Chapter 5

GROWTH KINETICS AND ESTERASE/LIPASE PRODUCTION BY THERMOPHILIC FUNGI GROWN IN SOLID STATE FERMENTATION ON OLIVE CAKE

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Abstract

Three thermophilic fungal strains *Rhizopus homothallicus* 13a, *Rhizomucor pusillus* S5 and *Rhizomucor pusillus* S8 were grown in solid state fermentation on sugar cane bagasse-olive cake mixture (50% each) at 47°C for 48 h. Humidity and pH increased during fermentation, from 5.1-5.2 to 5.5-6.1 and from 64% to 69% respectively. For all strains, O_2 uptake rates reached a maximum of 1.7-2.2 ml/h/g IDM (initial dry matter) at an early stage of cultivation (7.5-9 h) and high respiratory activities (μr) were obtained (1.2-1.4 h^{-1}). The maximal O_2 uptake at 48h of culture was 28.1, 31 and 33.2 ml/g IDM for strains 13a, S5 and S8 respectively. All strains produced lipolytic activities, which varied from 15 U/g IDM for strain S8 to 47 U/g IDM for strain 13a when olive oil was used as enzyme substrate. The lipolytic activity of crude enzyme extracts from strain S8 was specific of mid-chain length fatty acid esters. There was evidence of lipase activity (active on long chain fatty acid esters) and esterase activity (active on short chain fatty acid esters) for strain 13a. The latter one was present at 24h but not at 48h of culture. The specificity pattern of strain S5 extract looked similar to that of strain 13a. After incubation in *n*-heptane for 8 days at 37°C, the enzyme extracts retained 65%, 66% and 84% of initial activity for strains 13a, S5 and S8 respectively.

Keywords: *Rhizopus homothallicus, Rhizomucor pusillus*, thermophilic fungi, solid state fermentation, lipase, esterase, organic solvent.

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Introduction

Solid state fermentation (SSF) processes involve the growth and metabolism of microorganisms on moist solid substrates in the absence or near absence of free-flowing water (**1**). These processes offer many advantages over submerged cultures (SMC) mainly simplicity of fermentation medium, reduced energy demand, easier scale-up of processes, greater product yields and easier control of contamination due to the low moisture level in the system (**2**).

Filamentous fungi have a strong capability to grow in the absence of free water (**1**) since these conditions are similar to their natural habitat (**3**). Therefore, they are often most suited to SSF for the production of several valuable metabolites. Many kinds of enzymes are produced in SSF: pectic enzymes (**4,5**), amylases and glucoamylases (**6,7**) and cellulases (**8,9**).

Esterases (EC 3.1.1.1) and lipases (EC 3.1.1.3) are enzymes with great potential for biotechnological applications such as esters synthesis as flavors for the food industry (**10**), the modification of physico-chemical properties of triglycerides in the fat and oil industry (**11**) and for the preparation of optically pure bioactive molecules for the pharmaceutical industry (**12,13**).

Despite these potential applications, few studies have been reported about esterases and lipases production in SSF using filamentous fungi (**14 -20**).

In a previous work, Cordova et *al.* (**21**) reported the production of lipase by the thermophile fungi *Rhizomucor pusillus* A16 and *Rhizopus rhizopodiformis* 13a grown on sugarcane bagasse-olive cake mixture.

The aim of this study was to investigate the esterase/lipase production and the respiratory activity of three filamentous fungi strains grown on a sugarcane bagasse-olive cake mixture. The specificity and the stability of the crude enzyme extracts in *n*-heptane were also investigated.

Materials and Methods

Chemicals

Bactopeptone and Potato Dextrose Agar (PDA) were purchased from Difco Laboratories (Detroit, USA). Tris and KH_2PO_4 were purchased from ACP Chemicals (Montreal, Canada), $NaNO_3$ and $CaCl_2$ were obtained from BDH Chemicals (Montreal, Canada), $MgSO_4$ was purchased from (Fisher Scientific Nepean, Canada), *p*-Nitrophenol was obtained from American Chemicals (Montreal, Canada), 2-propanol was purchased from MAT (Beauport, Canada). Pyridine was from Omega Chemical company (Levis, Canada). Cupric-acetate-1-hydrate, gum arabic, heptane, isooctane, oleic acid (99%), olive oil, *p*-nitrophenyl esters, polyvinyl alcohol, tricaproin and Tween80 were obtained from Sigma (St-Louis, USA).

