. Nickel nitriloacetate was from Invitrogen. Tributyrin (99%; puriss) and benzamidine were from Fluka (Buchs, Switzerland); sodium deoxycholic acid (NaDC), yeast extract and ethylene diamine tetraacetic acid (EDTA) were from Sigma Chemical (St. Louis, USA); casein peptone was from Merck (Darmstadt, Germany); gum arabic was from Mayaud Baker LTD (Dagenham, United Kingdom); marker proteins and supports of chromatography used for lipases purification: Sephacryl S-200, Mono S-Sepharose and Mono Q-Sepharose gels were from Pharmacia (Uppsala, Sweden); pH-stat was from Metrohm (Switzerland).

2.2. Strains, media and plasmids

- *E. coli* strain DH5α was used as a cloning host for the gene part encoding the mature lipase. For expression, the *E. coli* strain BL21 (DE3), which contains the structural gene for T7 RNA polymerase under control of the lac promoter, was used. *E. coli* strains were grown in Luria-Bertani medium, supplemented with 100 μg/ml ampicillin whenever plasmid maintenance was required.
- *S. xylosus* was grown in medium containing: 17 g/l casein peptone, 5 g/l yeast extract, 2.5 g/l glucose. The culture was incubated in an orbital shaker (Certomat H/HK, Germany, Melsungen) at 200 rpm at 37 °C and at an initial pH of 7.4.
- The plasmid pCR® 4Blunt-TOPO® (Invitrogen corporation) was used as cloning vector. The plasmid pET-14b (Invitrogen), under the control of the T7 promoter, was used for the over-expression of SXL2. PCR products were purified using Wizard PCR preps DNA Purification System (Promega). All enzymes used in DNA manipulations were bought from Promega. Oligonucleotides were synthesised by GENOME Express (Grenoble, France).

2.3. Screening of lipolytic micro-organisms

Initial screening of lipolytic microorganisms from various Tunisian biotopes was carried out on a solid medium containing 1‰ olive oil, 1% nutrient broth, 1% NaCl, 1.5 g agar and 1‰ Rhodamine B. The culture plates were incubated at 37 °C, and colonies giving rise to widespread clearing around them were regarded as putative lipase producers. In order to select the best lipase producer for enzyme purification and characterization, strains were cultured in different liquid medium and lipase activity was determined with a pH stat using olive oil or TC_4 as substrates. After extensive screening of lipase producers, only one bacterial colony, isolated from the soil of a conditioning oil seed industry, was retained. The identification of this strain has been kindly determined by Dr. Hafedh Dhouib (Centre de biotechnologie de Sfax, Sfax, Tunisia). Based on the biochemical properties and the morphological aspect, the identification of this microorganism showed 99.7% of identity to *S. xylosus*.

2.4. Lipase and phospholipase activities assay

- The lipase activity was measured titrimetrically at pH 8.5 and 55 °C with a pH-stat under standard conditions using tributyrin (0.25 ml) in 30 ml of 2.5 mM Tris–HCl pH 8.5, 2 mM $CaCl_2$, 2 mM NaDC or olive oil emulsion (10 ml of oil emulsion in 20 ml of H_2O NaCl pH 8.5, 2 mM $CaCl_2$, 2 mM NaDC) [18]. When measuring the lipase activity in the absence of $CaCl_2$, EDTA or EGTA was added to the lipolytic system.
- The phospholipase activity was checked titrimetrically at pH 8.5 and 55 °C with a pH-stat using 0.2 g of phosphatidyl choline mixed in 30 ml of 150 mM NaCl, 7 mM $CaCl_2$ and 3 mM NaTDC.

Lipolytic activity was expressed as units. One unit corresponds to 1 μmol of fatty acid released per minute.

2.5. DNA manipulation

2.5.1. DNA preparation and transformation procedure

Staphylococcal DNA was prepared as described previously with slight modifications [19]. *S. xylosus* strain was cultured on LB medium agar at least for 14 h at 37 °C. Single colony was used to inoculate 10 ml of LB and then incubated at 37 °C for 12 h. Cells were harvested from the broth cultures by centrifugation at 3000 × *g* for 15 min. The pellet was re-suspended in 0.1 ml of TE buffer (10 mM Tris, 1 mM EDTA, pH 8) with lysozyme (5 mg/ml) and lysostaphin (4.5 U/ml). The mixture was incubated for 1 h at 37 °C. After addition of 0.5 ml of guanidine thiocyanate (0.5 M) and 0.25 ml of ammonium acetate (7.5 M), the mixtures were maintained on ice for 10 min. Chloroform–isoamyl alcohol (24:1) (0.5 ml) was added to the suspension, mixed and centrifuged at 13,000 × *g* for 10 min at room temperature. The upper phase was collected and DNA was precipitated by addition of 0.7 ml cold isopropanol and centrifuged for 5 min at 13,000 × *g*. The DNA was re-suspended in 0.5 ml of TE. DNA samples were treated with RNAse (10 mg/ml) at 37 °C for 30 min. DNA was extracted with an equal volume of chloroform–isoamyl alcohol, vortexed and centrifuged for 5 min at 11,000 × *g*. The upper phase was collected and a double volume of 90% (v/v) ethanol was added. DNA was pelleted by centrifugation for 10 min at 11,000 × *g*. The DNA obtained was resupended in TE. The quality of the DNA was checked by electrophoresis in a 1% agarose gel containing ethidium bromide.

E. coli was transformed using the $CaCl_2$ method. Cloning enzymes are purchased from Promega, Boehring Manheim, BRL or Pharmacia LKB. Assay conditions were in agreement with supplier recommendations.

