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Tailoring chain length selectivity of a solvent-tolerant lipase activity from *Aspergillus niger* MYA 135 by submerged fermentation

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ABSTRACT

The use of biocatalysts in fuel industry is an interesting and greener alternative. In this connection, it was found that the chain-length selectivity profile of a solvent-tolerant lipase activity from *Aspergillus niger* MYA 135 determined in both hydrolytic and synthetic reactions depended on the way that the enzyme was prepared. Indeed, a mycelium-bound (Mb) lipase activity obtained either in presence or absence of 2% olive oil as well as a lyophilized supernatant extract obtained in presence of 2% olive oil showed different specificity constants ($1/\alpha$). Thus, the highest substrate specificity in hydrolysis reaction was observed toward a long-chain fatty acid (C18; $1/\alpha = 1.0$) with the constitutive Mb-lipase in organic medium. In addition, this lipase preparation was specific toward the synthesis of methyl palmitate during esterification ($1/\alpha = 1.00$) and ethyl palmitate in transesterification ($1/\alpha = 0.93$). Interestingly, the induced Mb-lipase was a highly reactive biocatalyst preparation in both transesterification (58% of the reactions displayed $1/\alpha > 0.5$) and esterification (88% of the reactions displayed $1/\alpha > 0.7$) reactions. On the contrary, the induced lyophilized supernatant was the most specific enzymatic system showing a clear preference for linoleic acid in esterification reactions ($1/\alpha$ around of 0.77 for all acyl acceptors tested).

1. Introduction

Selective conversions of natural and/or synthetic substrates into useful products using enzymes have been gaining popularity at the industrial level [1]. Hydrolytic enzymes such as lipase (triacylglycerol ester hydrolase, EC 3.1.1.3) are also used in biosynthetic reactions. For example, lipases have been researched as esterification/transesterification catalysts for the production of biodiesel [2]. In addition, biodiesel production through the hydroesterification route catalyzed by lipases has also been explored [3]. This procedure involves two steps. First, all of the material is hydrolyzed to free fatty acids (FFAs) and glycerol, and then FFAs are esterified with a short-chain alcohol, generating biodiesel and water.

Compared with conventional acid and base catalysts, lipases offer the advantage of a cleaner product more readily isolated, coupled with a significant reduction in solid and liquid waste streams. Unfortunately, lipases tend to be expensive due to purification steps, making the process inefficient [4,5]. To extend the life of the enzyme and consequently reduce costs, a number of researchers have "immobilized" lipases in or on a physical structure to stabilize the enzyme and allow its reuse. Silica gels have proven to be the most effective immobilization supports, greatly extending lipase life without losing yield [6]. However, construction of the gel–lipase structure can be time-consuming and expensive [7]. An alternative to purified lipase is the use of organisms that produce the enzyme. In essence, intracellular enzymes expressed on the cell wall or membranes are used as "whole-cell" biocatalysts instead of extracellular enzymes that require extraction and purification from the culture medium. If organisms that produce lipases can be readily and cheaply cultured in large quantities, such whole-cell systems could become promising and inexpensive biocatalysts [8]. Thus, among the established whole-cell biocatalyst systems, filamentous fungi have arisen as the most robust whole-cell biocatalyst for industrial applications [9].

Lipase specificity has been studied extensively. In most cases, different kinds of specificity or selectivity can be distinguished during hydrolysis or synthesis reactions [10]. Chain-length specificity has been related to structural features of lipases [11,12], but to date no lipase has been found to be strictly specific for a given chain length.

Knowledge about substrate specificity would simplify the choice of a specific biocatalyst for a certain reaction. This is also the case in the production of biodiesel. These days, commercial biodiesel consists of fatty acid methyl esters produced from methanol. However, the increase in the production of bioethanol has made this alcohol the substrate of choice for enzymatic synthesis of fatty acid ethyl esters (FAEE) [13]. In addition, propanol, buthanol and amyl alcohol were also used in the biodiesel production due to good pouring characteristics of their fatty esters at low temperatures [14].