Microorganisms

Three thermophilic fungal strains were used in this study: strain *Rhizopus rhizopodiformis* 13a was obtained from ORSTOM culture collection (Montpellier, France) (**21**) and identified as *Rhizopus homothallicus* by the National Fungal Identification Service NFIS (Ottawa, Canada). Strains S5 and S8 were obtained from IAV (Rabat, Morocco) and identified by the NFIS as *Rhizomucor pusillus*. The strains were maintained on PDA agar slants at 4°C and subcultured every 4 months.

Substrate pretreatment

Sugarcane bagasse was obtained from the sugar refinery Dar El Gadari (Morocco) and olive cakes were obtained from various traditional olive oil extraction plants (Maâsra in Morocco). Both substrates were milled to 2.0 and 0.71 mm particles size range. Sugarcane bagasse was washed with 15 L of distilled water and then both substrates were dried at 60°C for 24 h for olive cake and 2-3 days for sugarcane bagasse before storage at 4°C. Before use, a mixture of these substrates was prepared (50:50) and incubated at 110°C for 15-21 h to reduce contaminants. The chemical composition of these substrates was as described by Cordova *et al.* (**21**).

Culture medium and conditions

The composition of the medium used was (/L): Bactopeptone 50 g, glucose 20 g, KH_2PO_4 1 g, $NaNO_3$ 1 g and $MgSO_4$ 0.5 g. Glucose was prepared separately (11.2% (w/v) aqueous solution) and sterilized at 121°C during 15 min.

The culture medium without glucose was prepared as a 2x medium and subsequently added to substrates mixture allowing 35% as moisture content. Sterilization was done at 121°C for 15 min. After cooling, the moisture content was increased until 64-66% by addition of spores suspension, glucose solution and sterile distilled water. Spores were collected by adding Tween 80 solution (0.1% (w/v)) to a one-week-old PDA agar culture followed by filtration through 0.19 mm filter. The inoculation rate was $2x10^7$ spores/g dry matter. The initial pH was 5.1-5.2.

The inoculated solid medium was transferred (26 g per unit) to sterile glass incubators (diam. 3 cm, length 19 cm) as described by De Araujo *et al.* (*22*) with little modification. The solid state fermentation was monitored in a similar apparatus as described by Cordova *et al.* (**21**). The incubators were maintained at 47°C and aerated with 26 ml/min water-saturated air. Growth was monitored by continuous analysis of carbon dioxide and oxygen by infrared and electrochemical sensors respectively using 3600 model CO_2-O_2 analyzer (Illinois Instruments, Ingleside, USA) coupled to automatic sampling. Before injection, outer air was dried using silica gel. Non-inoculated solid medium was used as a control and incubated in the same conditions. Each culture was done in triplicates.

Enzyme Assay

Spectrophotometric assay with triglycerides as substrate. The enzyme activity was measured using the formation of copper soaps for detection of free fatty acids according to Kwon and Rhee (**23**) with some modifications: The substrate solution contained olive oil or tricaproin (0.164 g/mL) emulsified with domestic blender during 15 min at 4°C in Tris-HCl 50 mM pH 8.7 containing 10% (w/v) gum arabic and 9.1 mM $CaCl_2$. Cupric-acetate-1-hydrate aqueous solution (50 mg/mL) adjusted to pH 6.1 with pyridine, was used as copper reagent.

The reaction was started by adding fermented substrates (0.25-0.5 g) to 10 ml of substrate emulsion preheated at 47°C. Enzyme reaction was carried out under shaking for 10-15 min and the reaction was stopped with 12N HCl. Fatty acids were extracted with 5 ml of isooctane and vigorous vortexing for 1 min 30 s. After a 10 min incubation in boiling water, phase separation was achieved by centrifugation (7810 g, 30 min). The organic phase was collected and added to 1 ml of copper reagent. After vigorous vortexing for 1 min 30 s., the absorption was measured at 715 nm versus a blank containing an autoclaved fermented substrate. Each measure was done in triplicate.