2.5.2. Cloning of the mature lipase gene region

The gene part encoding the mature lipase was amplified by PCR from genomic DNA of *S. xylosus* with primers, 5′-ATCGAATTCATATGTTAAAAGCGAATCAAGTACAA-3′, and 5′-GATCGAATTCGGATCCTTAACTTGCTTTCAATTGTGTT-3′. The primers were predicted from the N-terminal sequence of the purified wt-SXL2 and the C-terminal sequence of *Staphylococcus*

simulans lipase [20], respectively. The predicted primers contain the nucleotides corresponding to the *Eco*RI, *Bam*HI and *Nde*I sites used to target the gene in the right direction. The 50 μl PCR mixture contained 50 pmol of both primers, 20 pmol of each deoxynucleoside triphosphate, approximately 1 μmol genomic DNA as template, polymerisation buffer, and 5 U Taq polymerase (Amersham Pharmacia biotech). The thermal profile involved 35 cycles of denaturating at 94 °C for 45 s, primer annealing at 55 °C for 1 min, and extension at 72 °C for 2 min (10 min in the last cycle).

The PCR product (1.2 kb) was isolated and ligated into the *Eco*RI-linearised and dephosphorylated pCR® 4Blunt-TOPO® vector, using the pCR® 4Blunt-TOPO® blunt, according to the manufacturer's protocol (Invitrogen Corporation). Protoplasts of *E. coli* DH5α were transformed with the ligation mixture. The resulting recombinant plasmid was named pSXL2. The presence of the appropriate insert was determined by PCR and by digestion analysis. DNA products were analysed on a standard 1% agarose gel containing ethidium bromide. DNA sequences were determined using the dideoxynucleotide chain termination method according to a cycle sequencing protocol using thermosequenase (Amersham Pharmacia Biotech).

The sequencing reactions were analysed with the DNA sequencer (Genom express, Grenoble, France). The sequencing was performed three times, using the recombinant vector (pSXL2) from three clones as template with M13-sens primer and the M13-antisens primer (Amersham Pharmacia Biotech).

The nucleotide sequence determined in this study has been deposited in the Gene Bank database under access number: HM536978.

2.5.3. Construction of the recombinant plasmids

The recombinant plasmid (pSXL2) was double digested by *Nde*I/*Bam*HI. The double digestion product was purified and ligated into a previously *Nde*I/*Bam*HI linearised and dephosphorylated pET-14b vector. *E. coli* DH5α competent cells were transformed with the constructed pET-14b-SXL2 plasmid. The positive colony with gene inserts in the plasmid was identified by single restriction digestion of the plasmid with *Eco*RI, followed by agarose gel analysis. The identified positive colony was grown in LB medium containing ampicillin (100 μg/ml), and the plasmid pET-14b-SXL2 was isolated from bacteria cells using a plasmid extraction Kit. The isolated pET-14b-SXL2 plasmid was then used to transform *E. coli* strain BL21 (DE3) competent cells for expression purposes.

2.5.4. Expression and production of the recombinant lipase

The *E. coli* BL21 (DE3) strains containing the recombinant plasmids were grown at 37 °C in 50 ml LB medium containing 100 μg/ml ampicillin to an OD_{600} of 0.6. The culture was then adjusted to 0.4 mM IPTG (data not shown) and incubation continued at 37 °C for 14 h. Cells were harvested by centrifugation at 6000 rpm for 10 min and washed two times with buffer C (20 mM Tris–HCl, pH 8, 50 mM NaCl, 20 mM immidazole). The cells collected by centrifugation were resuspended in the buffer C and sonicated with 6 mm diameter tip, to release intracellular proteins. The cell free extract was centrifuged at 10,000 rpm for 20 min to remove cell debris and assayed for lipase activity. The culture broth obtained from the first centrifugation step was collected and assayed for secreted or intracellular lipase activity.

2.6. Procedure of lipases purification

2.6.1. Purification of the r-SXL2

As mentioned above, the clear supernatant, obtained after the last step of centrifugation, was mixed with 15 ml of the Ni^{2+} nitriloacetate (NTA) resin (Qiagen, CA, USA) equilibrated with buffer C. The crude extract–NTA mixture was loaded into a chromatographic column and washed with 200 ml buffer C. Lipases were eluted with a linear imidazole gradient (200 ml of 20–500 mM in buffer C).

2.6.2. Purification of the wt-SXL2

240 ml of culture medium, obtained after 24 h of cultivation, was centrifuged for 15 min at 8500 rpm to remove the cells. The supernatant containing extracellular lipase was used as the crude enzyme preparation.

- *Ammonium sulphate precipitation*: The cell-free culture supernatant was precipitated using solid ammonium sulphate to 65% saturation. The pellet obtained after centrifugation (30 min at 8500 rpm) was dissolved in 10 ml of buffer A (20 mM sodium acetate pH 5.4, 20 mM NaCl, and 2 mM benzamidine). Insoluble material was removed by centrifugation at 13,000 rpm during 5 min.
- *Heat treatment*: The supernatant obtained (10 ml) was incubated at 55 °C for 15 min. Insoluble material was removed by centrifugation at 13,000 rpm during 5 min.
- *Filtration on Sephacryl S-200*: The enzyme solution (10 ml) was applied to a Sephacryl S-200 column (2.5 cm × 150 cm) previously equilibrated in buffer A at a rate of 35 ml/h. The fractions containing the lipase activity (eluted at 1.4 void volume) were pooled.
- *Cation exchange chromatography*: Active fractions eluted from Sephacryl S-200 column were poured into a Mono S-Sepharose cation exchanger equilibrated in buffer A. The column (2 cm × 30 cm) was rinsed with 400 ml of the same buffer. No lipase activity was detected in the washing flow. Adsorbed material was eluted with a linear NaCl gradient (300 ml of 20–500 mM in buffer A) at a rate of 45 ml/h. The wt-SXL2 activity was eluted between 125 and 310 mM NaCl.
- The fractions containing the lipase activity were pooled, concentrated and washed three times with (3× 100 ml) of buffer B (25 mM Tris–HCl, pH 8) to remove NaCl. The enzyme was finally diluted in 50 ml of buffer B and poured into a Mono Q-Sepharose anion exchanger equilibrated in buffer B. The column (2 cm× 30 cm) was washed with 400 ml of the same buffer. No lipase activity was detected in the washing flow. Adsorbed material was eluted with a linear NaCl gradient (100 ml of 20–500 mM in buffer B) at a rate of 45 ml/h. The wt-SXL2 activity was eluted between 175 and 250 mM NaCl.

