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Comparison among immobilised lipases on macroporous polypropylene toward biodiesel synthesis

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Abstract

Eight commercial lipases were characterised (protein content and specific activity) and immobilised on macroporous polypropylene via physical adsorption. The lipases showed a different level of adaptation to the support as determined by the comparison of their catalytic efficiencies (activity/loading). The immobilised lipases were compared toward methanolysis of vegetable oil to obtain biodiesel in solvent-free conditions. Immobilised *Pseudomonas fluorescens* lipase was the most active biocatalyst (ester yield = 58 mol% after 22 h), followed by immobilised *Pseudomonas cepacia* lipase (ester yield = 37 mol% after 51.5 h), whereas all the other lipases (from *Rhizopus oryzae*, *Candida rugosa*, *Mucor javanicus*, *Penicillium roqueforti*, *Aspergillus niger*, *Penicillium camembertii*), were inactive toward biodiesel synthesis. The effect of triglyceride feedstock, reaction temperature, water content, and enzyme loading was determined. Under the optimal conditions, i.e. soybean oil, $T = 30\,^{\circ}C$, water content = 0.5 mg water/mg of biocatalyst, loading = 600 mg lipase AK/g support, an ester yield of 98 mol% after 70 h was obtained.

Keywords: Biodiesel; Macroporous polypropylene; *Pseudomonas fluorescens* lipase; Vegetable oil; Methanol

1. Introduction

Biodiesel is a natural substitute of diesel fuel that comes from renewable sources, such as vegetable oils [1]. Biodiesel presents several environmental advantages. It does not contribute to the greenhouse effect since the amount of CO_2 produced during its combustion is the same absorbed by the vegetable species during their growth. Moreover polluting gas emissions, such as CO, SO_2, HC and soot particulate are substantially diminished. Also heavy metals (lead, vanadium and cadmium) are not present. According to the Kyoto protocol several countries have decided to partially substitute fossil fuels with biofuels. For this reason the global biodiesel industry grew very fast in the last years (2000–2005), and even a faster growth rate is foreseen in the years up to 2008 and beyond [2].

From the chemical point of view, biodiesel is a mixture of fatty acid methyl esters (FAME) that are obtained by alcoholysis of triglycerides [3].

The industrial reaction for biodiesel production uses homogeneous alkaline catalysis. The basic catalyst, i.e. potassium hydroxide, reacts with methanol to yield sodium methoxide which then reacts with the triglyceride to produce the FAME. The reaction products are a mixture of esters and several by-products, the main one being glycerol that, after purification, can be used for cosmetic and pharmaceutical purposes. Partial alcoholysis of triglycerides gives di- and mono-glycerides, these by-products must be separated and pumped back to the reactor. Free fatty acids, water and unreacted alkaline catalyst are also present. Moreover, when the acidity of a feedstock is high the reaction between the free fatty acids and the basic catalyst produces soap. Thus complicated purification processes are needed in order to obtain a pure biodiesel achieving the standard requirements. In the presence of a high content of free fatty acids an acid-catalysed process is used. This employs strong acids, as well as high temperatures and pressures, and requires longer reaction times than the alkaline process. Industrial plants where either alkaline or acid processes are performed require that reactors and accessories are resistant to these aggressive agents; moreover, high safety standards are needed. All these drawbacks go toward the direction of partially deleting the benefits related to the use of biofuels. For this

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reason new "green" methods for biodiesel synthesis are being searched.

The use of immobilised lipases may be a solution to this problem [4]: indeed they are able to catalyse alcoholysis reactions in solvent-free media composed only by a mixture of the reagents giving exclusively FAME and glycerol. These products are easily separated due to their reciprocal immiscibility. Moreover, lipases work at low temperature, atmospheric pressure and are not aggressive to reactors and accessories.

Much research efforts have been done in this field [5]. The main problem to overcome is the inactivation of the biocatalyst – i.e. the widely used Novozyme 435 (immobilised lipase B from *Candida antarctica*) [6] – due to the inhibitory effect of methanol. Several studies related to the minimization of methanol inactivation have been reported [7–10]. For example a process where methanol was successively added in three steps was proposed [6]. In the first step only 1/3 of the stoichiometric amount was used obtaining a methanol, FAME, mono- and di-glyceride mixture that, with respect to the initial conditions where only methanol and the oil are present, is less aggressive toward the enzyme [4]. Alternatively, C_3–C_5 alcohols were proposed either for the biocatalyst regeneration – through washing solid particles of the immobilised lipase from *C. antarctica* [11] – or as reagents in place of methanol [12,13]. Finally, instead of focus on the most popular immobilised lipase (Novozyme 435), that is likely not the best for this application, other authors screened several commercial lipases toward triglyceride alcoholysis. This allowed to identify *Pseudomonas cepacia* lipase as the most methanol-resistant enzyme [14,15].