For calibration, pure oleic acid dissolved in isooctane to get final concentrations from 0 µmol/ml to 10 µmol/ml and added to copper reagent. One unit was defined as the amount of enzyme, which caused the release of 1 µmol oleic acid per minute under the given conditions.

Spectrophotometric assay with p-nitrophenyl esters as substrates. Activity was assayed by measuring the rate of hydrolysis of p-nitrophenyl esters at 37°C. One volume of 16.5 mM of substrate in 2-propanol was mixed with 9 volumes of 50 mM Tris-HCl pH 8.0, containing 0.25% (w/v) polyvinyl alcohol and 0.174% (w/v) $CaCl_2$. To 180 µL reaction mixture equilibrated at 37°C in a microplate well were added 20 µL of the enzyme solution. The absorbance was read at 405 nm using a microplate reader PowerWave X (BIO-TEK INSTRUMENTS, Vermont, USA) against a blank without enzyme and monitored continuously at 405 nm. The concentration of liberated *p*-nitrophenol (pNP) was calculated using an extinction coefficient of 8.6 10^6 cm^2/mol. This value was determined using standard solutions of pNP. For each assay, three different enzyme quantities were tested and activity was calculated from the slope of the curve of absorbance variation per min against enzyme amount. One enzyme unit was the amount of enzyme liberating one µmole of pNP per min in the conditions used. Fatty acid specificity was studied by the measure of hydrolysis of *p*-nitrophenyl esters from pNPC2 to pNPC16 as described above. This experiment was done in duplicate.

Assay techniques

The relative humidity and dry matter were determined by drying samples of solid medium (5 g) at 110°C for 24 h. The pH value of 10 fold diluted samples in distilled water was assayed by using a 718 Stat Titrino (Metrohm, Herisau,

Switzerland). Protein content of crude enzyme extracts was determined with the bicinchoninic acid (BCA) protein assay kit (Pierce, Instructions 23225).

Crude enzyme extract preparation

At the end of the culture, the contents of three columns were pooled and diluted 5 fold in Tris-HCl 50 mM pH 8.7. The matter was ground with a domestic blender to obtain fine powders (6 cycles: 1 min of mix and 2 min of pause) and diluted 2-fold before mixing with an Ultra-Turrax T25 homogenizer (Janke and Kunkel) (6 cycles: 1 min of mix and 1 min of pause). The supernatant was collected by centrifugation (14,300 g, 30 min, 4°C) and dialyzed overnight against Tris-HCl 50 mM pH 8.7 and finally lyophilized and stored at 4°C until use.

Stability in organic solvent

Thirty mg of crude enzyme extract with known activity were mixed with 10 ml of *n*-heptane in 40 ml glass bottles. The bottles were shaken (250 rpm) at 37°C during 8 days. The powder was collected by filtration, washed with acetone at -20°C and dried with P_2O_5. Each experiment was done in triplicates. The residual activity was determined by the measure of the hydrolysis of *p*-nitrophenyl laurate in standard conditions.

Data analysis

The O_2 data of the cultures were fitted with Tablecurve program. The correlation coefficients obtained, for the mean values of all the cultures, were between 0.93 and 0.98 for O_2 uptake rates and between 0.96 and 0.98 for cumulatives O_2 uptake. The respiratory activity (μr) values were directly calculated from the crude data of each culture and the mean values were calculated with standard deviation less than 7%.

Results and discussion

Growth kinetics of strains

Strains were grown in SSF on sugar cane bagasse-olive cake mixture (50% each) at 47°C for 48h as described in the Materials and Methods section. Humidity and pH were determined at initial and final times of culture. In this study, O_2 consumption was used as an indirect measure of the growth of the strains. Oxygen values (%) obtained were used to calculate the O_2 uptake rates from which, after integration, cumulative O_2 uptake was obtained.