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The goal of this research was to determine chain-length selectivity of whole cells with lipase activity and extracellular lipases in lyophilized supernatant from *A. niger* MYA 135 in hydrolysis, transesterification and esterification reactions. Fatty acids, fatty acid esters, water-miscible and immiscible alcohols were used as substrates, and lipase specificity was examined regarding the length of the carbon chain of the fatty acid and the type of alcohol. As well, the production of a biodiesel component catalyzed by whole-cell biocatalysts was demonstrated.

2. Materials and methods

2.1. Materials

Sucrose was purchased from Biochem S.R.L. (Argentina); KH_2PO_4 anhydrous 99.99% Suprapur, $MgSO_4 \cdot 7H_2O$, $CuSO_4 \cdot 5H_2O$, Na_2CO_3 anhydrous 99,99% Suprapur, and NaOH were obtained from Merck (Germany); NH_4NO_3 ACS reagent ≥ 98%, *p*-nitrophenyl caprate, *p*-nitrophenyl laurate, *p*-nitrophenyl palmitate, *p*-nitrophenyl stearate and palmitic acid minimum 99% were acquired from Sigma-Aldrich (USA); caproic acid purum 98% ≥ (GC), caprylic acid purum 98% ≥ (GC) and linoleic acid 65–75% were purchased from Fluka (Germany); *n*-hexane for synthesis, acetic acid 99–100% for synthesis, methanol for HPLC, absolute ethanol, propanol, butanol for HPLC, hexanol for synthesis and heptanol for synthesis were acquired from Merk (USA).

2.2. Microorganism and maintenance

Aspergillus niger ATCC MYA 135, formerly *A. niger* 419 from the PROIMI culture collection, was used throughout this project. It was maintained by monthly transfers onto glucose–potato agar slants, incubated at 30 °C and stored at 4 °C.

2.3. Fermentation medium

The fermentation medium was as follows (in g/l): sucrose, 10.0; KH_2PO_4, 1.0; NH_4NO_3, 2.0; $MgSO_4 \cdot 7H_2O$, 2.0; $CuSO_4$, 0.06. The initial pH was adjusted to 7.0 with NaOH.

2.4. Enzyme production

Fermentation was carried out at 30 °C in 500 ml shake flasks (250 rpm) containing 100 ml of fermentation medium. Culture flasks were inoculated with 10 ml of a conidial suspension (about 10^6 conidia/ml) from a stock culture. After 24 h of incubation, the culture was transferred to another 500 ml shake flask containing either 50 ml 3% (v/v) olive oil or distilled water and further incubated for 4 days under the same conditions. The mold developed a pelleted growth form. Mycelium was collected, washed with acetone and centrifuged at 6000 *g* at 4 °C for 3 min; cells were used as the enzyme source. Supernatant obtained after filtration of olive oil containing medium was lyophilized and also used as the enzyme source. Calibration curves were generated with wet and dry mycelium grown in medium without olive oil ($R^2 = 0.973$) or supplemented with 2% olive oil ($R^2 = 0.982$). Protein content was determined according to Bradford [15].

2.5. Enzyme assaying

2.5.1. Hydrolysis reaction

Hydrolytic activity was measured in aqueous and organic medium with *p*-nitrophenyl derivatives solubilized in acetone as the substrate: *p*NPCa (*p*-nitrophenyl caprate; C_{10}), *p*NPL (*p*-nitrophenyl laurate; C_{12}), *p*NPP (*p*-nitrophenyl palmitate; C_{16}) and *p*NPS (*p*-nitrophenyl stearate; C_{18}).

Reactions in aqueous medium were prepared by adding about 0.010 g of wet mycelium or lyophilized supernatant to 1 ml of 100 mM phosphate buffer (pH 7.0), containing 2 mM *p*-NP derivative, 0.1% (w/v) gum Arabic and 0.4% (w/v) Triton X-100 [16].The molar extinction coefficient of *p*-nitrophenol (*p*-NP) under the given assay conditions was 0.0103 $\mu M^{-1}\,cm^{-1}$.