2.7. Analytical methods

- The fractions containing lipase activities (wt-SXL2 or r-SXL2) were analysed by analytical polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate (SDS–PAGE) following the method of Laemmli [21].
- The protein concentration was determined as described by Bradford [22] using BSA as standard.
- The N-terminal sequence of purified wt-SXL2 was kindly determined by Pr Hafedh MEJDOUB with automated Edaman's degradation, using an Applied Biosystems Protein Sequencer Procise 492 CLC. Samples for sequencing were electroblotted according to Bergman and Jörnvall [23]. Protein transfer was performed for 1 h at 1 mA/cm^2 at room temperature.

2.8. Effect of pH and temperature on enzyme activity and stability

- Lipase activity was tested at different pHs (5–9.5) at 55 °C. The pH stability of the two purified lipases was determined by incubating the enzyme in glycine (pH 3–4), sodium acetate (pH 5–6), phosphate (pH 7), Tris–HCl (pH 8–9) and borate (pH 10–11) for 24 h at room temperature. The residual activity was determined,

after centrifugation, under standard assay method.
- The optimum temperature for the lipase activity was determined by carrying out the enzyme assay at different temperatures (37–60 °C) at pH 8.5. The effect of temperature on lipase stability was determined by incubating the enzyme solution at different temperatures (37–60 °C) for 60 min. The residual activity was determined, after centrifugation, under standard conditions.

2.9. Esterification reaction

The wt-SXL2 immobilization was made onto $CaCO_3$ as previously described [30]. The esterification reaction was performed, using immobilized wt-SXL2, in screw-capped flasks. The reaction mixture containing different substrate concentrations and different amount of immobilized lipase was carried out at different temperatures with shaking (200 rpm). Aliquots of the reaction mixture were withdrawn periodically. The residual acid content was assayed by titration with sodium hydroxide 3.5 g/l using phenolphthalein as indicator and 2 ml of ethanol as quenching agent. The conversion percentage in ester synthesis was based on the amount of the consumed acid.

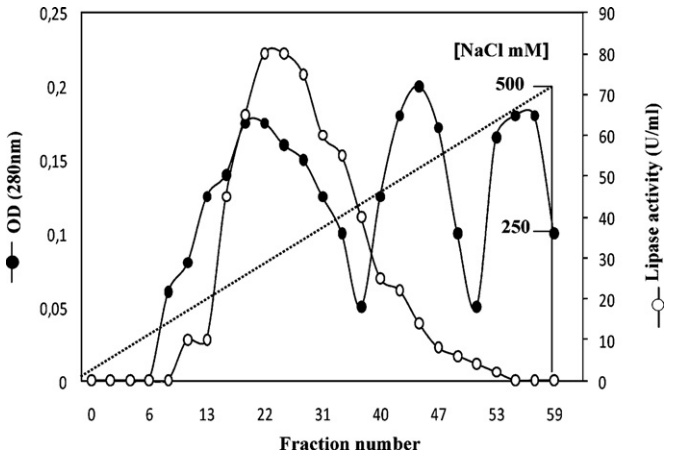

Fig. 1. Chromatographic profile of wt-SXL2 on Mono-S Sepharose. The column (2 cm × 30 cm) was equilibrated with buffer A. The elution of the adsorbed proteins was then performed with a linear gradient of NaCl (20–500 mM) at a rate of 45 ml/h. wt-SXL2 activity was eluted between 125 and 310 mM NaCl.

3. Results and discussion

3.1. Screening of lipolytic microorganisms

Screening and isolation of microorganisms for lipase activity is most frequently carried out employing agar plates containing triacylglycerol or Tweens. Lipase catalysed hydrolysis gives rise either to clearing or opacity zones developed around colonies of lipolytic organisms.

An initial screening of 120 strains from various Tunisian biotopes was carried out. Colonies giving rise to widespread clearing around them and were regarded as putative lipase producers. About 20 strains in the first stage were selected for their ability to growth at high temperature (45 and 55 °C) and alkaline conditions (8–9).

S. xylosus strain was chosen for lipase production since it produced the highest lipase activity (220 U/ml) at elevated temperature and alkaline conditions. The maximal lipase activity was obtained at the beginning of the stationary phase, after 24 h of growth in a medium A (data not shown). A decrease in lipase activity was observed during the late stationary phase, probably due to the presence of proteases in the culture medium. The lipase production by *S. xylosus* 2 (220 U/ml) was approximately 8-fold higher than the one produced by the *S. xylosus* 1 (30 U/ml), previously isolated [24]. It is noteworthy that the two strains were cultivated under the same conditions and the activity was measured at the optimal conditions of each lipase using tributyrin as substrate. In addition to the difference in the activity level, the two lipases (SXL1 and SXL2) showed other biochemical differences especially in the temperature and pH stability.