As written above, enzymatic biodiesel synthesis is carried out in a non-aqueous medium composed by a mixture of the reagents (triglyceride and alcohol). In this kind of media, where enzymes are not soluble, the small amount of water added to increase the catalytic activity favours the aggregation of enzyme particles. Hence, only a small fraction of enzyme molecules, those present on the aggregate surface, can work. Porous materials permit the effective dispersion of enzyme molecules in a high surface area, allowing to a bigger number of enzyme molecules to express their catalytic potentiality [16].

Previous works concerning biodiesel synthesis through immobilised lipases used mainly commercial preparations [6,9,13,17–20]. More recent works, instead, focused on the importance of the immobilisation procedure. Indeed, a hydrophobic sol–gel support [14], a silica aerogel [21], celite [15], hydrotalcite [22] and different types of zeolites [22,23] were used to immobilise lipases for biodiesel synthesis.

The present work is aimed to investigate macroporous polypropylene powder – a widely used support for lipase immobilisation [24–28] – to tailor an immobilised biocatalyst for the synthesis of biodiesel. This was previously done in a non-systematic way only for lipase AK [29] and lipase PS [30]. The first part of the work deals with the characterisation and the immobilisation of eight commercial lipases onto macroporous polypropylene. In order to find a methanol-resistant immobilised lipase, a screening toward biodiesel synthesis using a high methanol:oil (8:1) ratio was performed. The latter part deals with the use of the best immobilised lipase for biodiesel synthesis. Some parameters (oil source, reaction temperature, water content, and enzyme loading) affecting the methyl esters yield were optimised.

2. Experimental

2.1. Chemicals

Lipases (triacylglycerol acyl hydrolases, E.C. 3.1.1.3) from *Aspergillus niger* (lipase A), *P. cepacia* (lipase PS), *Mucor javanicus* (lipase M), *Pseudomonas fluorescens* (lipase AK), *Penicillium roqueforti* (lipase R), *Penicillium camembertii* (lipase G), *Candida rugosa* (lipase AY), *Rhizopus oryzae* (lipase F) were purchased from Amano Enzymes (Nagoya, Japan). The polypropylene powder MP1004 was a gift of Membrana GmbH Accurel Systems, (Obernburg, Germany). Bradford reagent, bovine serum albumin (98%), tributyrin 98%; triolein 65%, glycerol 99%, potassium hydroxide 98%, sodium sulfate 99.7%, sodium deoxycholate >97%, methyl ester GC-standards (methyl palmitate >99%, methyl stearate >99%, methyl linoleate >99%, and methyl linolenate >99%) were from Sigma (Milan, Italy). Methyl oleate 99%, ethyl oleate 99%, and gum arabic (acacia powder) were from Aldrich (Milan, Italy). Disodium hydrogen phosphate >99.5%, sodium dihydrogen phosphate >99.0%, potassium dihydrogen phosphate >99.0%, ethanol 95% and methanol 99.5% were from Fluka (Milan, Italy). Sodium chloride 99.5%, hexane >98% and sodium hydroxide standard solution 10 mM were from Merck (Darmstadt, Germany). Soybean and sunflower oils were purchased in a supermarket whereas fried waste oil was of household provenience.

2.2. Determination of protein content

Determination of protein content was carried out according to the Bradford assay [31]. A volume of 50 μL of the lipase solution was mixed with 950 μL of phosphate buffer solution 0.1 M at pH 8 and 1 mL of the Bradford reagent. After exactly 6 min, absorbance was read by mean of a Cary 50 spectrophotometer at $\lambda = 595$ nm. The blank was obtained by mixing 1 mL of the buffer solution with 1 mL of the Bradford reagent. Protein content was estimated by mean of a calibration curve obtained using BSA (98%) as protein standard.

2.3. Lipase activity assays

Tributyrin and triolein hydrolysis were measured using a 718 Stat Titrino equipment from Metrohm (Herisau, Switzerland) as follows.

2.3.1. Tributyrin assay

A sample of 50–250 μL of lipase solution, or 20–50 mg of the immobilised preparation, was added to a gum arabic-stabilised emulsion of tributyrin in distilled water at 25 °C. The pH was maintained at 7.0 by titration with 10 mM sodium hydroxide solution. The substrate emulsion was prepared by homogenising a mixture of tributyrin (3 mL), distilled water (47 mL), and

an emulsification reagent (10 mL) at 18,000 rpm for 1 min by an Ultra-Turrax homogeniser. The emulsification reagent was prepared by dissolving gum arabic (6.0 g), glycerol (54 mL), NaCl (1.79 g) and KH_2PO_4 (0.041 g) in distilled water (40 mL).

2.3.2. Triolein assay

The assay mixture contained 5 mL of the substrate emulsion, 50 μL of 10 mM sodium deoxycholate and 0.89% NaCl solution added to reach the final volume of 10 mL. The pH was adjusted to 7.0 by the addition of 10 mM NaOH, then 50–250 μL of lipase solution, or 20–40 mg of the immobilised preparation, were added. The continuously stirred mixture was incubated at 25 °C. The release of oleic acid was monitored by titration with 10 mM NaOH in the pH stat equipment. The substrate emulsion was prepared by mixing 500 mg of triolein 65%, 9.5 ml of 0.89% NaCl solution, and 500 mg gum arabic (added slowly to the liquids during Ultra Turrax mixing). Mixing was continued for 1 min and the emulsion was used immediately.