At the end of cultures, medium pH increased slightly from 5.1 to 5.5 in strains 13a and S8 cultures while it reached 6.1 in the culture of strain S5. Relative humidity increased slightly from 64-66% to 68-69% at 48 h of culture. The figure 1 showed the growth kinetics of the strain *Rhizopus homothallicus* 13a on bagasse-olive cake mixture. The accelerated growth phase started after a 3h lag phase.

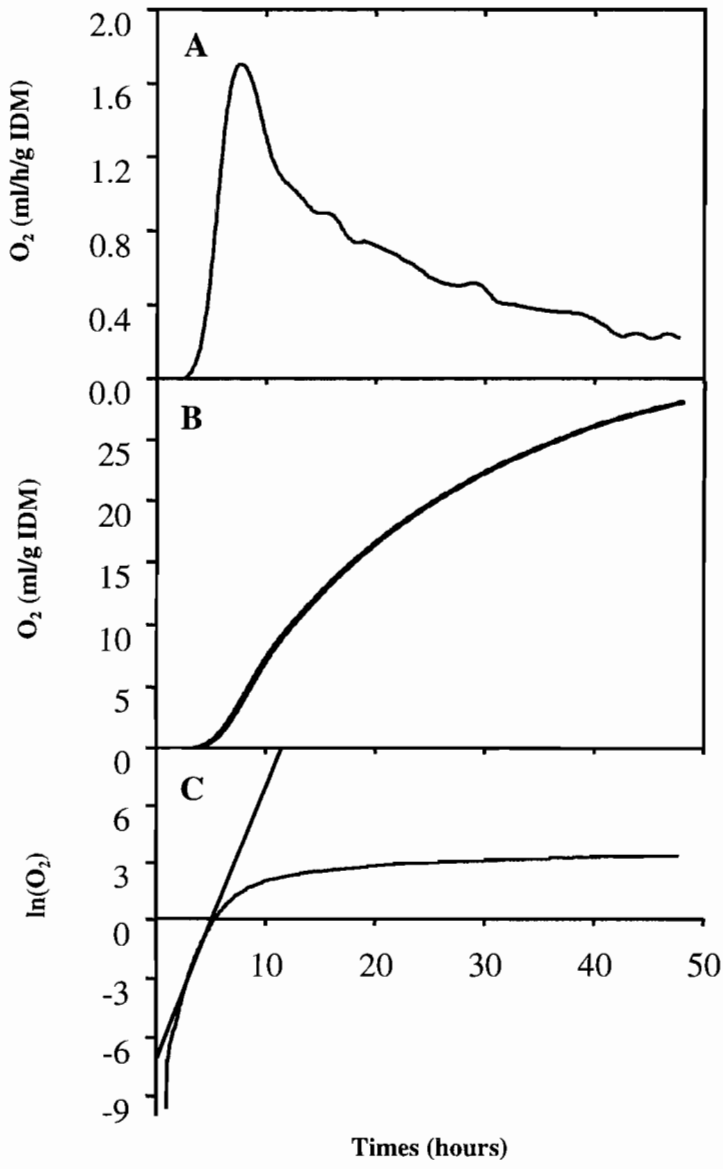

Figure 1: Growth kinetics of the strain *Rhizopus homothallicus* 13a grown in SSF on sugar cane bagasse-olive cake mixture. (**A**) O_2 uptake rate, (**B**) cumulative O_2 uptake and (**C**). Respiratory activity (μr) measurement.

Maximum O_2 uptake rate (1.7 ml/h/g IDM) was obtained at 7.5-8.0 h of incubation (Fig. 1A). At 48 h of culture, O_2 uptake reached 28.1 ml/g IDM (Fig. 1B).

Respiratory activity (μr) calculated at the beginning of incubation (0-15 h) according to Saucedo-Castaneda *et al.* (**24**) from O_2 data was 1.4 h^{-1} (fig 1C). This high value showed a fast growth of this strain in the conditions tested. This value was higher than those obtained with mesophilic strains like *Aspergillus niger* (**25**) and *Schwanniomyces castellii* CBS 2863 (**24**).