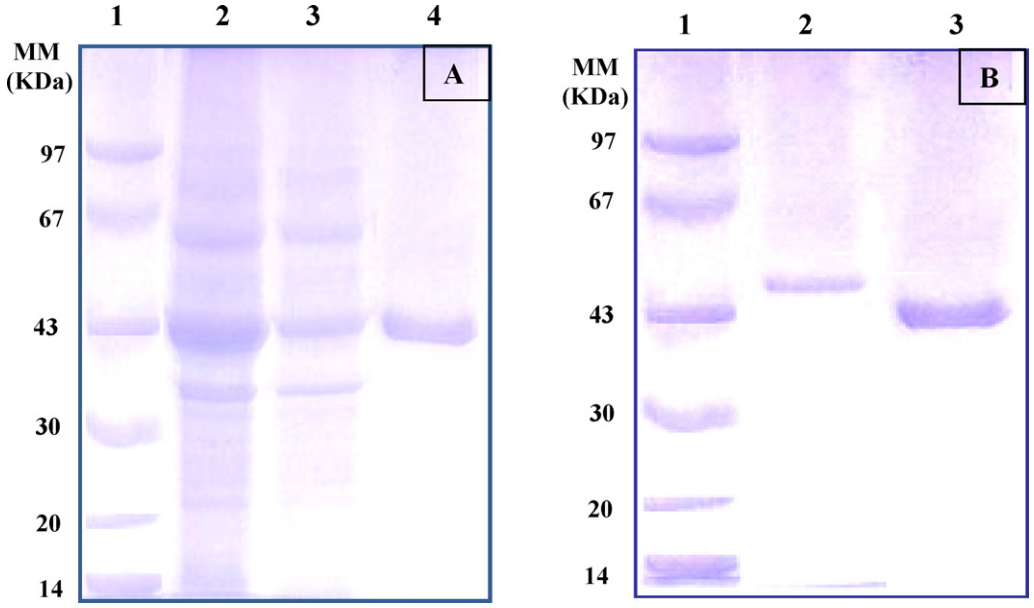

Fig. 2. Analysis of the purified wt-SXL2 and r-SXL2 by SDS/PAGE (12%). (A) Lane 1, molecular mass markers; lane 2, profile of the wt-SXL2 (20 μg) obtained after Sephacryl S-200; lane 3, profile of wt-SXL2 (15 μg) obtained after Mono-S chromatography; lane 4, 10 μg of purified SXL2. (B) Lane 1, molecular mass markers; lane 2, 10 μg of purified r-SXL2; lane 3, 10 μg of purified wt-SXL2.

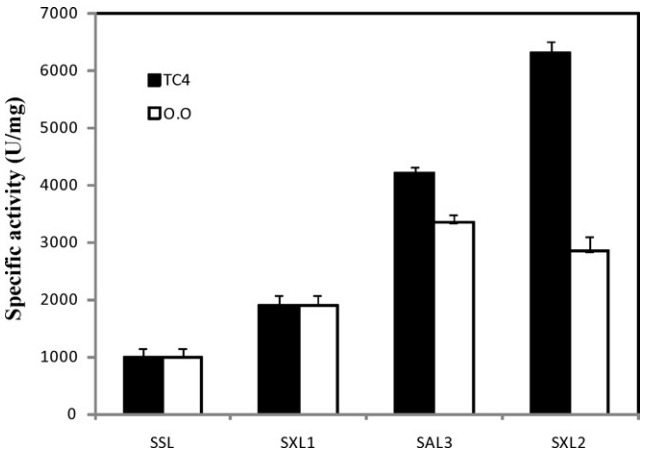

Fig. 3. Specific activities of *S. simulans*, *S. xylosus* 1, *S. aureus* and *S. xylosus* 2 lipases using tributyrin or olive oil as substrate. The activities were determined at the standard conditions of each enzyme.

3.2. Purification of the wt-SXL2

After 24 h of culture, the wt-SXL2 was purified according to the procedure described in Section 2. The elution profile of the wt-SXL2 obtained after the cation exchange chromatography (Mono-S Sepharose) is shown in Fig. 1. This figure shows that the wt-SXL2 was eluted between 125 and 310 mM NaCl.

The results of the SDS–PAGE analysis of the pooled fractions of the wt-SXL2 and after the last step of chromatography are given in Fig. 2A. This figure shows that the wt-SXL2 exhibited one band corresponding to a molecular mass of about 43 kDa.

The purification flow sheet is shown in Table 1. The specific activity of the pure lipase reached 6300 U/mg using TC_4 as substrate in the presence of 2 mM $CaCl_2$, 2 mM NaDC at pH 8.5 and 55 °C, with a recovery of 11%. Under the same conditions, a specific activity of 2850 U/mg was obtained when using emulsified olive oil as substrate. The specific activity of the wt-SXL2 on TC_4 or olive oil was compared to other purified staphylococcal lipases previously described (Fig. 3). From the data presented in this figure, one can note that, in contrast to SSL and SXL1 which hydrolyse triacylglycerols irrespective of their chain length, the wt-SXL2 has a preference for the short chain substrate (TC_4). Moreover, the wt-SXL2 is the most active lipase in the staphylococcal family. It presents a specific activity 6-fold and 3-fold higher than the SSL [20] and SXL1 [24], respectively.

It is well known that the SHL, in addition to its hydrolytic activity on triacylglycerols, efficiently hydrolyses phospholipids [25]. In order to test if the wt-SXL2 and the r-SXL2 are able, like SHL, to hydrolyse phospholipids, the purified lipases were incubated with different phospholipids (egg-PC, PG, PE) at different pHs and temperatures. Our results show that, in contrast to SHL, the two lipases did not present a phospholipase activity on all used phospoholipids (data not shown).

Table 2
N-terminal sequence comparison of wt-SXL2 with SSL and SXL1.

Lipases	Sequence N-terminal
SXL2 [present study]	LKANQVQPLNKYPVVFVH
SAL3 [14]	LKANQVQPLNKYPVVFVH
SSL [20]	ANQVQPLNKYPVVFVHGFLGLVG
SXL1 [24]	ANQVQPLNKYPVVFVHGFLGLVG

3.3. NH_2-terminal sequence of wt-SXL2

The 18 NH_2-terminal amino acids of the wt-SXL2 determined by Edman degradation were: L-K-A-N-Q-V-Q-P-L-N-K-Y-P-V-V-F-V-H. The above mentioned N-terminal sequence of the wt-SXL2 exhibits a high identity with lipases of the same genus previously characterized [14,20,24] (Table 2). Two additional amino acid residues (LK) are present at the N terminus of purified lipase from *S. xylosus* 2 as compared to SXL1 [24]. The presence of the additional dipeptide Leu-Lys at the N terminus of the wt-SXL2 might originate from a different proteolytic cleavage during processing of the mature enzyme. These residues might be responsible for the differences observed in the biochemical properties of the two lipases. In previous work we have shown that the insertion of two residues (LK) at the N-terminus of the SXL1 was accompanied by an improvement of the temperature action and the stability of the modified enzyme [26].