Reactions in organic medium were prepared by adding about 0.010 g of wet mycelium or lyophilized supernatant to 1 ml of *n*-hexane containing 2 mM *p*-nitrophenyl derivative [17]. All reaction mixtures were incubated by shaking (150 rpm) at 37 °C. The absorbance of the supernatant containing *p*-NP was determined at 405 nm. *p*-NP was extracted from *n*-hexane with 1 ml 0.25 M Na_2CO_3 before measurement. The molar extinction coefficient of *p*-NP under these assay conditions was 0.0205 $\mu M^{-1}\,cm^{-1}$.

2.5.2. Transesterification reaction

Enzymatic transesterification was carried out as follows: *p*-nitrophenyl derivatives dissolved in acetone were added at a final concentration of 2 mM, and 100 µl of each alcohol and about 0.010 g of wet mycelium or lyophilized supernatant were added to 800 µl of *n*-hexane. The reaction mixture was shaken (150 rpm) for 1 h at 37 °C, and *p*-NP in the supernatant was measured as above. A reaction mixture without alcohol served as hydrolysis control. In the absence of a biocatalyst, no reaction was observed.

2.5.3. Esterification reaction

Enzymatic esterification was carried out in 10 ml of *n*-hexane. Acetic, caproic, caprylic, palmitic and linoleic acid were used at a final concentration of 2 mM. To each reaction mixture, 1000 µl of different alcohols were added. About 0.10 g of each biocatalyst was used. The reaction mixture was shaken (150 rpm) for 24 h at 37 °C. The ester content was quantified by alkalimetric titration of unreacted acid with 0.1 N NaOH directly measuring the pH with a pH-meter [18].

2.6. Specificity constants determination

The results are expressed as specificity constants, $1/\alpha$, according to Rangheard et al. [19]. For a given substrate *x* and a reference substrate the equation is as follows: $1/\alpha = \log([x]_t/[x]_0) \,/\, \log([\text{reference}]_t/[\text{reference}]_0)$ where $[x]_0$ and $[x]_t$ are molar concentrations at times 0 and *t*, respectively. The reference substrate is that with the highest conversion rate. Hence, the specificity constant of the reference substrate is 1, whereas the values for the other substrates range between 0 and 1. Data given are the means of two or three independent experiments. Standard deviations were ≤10% of the mean for reaction rates and specificity constants.

2.7. GC-MS analysis of synthesis product

The selected product after esterification and transesterification, ethyl palmitate, was analyzed with Gas Chromatography-Mass Spectroscopy (GC-MS), using a Shimadzu QP 5050 equipped with a Carbowax 20 M column and applying the following temperature program: 150 °C (2 min), then from 150 °C to 180 °C (2 °C/min) and 180 °C (7 min). The injector and mass spectrometer were maintained at 250 °C. Carrier gas flow rate was 0.6 ml/min throughout the process. Total ion chromatograms were generated using ionization energy of 70 eV.

The identity of the compounds was confirmed by comparison of the retention times of the samples with reference standards from the Wiley library.

3. Results and discussion

The cost of lipases is one of the major obstacles that limit their applicability for the bulk production of fuels and chemicals as opposed to the conventional base-catalyzed process. While research has mainly focused on biocatalytic reactions using extracellular lipases, relatively few studies have dealt with biotransformation using whole-cell biocatalysts. Thus, the use of microorganisms such as bacteria, yeast and

filamentous fungi as whole-cell biocatalysts has been proposed in attempts to improve the cost effectiveness of the bioconversion processes. Tamalampudi et al. [20] reported that whole cells of lipase producing *Rhizopus oryzae* immobilized onto biomass support particles catalyze the alcoholysis of Jatropha oil more effectively than Novozym 435. On the other hand, Vaysee et al. [10] investigated the chain-length selectivity profile of seven lipases (two purified extracts and five commercial preparations) during hydrolysis, esterification and alcoholysis in biphasic aqueous medium. In our work, the chain-length specificity of three preparations with lipase activity obtained by submerged fermentation using *A. niger* MYA 135 was evaluated in hydrolysis and synthetic reactions.