Strains S5 and S8 exhibited metabolic activities similar to strain 13a (Table 1). Maximal O_2 uptake rates were 2.0 and 2.2 ml/h/g IDM for both strains. The overall O_2 uptakes were slightly higher than that of the strain 13a. However, the respiratory activities were slightly lower than that obtained from the strain 13a.

Table 1: Growth parameters of strains S5 and S8 grown in solid state fermentation on sugar cane bagasse-olive cake mixture.

Growth parameters	Strains	
	S5	S8
Lag phase duration (H)	3.5-4.0	3-4
Maximal O_2 uptake rate (ml/h/g IDM)	2.0	2.2
Max. Time (H)*	8.0-9.0	7.5-8.0
Cumulative O_2 uptake (ml/g IDM)	31	33.2
Respiratory activity (μr)	1.3	1.2

* refers to time of maximal O_2 uptake rates.

The three strains showed the same ability to grow on a bagasse-olive cake mixture. Growth of the strains was fast despite the high temperature of culture (47°C) and the low moisture content (initial humidity was 64%).

Production of Lipolytic activities

Cordova *et al.* (**21**) reported that strain 13a, when grown on bagasse-olive cake mixture, produced a lipolytic activity assayed by the measure of olive oil hydrolysis. This result showed the occurrence of a lipase in the extracts but did not inform about esterases since they could not act on olive oil. In this study, the tricaproin (C6:0) was also used as enzyme substrate. Tricaproin is a partially soluble substrate and could be hydrolysed by both esterases and lipases. At 48h of culture, fermented matter was used as enzyme source and the activity was assayed at 47°C. The results are shown in Figure 2.

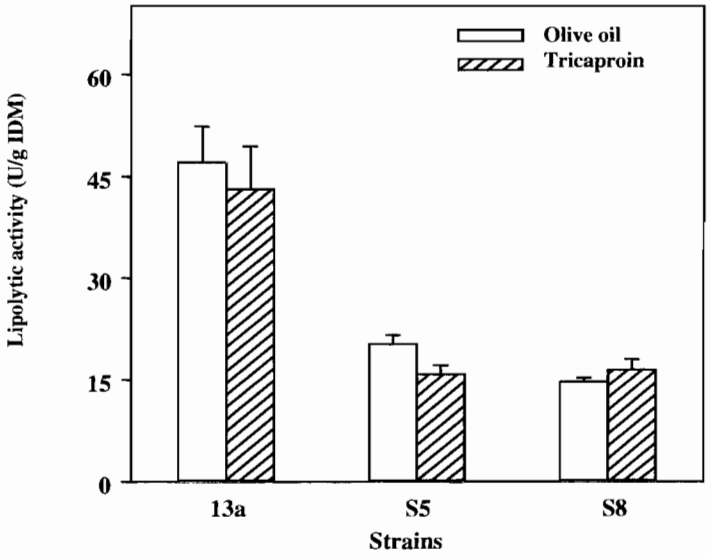

Figure 2: Lipolytic activity on olive oil and tricaproin of *Rhizopus homothallicus* 13a, *Rhizomucor pusillus* S5 and *R. pusillus* S8 grown in SSF on sugar cane bagasse-olive cake mixture at 47°C during 48h.

All strains produced lipolytic activities on bagasse-olive cake mixture. When olive oil was used as enzyme substrate, the enzyme activity was 15, 20 and 47 U/g initial dry matter (IDM) for strains S8, S5 and 13a respectively. Cordova *et al.* (**21**) reported that the lipase production by strain 13a was maximal at 24 h of culture (80 U/g IDM). When tricaproin (C6:0) was used as enzyme substrate, almost similar activities to those on olive oil were obtained. The ratios of activity on olive oil versus activity on tricaproin were 1.1, 1.3 and 0.9 for strains 13a, S5 and S8 respectively. Usually, lipase activity is maximal on long chain fatty acid esters and insoluble like olive oil (**26**) and esterase action is maximal on short chain fatty acid esters (carbon number from C2 to C5). In the present case, the similarity of activity on olive oil and tricaproin suggested the presence of several enzymes (esterases and/or lipases) responsible of the lipolytic activity of the strains or the occurrence of one enzyme with maximal activity on substrate other than olive cake and tricaproin.