3.4. Cloning and sequencing of the mature lipase gene region

The PCR product corresponding to the gene part encoding the mature SXL2 was cloned and sequenced. It revealed a sequence of 1191 nucleotides (Fig. 4). The deduced polypeptide sequence, corresponding to the mature protein, comprises 397 amino acids with a molecular mass of 43 kDa. As revealed by sequence similarity with *Staphylococcus hyicus* lipases [27], Ser 119, Asp 310 and His 352 are the catalytic triad of SXL2. The amino acid sequence of this mature lipase shows a high identity (98.7%) with the mature amino acid sequence of SSL [20]. The comparison of the two amino acids sequences shows that the wt-SXL2 is identical to the SSL with the addition of two amino acid residues (LK) at the N terminus and the substitutions Tyr 39 by Phe, Asn 247 by Asp and Val 311 by Gly.

The lipase expression vector, pET-14b, was constructed by legating the 1191 pb *Nde*I/*Bam*HI fragment pCR® 4Blunt-TOPO® vector into *Nde*I/*Bam*HI digested pET-14b. This construction places the lipase gene under of a T7 promotor and in frame with N-terminal region coding for six histidines residues, which greatly facilitate the protein purification process.

Table 1
Flow sheets of the wt-SXL2 and the r-SXL2 purification.

Purification step	Total activity (Units)[a]	Proteins[b] (mg)	Specific activity (U/mg)	Activity recovery (%)	Purification factor
wt-SXL2					
Culture supernatant	48,000	247.42	194	100	1
$(NH_4)_2SO_4$ precipitation	33,000	77.1	428	69	2.2
Heat treatment (15 min at 55 °C)	27,000	51	529	57	2.72
S-200 chromatography	16,500	18	916	35	4.72
Mono-S chromatography	7000	2.69	2600	14.6	13.4
Mono-Q chromatography	5300	0.85	6300	11	32.5
r-SXL2					
Culture supernatant	16,500	13.75	1200	100	1
Ni–NTA affinity chromatography	11,050	2.7	4100	67	3.41

[a] 1 Unit corresponds to 1 μmol of fatty acid released per min using tributyrin as substrate.
[b] Proteins were estimated by the Bradford method [21].