3.1. Hydrolysis

Previously, it was reported an induced extracellular lipolytic extract from *A. niger* MYA 135 that is very stable in the presence of 50% water-miscible organic solvents. This lipase activity also retains around of 80 and 60% of its hydrolytic activity in aqueous medium after incubation for 1 h at 37 °C with *n*-butanol and *n*-hexanol, respectively [21]. In addition, both the induced and the constitutive mycelium bound (Mb) lipases are very stable in reaction mixture containing methanol and ethanol. In fact, the constitutive Mb-lipase maintaining almost 100% of its hydrolytic activity in aqueous medium after exposure by 1 h at 37 °C in ethanol [22]. This is an important result because hydrophilic solvents often destabilize lipase activity.

Taking account of the importance of hydrolytic reactions in some processes of industrial fuels, additional experiments were performed. Although the highest substrate specificity in hydrolysis reactions was observed toward a long-chain fatty acid (C18; $1/\alpha = 1.0$) with the constitutive Mb-lipase in organic medium, the hydrolysis was also defined by the medium in which the reactions were carried out. In organic medium, specificity of the three biocatalysts toward at least one long-chain fatty acid ester (C16 or C18) was observed; while in aqueous medium, selectivity was toward middle-chain fatty acid esters (C10 or C12) (Table 1). In addition, the highest specificity constant in aqueous medium was also observed with the constitutive Mb-lipase in the presence of *p*-NPCa (C10; $1/\alpha = 0.62$). In a very interesting and useful work, Vaysse et al. [10] reported a lipase from *Candida parapsilosis* that also shows specificity toward a C10 substrate ($1/\alpha = 0.80$) in aqueous medium, however this purified extract does not catalyze any residual hydrolysis in the presence of methanol.

On the other hand, Pencreac`h and Baratti [23] suggested a strong effect of diffusional limitation on lipase activity in organic medium. In our case, the induced Mb-lipase (insoluble enzyme) and the induced lyophilized supernatant (soluble enzyme) displayed almost similar specific constants toward the corresponding esters in aqueous medium. As it was mention before, in organic medium both biocatalysts also showed a same specific constant profile.

Thus, the wide reactivity of these lipase preparations could be useful in a two-step biocatalytic production of biodiesel, hydrolysis of the trygliceride followed by esterification [24].

Table 1
Specificity constants ($1/\alpha$) and absolute hydrolysis rate of C10–C18 esters in aqueous and organic medium (*n*-hexane) in the presence of different biocatalyst preparations with lipase activity.

Biocatalyst / Substrate	Aqueous medium	Organic medium
Constitutive mycelium-bound lipase		
C10	0.62	0.48
C12	0.43	0.31
C16	0.16	0.13
C18	0.13	1.00
Induced mycelium-bound lipase		
C10	0.37	0.18
C12	0.51	0.29
C16	0.24	0.42
C18	0.14	0.22
Lyophilized induced supernatant		
C10	0.36	0.33
C12	0.51	0.33
C16	0.24	0.62
C18	0.14	0.48
Absolute rate for the substrate with highest reaction rate (μmol/min)	0.64 ± 0.007	

3.2. Transesterification

In initial experiments, the performance of both the constitutive and the induced Mb-lipases in transesterification of different alcohols using *p*-nitrophenyl palmitate was evaluated [22,25]. Additionally, an entrapped extracellular purified extract was able to produce ethyl estearate in a solvent-free system with a transesterification activity of 0.78 ± 0.01 U/L [26].