The lipolytic activity obtained by strain 13a was higher than lipase production by *Aspergillus oryzae* (0.8 U/g of wet substrates) (**27**) and *Candida rugosa* on rice bran (27 U/ g) (**28**) and lower than the lipase production by *Aspergillus niger* on gingelly oil cake (363.6 U/g of dry substrate) (**19**) after 72 h of culture. *Rhizopus delemar* produced 96 U/g of Dextrin at 15 h of culture (**17**).

Substrate Specificity

Substrate specificity was studied to check whether lipases and/or esterases were responsible of lipolytic activities of the strains. Crude enzyme extracts were prepared as described in the Materials and Methods section and used as enzyme source. Instead of the triglycerides assay, activity was assayed with *p*-nitrophenyl esters because, with the former one, activity on triglycerides shorter than tricaproin (C6:0) could not be measured with a good reproducibility (**23**). A preliminary study showed a good correlation between these assays (results not shown).

Esters of different fatty acids from acetic acid (pNPC2) to palmitic acid (pNPC16) were used as enzyme substrates and the reaction rates were assayed under standard conditions.

Ratios of activity on a long chain fatty acid ester (*p*-nitrophenyl palmitate) versus activity on a short chain fatty acid ester (*p*-nitrophenyl caproate) were calculated. The values obtained were 1.2, 1.2 and 0.9 for 13a, S5 and S8 respectively. These ratios were equal or close to those calculated when the triglycerides assay was used (see paragraph 3.2). This result confirmed the correlation between the assays. The specificity results are shown in figure 3. The lipolytic activity of strain 13a (Fig. 3A) on long chain fatty acid esters (pNPC10 to pNPC16), was maximal on *p*-nitrophenyl laurate (pNPC12). On short and mid chain length of the fatty acid (pNPC2 to pNPC8) the activity was lower and the maximum was obtained on *p*-nitrophenyl caprylate (pNPC8). The relative activity on pNPC8 was 76% of the maximal activity found on pNPC12.

The lipolytic activity of the strain 13a seemed to agree with a lipase activity, which was higher on insoluble long chain fatty acids esters. When the enzyme extract obtained from a 24 h culture was used as enzyme source, activity on short chain fatty acid esters was higher than at 48h with maximal activity being on pNPC4 and pNPC6, while it remained stable on longer fatty acids esters with maximum on pNPC12 (data not shown).