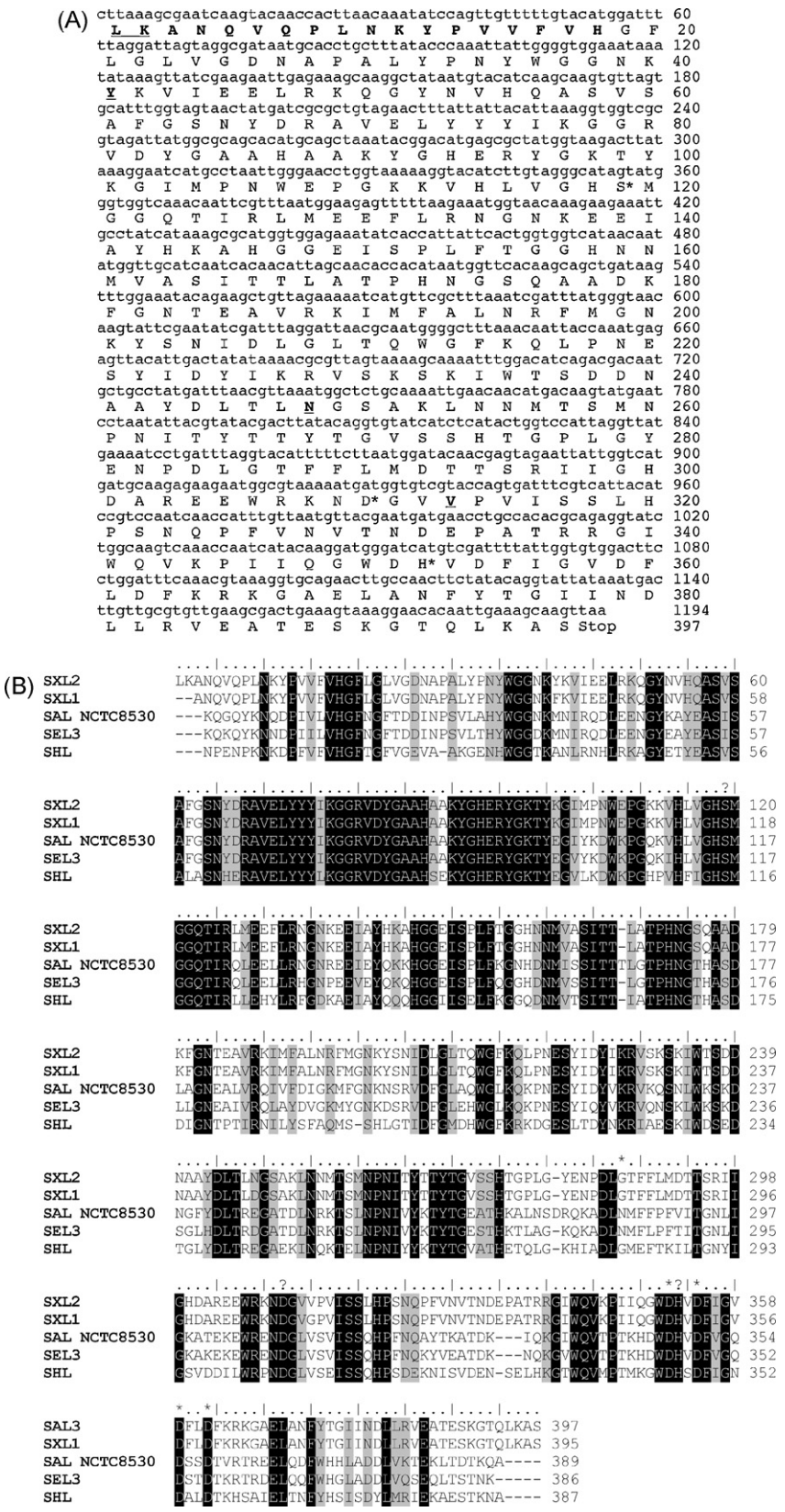

Fig. 4. (A) Nucleotide sequence of the SXL2 gene part encoding the mature lipase and the deduced amino acid sequence. Experiment was performed three times from three different clones, no difference was observed (see Section 2). The amino acid sequence obtained by sequencing of the pure wt-SXL2 N-terminal is shown bold. The differences between wt-SXL2 and SXL1 [23] are highlighted in bold and underlined. The wt-SXL2 presents two additional LK at the N terminus and three substitutions at the position 41 (Tyr substituted by Phe), Asn 247 by Asp and Val 311 by Gly. * indicates the catalytic triad (Ser, Asp and His). (B) Alignment of the amino acid sequences of the mature forms of SXL2 (present study, HM536978), SXL1, SAL NCTC8530, SEL and SHL. Boxes in black indicate positions at which the amino acids are identical in the five proteins. Boxes in gray indicate the location of similar residues of the five protein sequences. The closed triangles indicate the Ser, Asp and His which form the catalytic triad. Stars indicate the calcium-binding residues. The dashes represent gaps introduced during the alignment process. The alignment was generated with BioEdit software.

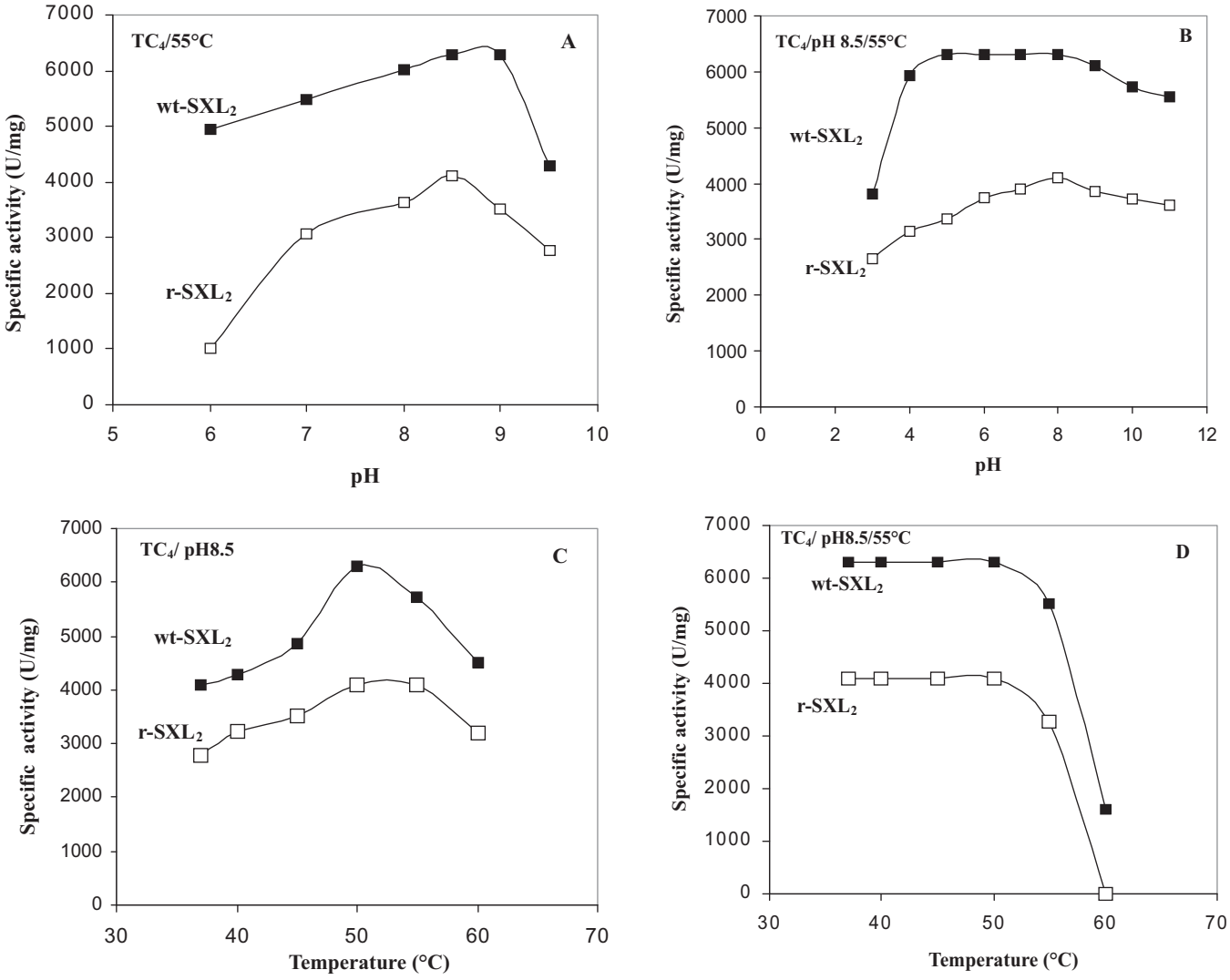

Fig. 5. pH effect on enzyme activity (A) and stability (B) of wt-SXL2 or r-SXL2. Optimal pH was determined with tributyrin at 55 °C under the standard conditions. Stability was analysed after preincubating the pure enzyme for 24 h in different buffer solutions at various pHs ranging from 3 to 12. Temperature effect of on wt-SXL2 and r-SXL2 activity (C) and stability (D). The stability was evaluated after incubation of the pure enzyme at various temperatures for 1 h then the remaining activity was measured under standard conditions.