Table 2 shows the specific constants and the absolute transesterification rates of C10–C18 esters with different alcohols in *n*-hexane using the three lipase preparations obtained by submerged fermentation. For methanolysis the lyophilized induced supernatant showed a clear preference for C10 ($1/\alpha = 0.89$) and C12 ($1/\alpha = 1.00$) substrates, whereas C18 was preferentially propanolyzed or butanolized in the presence of induced Mb-lipase. For ethanolysis, constitutive Mb-lipase displayed specificity toward C16 ($1/\alpha = 0.93$); thus, under this reaction condition ethanol seems to be a better acyl acceptor than water for *p*-NPP substrate (Table 1).

At present, methanol, ethanol, propanol, butanol and amyl alcohol were used for the production of biodiesel. Higher and secondary alcohols are interesting due to the good pouring characteristic of their fatty esters at low temperatures [14]. On the other hand, the use of ethanol in the biodiesel synthesis is very up to — date due to the possibility of its production from biorenewable sources [27]. Similarly, biobutanol has recently gained some attention because it also can be produced from renewable sources [28,29]. So, a completely biofuel can be obtained by either ethanolysis or butanolysis.

3.3. Esterification

The specificity profile obtained for esterification reactions showed some interesting aspects as outline bellow.

Esterification carried out with constitutive Mb-lipase showed specificity toward synthesis of methyl palmitate (Table 3). A global observation about esterification by this biocatalyst was that the reaction rate (ester production in μmol per min) was lower than the hydrolysis rate (fatty acid release in μmol per min) (Table 1). This difference can be attributed to the competition between water and alcohol, which is absent in hydrolysis, as well as properties of the acyl donor (fatty acids instead of esters). A notable difference between fatty acids and esters could be the repulsive interaction of dissociated fatty acids with negative charges of the active enzyme sites [30,31].

No specificity was found in esterification catalyzed by induced Mb-lipase. This biocatalyst was able to catalyze most reactions with a high specificity constant (0.75–1.00).

Induced lyophilized supernatant demonstrated esterification specificity in the synthesis of propyl and butyl palmitate. In addition, synthesis of linoleic acid esters was obtained with all alcohols assayed (Table 3). No esterification was observed with short-chain fatty acids. This may be due to the higher solvation of short-chain fatty acids compared to long-chain fatty acids that would selectively shift the reaction equilibrium toward hydrolysis of short chains [32,33].

Table 2
Specificity constants ($1/\alpha$) and absolute transesterification rates of C10–C18 esters with different alcohols in *n*-hexane using different biocatalyst preparations with lipase activity.

Transesterification						
Biocatalyst	Substrate / Alcohol	C10	C12	C16	C18	Absolute rate for substrate with highest reaction rate (μmol/min)
Constitutive mycelium-bound lipase	Methanol	0.54	0.25	0.54	0.22	0.67 ± 0.01
	Ethanol	0.65	0.50	0.93	0.50	
	Propanol	0.72	0.42	0.49	0.50	
	Butanol	0.69	1.00	0.29	0.33	
	Hexanol	0.39	0.92	0.38	0.44	
	Heptanol	0.36	0.54	0.18	0.41	
Induced mycelium-bound lipase	Methanol	0.15	0.67	0.12	0.28	0.73 ± 0.007
	Ethanol	0.75	0.69	0.38	0.33	
	Propanol	0.63	1.00	0.51	0.81	
	Butanol	0.36	0.74	0.64	0.80	
	Hexanol	0.33	0.39	0.70	0.55	
	Heptanol	0.39	0.45	0.79	0.51	
Lyophilized induced supernatant	Methanol	0.89	1.00	0.36	0.13	4.10 ± 0.01
	Ethanol	0.00	0.01	0.16	0.03	
	Propanol	0.01	0.02	0.16	0.01	
	Butanol	0.01	0.02	0.01	0.02	
	Hexanol	0.02	0.02	0.07	0.03	
	Heptanol	0.40	0.42	0.27	0.10	

3.4. Comparison of hydrolysis, transesterification and esterification

Chain-length specificity profiles not only depend on the enzyme, but also on the type of reaction. This is at least partly true because the reactions assayed involved different substrates or co-substrates (ester or acid and water or alcohol).