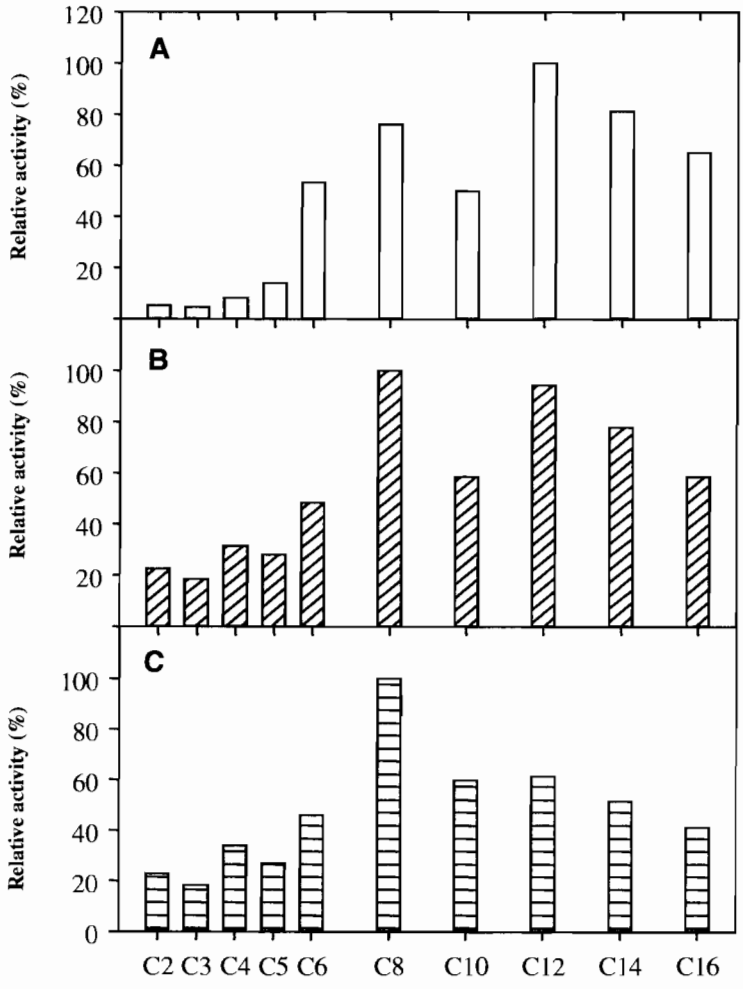

Figure 3: Effect of fatty acid chain length on specificity of lipolytic enzymes from *Rhizopus homothallicus* 13a (A), *Rhizomucor pusillus* S5 (B) and *R. pusillus* S8 (C). The initial rate of hydrolysis was assayed on substrate with fatty acid from acetic (pNPC2) to palmitic (pNPC16). The maximal activity was 0.98 U/mg protein for the strain 13a extract (on pNPC12), 0.16 U/mg protein for the strain S5 extract (on pNPC8) and 0.14 U/mg protein for the strain S8 extract (on pNPC8). Experiments were conducted in duplicate with standard deviation less than 10%.

This result showed that at least two enzymes were responsible for the lipolytic activity of the strain 13a, an esterase activity acting on short chain fatty acid esters and a lipase activity more active on insoluble fatty acid esters. The occurrence of at least two enzymes should be plausible for the lipolytic activity of the strain S5 (Fig. 3B). A specificity profile similar to that of strain 13a was observed. Activity was maximal on pNPC8 and pNPC12 (94%) and lower on short fatty acids esters (pNPC2 to pNPC6), pNPC10 and pNPC14 to pNPC16. Activity on short chain fatty acid esters was higher than that of strain 13a.

For strain S8 (Fig. 3C), the activity was maximal on pNPC8 and it decreased on shorter or longer fatty acids esters. This result suggested that at least one enzyme dominated in the S8 extract which could have a lipase activity like the lipases from *Mucor hiemalis f. hiemalis* (**29**), *Bacillus thermocatenulatus* (**30**) and *B. subtilis* (**31**) whose activities were maximal on pNPC8 or tricaprilyn. Esterases acted on short chain length fatty acids esters (C2 to C4) and few had maximal activity on mid chain length fatty acids (pNPC6 to pNPC8) like the esterase of *Bacillus acidocaldarius* (**32**) with maximal activity on pNPC6.

Stability in organic solvent

Stability of the enzyme extracts in heptane was determined to check the potential of the enzymes for biotechnological applications mainly ester synthesis. Known amounts of activity were incubated at 37°C in 10 ml heptane for 8 days with shaking. The residual activity of acetone washed extract was assayed at 37°C by the measure of hydrolysis of *p*-nitrophenyl laurate (pNPC12) in the standard conditions. The results are shown in Table 2.

Activity retention of 65%, 66% and 84% was obtained by the extracts of 13a, S5 and S8 respectively. This result showed that these enzyme extracts had a good stability in heptane. Similar result (75% of activity retention) was obtained when the mycelial lipase of *Rhizopus oryzae* was incubated for 8 days in heptane at 30°C (**33**). The lipozym of *Rhizomucor miehei* retained 90% of initial activity in 80% hexane after 1 h incubation at 25°C (**34**). Our result showed that these extracts might be useful for transesterification and ester synthesis.

Table 2 Stability of crude enzyme extracts in heptane (*).

Activity	Enzyme extracts		
Strains	13a	S5	S8
Relative activity (%)	66%	65%	84%

(*)Enzyme Extracts (30 mg) were incubated in 10 ml of heptane in shaking (250 rpm) at 37°C for 8 days. Activity was assayed by the measure of the hydrolysis of *p*-nitrophenyl laurate. Experiment was done in triplicate with standard deviation less than 5%.

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