3.5. Expression and purification of the r-SXL2

The expression of the *S. xylosus* lipase in *E. coli* BL21 (DE3) was carried out in the presence of different concentrations of IPTG (0.1–0.8 mM). The initial absorbance (OD) used for the r-SXL2 and wt-SXL2 production was 0.2. The lipase activity of r-SXL2, like the wt-SXL2, was detected to start soon after incubation and reaches its maximal (600 U/ml) at the end of the exponential phase corresponding to 10 h of cultivation time after induction with 0.4 mM IPTG (data not shown). Lipase activity of r-SXL2 (600 U/ml) was approximately 3-fold higher than that of the wt-SXL2 (220 U/ml). The difference in the activity level between the two lipases could be explained by the fact that the r-SXL2 was expressed in *E. coli* under the control of a strong T7 promoter which is induced by IPTG.

The purification of the r-SXL2 was facilitated by the presence of six histidine residues at the N-terminus of the enzymes. This allowed us to use only one step to purify the lipases using Ni–NTA affinity chromatography. The results of SDS–PAGE of the pooled fractions after the Ni–NTA affinity step are given in Fig. 2B. It was found that the purified recombinant lipase exhibits one band corresponding to a molecular mass of about 45 kDa. The difference of about 2 kDa between the wild type and recombinant tagged lipase is due to the presence of the His tag extension at the N-terminal of the mature lipase (Fig. 2B).

The purification flow sheet given in Table 1 showed that the r-SXL2 was purified to homogeneity with a high purification yield of 67%. One can note that the use of Ni–NTA gel allowed us to produce a high amount of the His tag lipase (2.7 mg). In contrast, a lower quantity (0.8 mg) of the pure r-SXL2 was obtained after three chromatographic steps.

Table 1 shows also that the specific activity of the r-SXL2 was 4100 U/mg or 1500 U/mg when using TC_4 or olive oil emulsion as substrates, respectively. The slight decrease in the specific activity of the r-SXL2 (4100 U/mg) compared to the wt-SXL2 (6300 U/mg) is probably due to the presence of six histidine residues at the N-terminus of the recombinant protein. Similar results were previously observed by Horchani et al. showing that the N-terminus His-tag affects the specific activity of the SAL3. The effect of the His-tag is more pronounced when using olive oil as sub-

strate. In contrast, with a partly water soluble substrate (TC_4) this above mentioned negative effect is minimum [28,29]. In the light of the data obtained in previous study, it can be assumed that independently from the negative effects of the recombinant expression process per se, the presence of an N-terminal tag extension decrease the catalytic activities of staphylococcal lipases by creating a steric hindrance during the interfacial binding step [28,29]. An alternative hypothesis is that the N-terminal tag extension process may be responsible for a change in the orientation of the lipase at the interface. One can also note that the wild type staphylococcal lipases are secreted into the culture medium, contrary to what occurs with the recombinant untagged or tagged lipases, which are produced in the form of intracellular proteins [14,20,24]. It has been reported that the rate of translation of a protein can affect its folding and activity. This hypothesis might therefore explain the differences observed here between secreted and intracellular staphylococcal lipases.

3.6. Comparison of the biochemical properties of the wt-SXL2 and the r-SXL2

3.6.1. Effects of pH and temperature on wt-SXL2 and r-SXL2 activity and stability

A great deal of research is currently going into developing lipases, which will work under thermostable and alkaline conditions as fat stain removers.

The activity of the wt-SXL2 and the r-SXL2 was investigated at different pHs using tributyrin as substrate (Fig. 5A). Results show that, the wt-SXL2 and the r-SXL2 remain active at alkaline pH ranging from 8 to 9.5 and their maximal activities were measured at pH 8.5.

To determine the pH stability, the two lipases were incubated at different pHs and the residual activity was determined under optimal conditions at pH 8.5 and 55 °C. Our results show that the two lipases were stable at a broad range of pH values between pH 4 and 12 after 24 h-incubating (Fig. 5B). The activity and the stability of lipases in alkaline media are very attracting, for example, lipase produced by *Acinetobacter radioresistens* has an optimum pH of 10 and it was stable over a pH range of 6–10; this enzyme has a great potential for application in the detergent industry [30].

The lipase activity was also measured at different temperatures (37–60 °C) under standard assay conditions (Fig. 5C). One can note that the wt-SXL2 and the r-SXL2 activity increased significantly with increasing the temperature to reach its maximum value at 55 °C. The thermostability of the wt-SXL2 and the r-SXL2 was also determined by measuring the residual activity after incubation (60 min) of the pure lipases at various temperatures (37–60 °C) (Fig. 5D). The wt-SXL2 and the r-SXL2 retained 90% of their initial activities after 60 min incubation at 55 °C.

Thermostable and alkaline lipases are therefore highly attractive [31] to the synthesis of biopolymers and biodiesel and used for the production of pharmaceuticals, agrochemicals, cosmetics, and flavour [32]. All these findings allow us to conclude that the wt-SXL2 and the r-SXL2 could be good candidates for industrial applications.

3.7. Effects of bile salts on wt-SXL2 and r-SXL2

It is well known that all detergents act as potent inhibitors of pancreatic and some microbial lipases [33]. In order to check if the purified wt-SXL2 and r-SXL2 are able to hydrolyse triacylglycerols in the presence of some surface-active agents like bile salts, the hydrolysis rate of TC_4 by wt-SXL2 and r-SXL2 was measured in the presence of various NaDC concentrations. Fig. 6 shows that the maximal activity for the wt-SXL2 or r-SXL2 is reached in the presence of 2 mM or 1 mM of NaDC using tributyrin as substrate,