Constitutive Mb-lipase was specific toward synthesis of methyl palmitate during esterification and ethyl palmitate in transesterification.

Interestingly, the induced Mb-lipase was a highly reactive biocatalyst preparation in both transesterification (58% of the reactions displayed $1/\alpha > 0.5$) and esterification (88% of the reactions displayed $1/\alpha > 0.7$) reactions. Thus, this biocatalyst was able to synthesize ethyl palmitate during both esterification and transesterification. As it was mention before, the use of ethanol to produce a biodiesel component is interesting because of the ecological advantages of this alcohol.

Induced lyophilized supernatant was the most specific enzymatic system. This specificity was influenced by the type of reaction. Esterification and transesterification showed an opposite behavior. During esterification specificity was observed toward long-chain fatty acids, but no reaction was observed with short or middle-chain fatty acids. In contrast, during transesterification specificity was observed toward middle-chain fatty acid esters. Acylation could be the limiting factor in the case of esterification of short and middle-chain fatty acids [34], because short-chain fatty acids, compared to their corresponding esters, are more soluble and hence the energy required for desolvation is higher. Solvation of the carboxylic group may be less important for long-chain fatty acids when compared with their corresponding esters. Also, due to the rigidity at the reaction temperature, the reactivity of long-chain fatty acids may be lower compared to the corresponding esters [34]. The difference between esterification and transesterification could be partly explained by the fact that the biocatalyst may contain more than one lipase. A lipase from *Candida antarctica* also showed specificity toward a middle-chain fatty acid ester (C10) in transesterification but its displays a low initial rate with this fatty acid during esterification [10].

The reaction rates in μmol of ester per min during esterification with constitutive Mb-lipase and induced lyophilized supernatant were interesting, because they were lower than those during transesterification (Tables 2 and 3). This difference may be due to the competition between alcohol and water, present during esterification as a reaction product, and the properties of the acyl donor (fatty acid instead of ester). An important difference between fatty acids and esters is the repulsive interaction of dissociated fatty acids and the negative charges of the active site of the enzyme [30].

Induced Mb-lipase showed a very interesting behavior. The same reaction rate (0.3 μmol/min) was observed when this biocatalyst catalyzed organic hydrolysis of *p*-NPP (C16) and alcoholysis of *p*-NPP (C16) with ethanol. This is likely due to the fact that this biocatalyst

Table 3
Specificity constants ($1/\alpha$) and absolute esterification rates of C2–C18 acids with different alcohols in *n*-hexane using different biocatalyst preparations with lipase activity.

Esterification							
Biocatalysts	Substrate / Alcohol	C2	C6	C8	C16	C18	Absolute rate for substrate with highest reaction rate (μmol/min)
Constitutive mycelium-bound lipase	Methanol	0.62	0.00	0.00	1.00	0.54	0.35 ± 0.002
	Ethanol	0.58	0.00	0.52	0.00	0.52	
	Propanol	0.00	0.49	0.00	0.00	0.52	
	Butanol	0.00	0.00	0.00	0.84	0.00	
	Hexanol	0.50	0.49	0.00	0.93	0.00	
Induced mycelium-bound lipase	Methanol	0.80	0.79	0.89	0.00	0.75	1.00 ± 0.03
	Ethanol	0.79	0.75	0.77	0.89	0.76	
	Propanol	0.76	0.00	0.98	0.77	0.75	
	Butanol	0.75	0.76	1.00	0.98	0.75	
	Hexanol	0.75	0.76	0.00	1.00	0.75	
Lyophilized induced supernatant	Methanol	0.00	0.00	0.00	0.00	0.78	1.50 ± 0.28
	Ethanol	0.00	0.00	0.00	0.00	0.77	
	Propanol	0.00	0.00	0.00	0.97	0.78	
	Butanol	0.00	0.00	0.00	1.00	0.77	
	Hexanol	0.00	0.00	0.00	0.00	0.77	

catalyzes direct alcoholysis whereas other lipases mainly proceed via hydrolysis of the donor ester followed by esterification of the free fatty acid released [35].