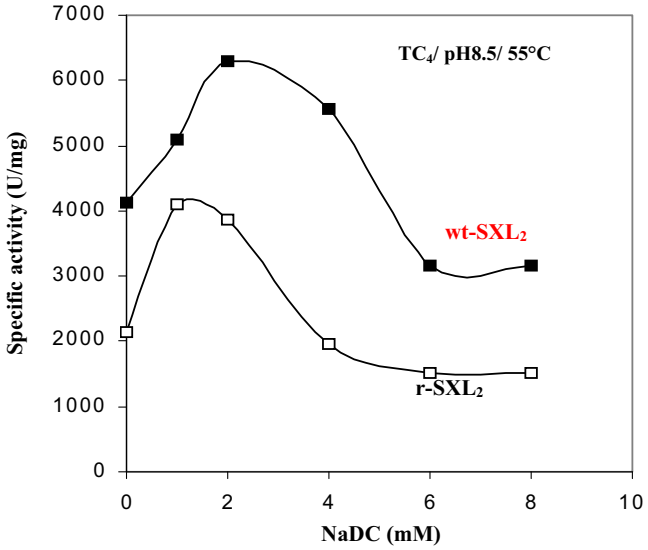

Fig. 6. Effect of increasing concentration of detergent NaDC (0, 1, 2, 4, 6, 8 mM) on the hydrolysis rate of tributyrin by wt-SXL2 and r-SXL2. Lipolytic activity was measured using tributyrin as substrate at optimal conditions of each lipase at pH 8.5 and 55 °C. Assay conditions: tributyrin (0.25 ml) in 30 ml of 2.5 ml Tris–HCl.

respectively. This activity drops 50% down at a concentration of bile salt between 5 and 8 mM. One can note that wt-SXL2 and r-SXL2 are able to hydrolyse efficiently triacylglycerols even in the presence of bile salts. Similar results were obtained with SSL [20], SXL1 [24] and SAL3 showing that these enzymes are stable at high concentrations of detergents. Hence, it can be deduced that SXL2 penetration power is higher than those of pancreatic and some microbial lipases since it can hydrolyze TC_4 even in the presence of amphiphilic proteins.

3.8. Effects of calcium on wt-SXL2 and r-SXL2 activity

Previously, it has been demonstrated that the activity of staphylococcal lipases may depend on the presence of Ca^{2+} ions [4]. We therefore studied the effect of various Ca^{2+} concentrations on the rate of hydrolysis of wt-SXL2 and r-SXL2. Our results showed that the wt-SXL2 activity requires the presence of Ca^{2+} to trigger the hydrolysis of triacylglycerols. A maximum activity was obtained in the presence of 2 mM $CaCl_2$ (data not shown). In contrast, the activity of the r-SXL2 can be detected in the absence of Ca^{2+}. In fact, a specific activity of 2050 U/mg was measured in the absence of Ca^{2+} and in the presence of 10 mM of chelator such as EDTA or EGTA, when using tributyrin as substrate. This result is in line with previous works showing that the enzymatic activity of staphylococcal lipases is stimulated by Ca^{2+} [4,20]. It has been also reported that lipases from *P. gluma* [34] and *S. hyicus* [35] contain a Ca^{2+} binding site which is formed by two conserved aspartic acid residues near the active-site, and that the binding of the Ca^{2+} ion to this site dramatically enhanced the activities of these enzymes. Both residues are conserved in wt-SXL2 as well as in all other staphylococcal lipases sequences, indicating that these lipases probably bind Ca^{2+} at the same site [34].

3.9. Enzymatic esterification

Interest in the staphylococcal lipases was originally stimulated by their ability to catalyse the esterification, interesterification, and transesterification in non-aqueous media. The obtained esters are versatile components of flavours, fragrances, antioxidants which are widely used in the food, beverage, cosmetic, and pharmaceutical industries. Therefore, the ability of the newly isolated

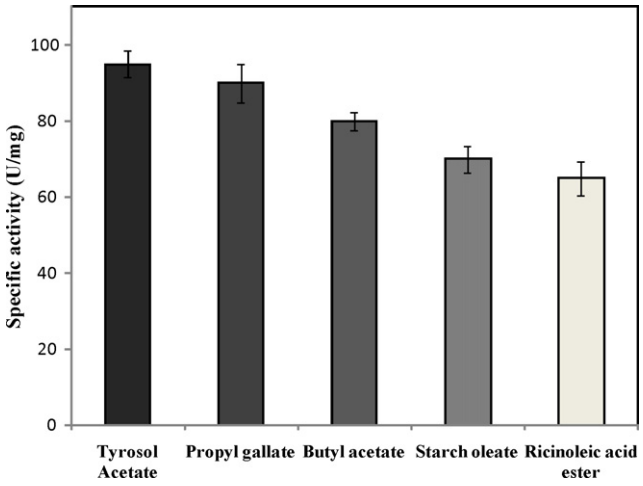

Fig. 7. Synthesis of tyrosol acetate, propyl gallte, butyl acetate, starch oleate and ricinoleic acid esters by immobilized *Staphylococcus xylosus* lipase (wt-SXL2).

immobilized wt-SXL2 to synthesize some esters such as propyl-gallate, starch oleate, butyl oleate, tyrosol acetate and ricinoleic acid esters was studied. After optimization of the conditions of the synthesis of such molecule, the conversion yields were determined at equilibrium and the results are shown in Fig. 7. One can note that the immobilized wt-SXL2 is able to synthesise antioxidants such as propyl gallate and tyrosol acetate with a high conversion yield of 90 ± 3.5 and $95 \pm 5\%$, respectively. It can also be used as biocatalyst to synthesize butyl acetate (pineapple flavour) with a high conversion yield (80 ± 2.5). Others esters were also synthesized using immobilized wt-SXL2 as biocatalyst by esterification of oleic acid with starch to produce fully biodegradable thermoplastic materials ($70 \pm 3.5\%$ of conversion yield) and by esterifiaction of ricinoleic acid to produce ricinoleic acid esters ($65 \pm 4.5\%$ of conversion yield). These achievements would help to save petrochemical resources and to find out new industrial uses of starches and castor oil.

4. Conclusions

In this study the lipase from *S. xylosus* 2 displayed encouraging properties for biotechnological applications which are different from other staphylococcal lipases. It presents a specific activity 6-fold and 3-fold higher than the SSL and the SXL1. The expression of this enzyme produces a quantity of purified lipase higher than that obtained with other over-producing bacteria. Many properties of wt-SXL2 were investigated, the high stability and activity at high temperature and at alkaline conditions and the ability to synthesise a high value molecules are especially encouraged.

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