Various factors are involved in chain-length selectivity of lipases. Substrate thermodynamic properties determine the chemical reactivity and reaction equilibrium. These properties notably depend on substrate chain-length, temperature, solvent and the composition of the reaction medium [36–38]. Enzyme structure and molecular dynamics are key determinants for substrate specificity. Influence of the lipase structure on the chain-length specificity has been studied by several authors [35,39]. Other authors have mentioned that selectivity depends on modulation of the substrate due to steric or electronic interactions between the alcohol co-substrate and the acyl chain in the lipase active site [40].

3.5. Use of a biocatalyst for selective synthesis of an ethyl ester

Induced Mb-lipase was chosen for synthesis of ethyl palmitate, because of its reactivity toward a long-chain fatty acid (C16) in the presence of ethanol during esterification and transesterification reactions.

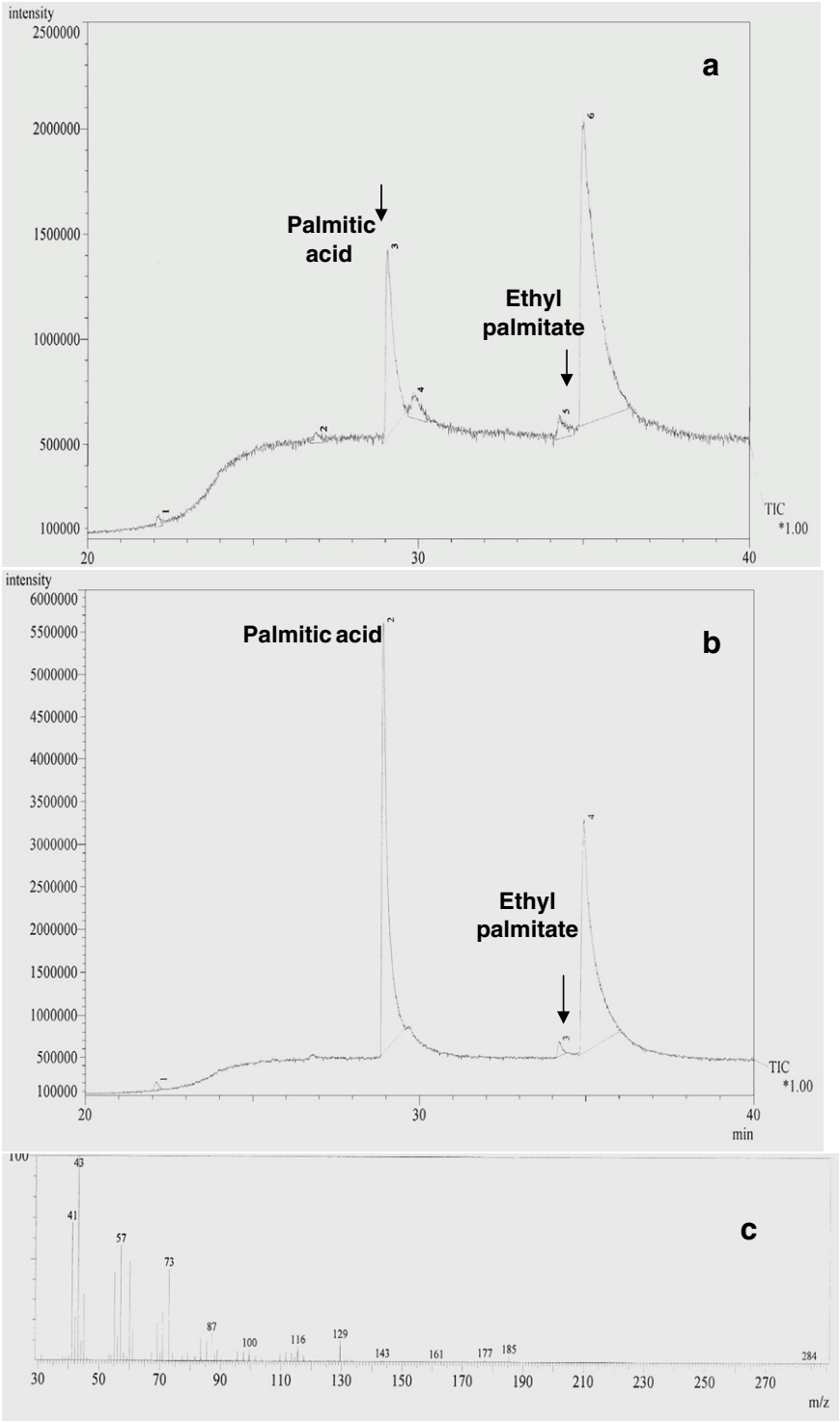

Fig. 1. GC-MS analysis of synthesis of ethyl palmitate from ethanol and *p*-nitrophenyl palmitate (*p*-NPP) (transesterification) and from palmitic acid (esterification); a) ethyl palmitate synthesized by esterification, b) ethyl palmitate synthesized by transesterification, and c) mass peaks (molecular ion peaks and characteristic fragments) of ethyl palmitate.

Ethanol is bigger and heavier than methanol which means that the biodiesel production yield per unit of oil is higher. There are also some other desirable attributes of ethyl esters over methyl esters such as: a significantly lower smoke opacity, lower exhaust temperatures and lower pour point. Ethyl esters tend to have more injector coking than methyl esters. Ethanol is also preferred over methanol in the transesterification process because it is derived from agricultural products, which are renewable and environmentally friendly [16]. In addition, some studies have observed better transesterification or esterification yields with ethanol rather than with methanol [8,41]. This may be due to the fact that lipases are more tolerant of ethanol than of methanol [13] or the fact that lipases act better on long-chain alcohols rather than on short-chain alcohols [42].

Because of the advantages described above, transesterification and esterification with ethanol and C16 were selected for product identification. The presence of ethyl palmitate and secondary or intermediate reaction products were confirmed by gas chromatography–mass spectrometry (GC-MS) and results are shown in Fig. 1. The esterification reaction showed two peaks with retention times of 29.0 and 34.25 min, which were identified as palmitic acid and ethyl palmitate, respectively (Fig. 1a). During transesterification a peak with a retention time of 34.9 min was observed, which could be part of the *p*-NPP molecule (retention time of *p*-NPP was 36.7 min). Two more peaks were found with retention times of 29.0 and 34.20 min and, like in esterification, were identified as palmitic acid and ethyl palmitate, respectively (Fig. 1b). In both esterification and transesterification the molecular structure of ethyl palmitate was confirmed after analysis of molecular ion peaks and characteristic fragments. GC-MS results showed m/z 284 (M^+, $C_{18}H_{36}O_2$), m/z 255 (loss of the ethyl group), m/z 241, m/z 239 (loss of the ethoxide ion), m/z 101 and m/z 88 (McLafferty ion) corresponding to ethyl palmitate (Fig. 1c).

4. Conclusions

The above study determined the chain-length specificity of whole-cells lipase activity and lyophilized supernatant from *A. niger* MYA 135. Specificity of the three biocatalysts assayed, constitutive or induced Mb-lipase and lyophilized induced supernatant, was different and depended on the type of reaction and the substrate (fatty acid or fatty acid ester and alcohol). Induced Mb-lipase was the most reactive, and lyophilized induced supernatant the most selective biocatalyst.

Induced Mb-lipase was selected for its reactivity in esterification and transesterification reactions with long-chain fatty acid esters in the presence of ethanol for identification of FAEE, one component of biodiesel. Thus, combining a versatile whole-cell biocatalyst system with its high ethanol tolerance, significant reduction in the cost of biodiesel production could be expected. Current studies are directed toward statistical optimization using an improved biocatalyst for biodiesel production from different triglyceride sources.

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