Hydrolytic activity was expressed in terms of "LU" (Lipase Units). 1 LU = 1 μmol of butyric (or oleic) acid formed per minute. Specific activity is the hydrolytic activity per mg of protein.

2.4. Lipase immobilisation by physical adsorption

Lipase immobilisation was carried out as follows. A weighed amount of polypropylene powder (250 mg) was placed in a 25 mL flask and wetted with 300 μL of 95% ethanol. The flask was filled with 20 mL of commercial lipase solution 5 mg/mL in sodium phosphate buffer (20 mM and pH 6.0). The activity of the lipase solution was measured before mixing with the support powder through the tributyrin assay. The flask was slowly rotated overnight then the solution was removed from the flask with a pipette and checked with the tributyrin assay to measure the residual activity. The support particles were washed with fresh buffer until the residual water did not exhibit any enzymatic activity. The adsorbed lipase preparation was dried overnight under vacuum. The enzyme loading was calculated according to the following equation:

$$L = \frac{A_i - A_r}{m_s}$$

where L is the enzyme loading; A_i is the initial enzyme activity; A_r is residual enzyme activity; m_s is the mass of support (g). Hence the loading is expressed as LU/g support.

In order to study the effect of loading, six different concentrations of commercial lipase AK (0.125, 0.625, 1.25, 2.50, 5.00, and 7.50 mg/mL) were prepared to obtain six different loadings (10, 50, 100, 200, 400, and 600 mg commercial lipase/g of support).

2.5. Determination of vegetable oil composition

An amount of vegetable oil equal to 0.5 g was mixed with 2 mL of 5 wt% KOH in methanol in a screw capped vial. The vial was shaken for half an hour at room temperature and the formed methyl esters extracted by adding 2 mL of hexane. The upper phase was dried with sodium sulfate and the methyl esters analyzed through gas chromatography (GC).

2.6. Gas chromatographic analysis

The methyl esters were analyzed on a Gas Chromatograph Varian 3900 equipped with a FID. A capillary column (internal diameter 0.25 mm, length 30 m, film thickness 0.20 μm) Supelco SP 2330 with helium as the carrier gas was used. The column oven temperature was set at constant temperature (190 °C) for the whole analysis duration (9 min). Both the temperature of the injector and of the detector were set at 220 °C. The retention times of the analyzed compounds were: methyl palmitate, 3.50 min; methyl stearate, 4.58 min; methyl oleate 5.02 min; ethyl oleate (internal standard) 5.30 min; methyl linoleate 5.79 min; methyl linolenate 6.89 min. The methyl esters formed were quantified according to calibration curves obtained with the internal standard method that allowed the calculation of analytical yield of fatty acid methyl ester as reported in Figs. 2–4 and 7. All analyses were performed in triplicate, with reproducibility always within 3%.

2.7. Enzymatic biodiesel synthesis

A typical substrate mixture was obtained by mixing 2 g of vegetable oil (soybean oil, sunflower oil, or waste fried oil), a weighed amount of methanol in the ratio (alcohol:oil) 8:1, and water (0–225 mg), in a 4 mL screw-capped vial with teflon-lined septum. The reactions were carried out at controlled temperature (30–60 °C) and started by adding 125 mg of immobilised lipase to the substrates mixture. Reaction vials were shaken through a horizontal shaking water bath at 100 oscillations/min. Samples (5 μL) were withdrawn at different times, and 40 μL of the internal standard solution 0.01 M (ethyl oleate in hexane) were added. The resulting mixtures were diluted to the final volume of 100 μL with hexane and analysed by GC. All reactions were performed at least in duplicate.

3. Results and discussion

3.1. Characterisation of commercial lipases

Eight microbial lipases were used in the present work namely: lipase F (from *R. oryzae*), lipase AK (from *P. fluorescens*), lipase PS (from *P. cepacia*), lipase AY (from *C. rugosa*), lipase M (from *M. javanicus*), lipase R (from *P. roqueforti*), lipase A (from *A. niger*), and lipase G (from *P. camembertii*).

Since they were commercial preparations with different purity grade, they were characterised regarding protein content and specific activity (Table 1). The protein content of the commercial preparations, determined through the Bradford assay [31], was in general quite low. Lipase M was the preparation with the highest protein content (5.6 wt%), followed by Lipase F (4.3 wt%), lipase A (3.31 wt%), lipase AK (2.0 wt%) and lipase AY (1.1 wt%). The other commercial preparations – lipase G, lipase R, lipase PS – had a protein content lower than 1 wt%, being 0.80 wt%, 0.64 wt%, and 0.55 wt%, respectively.

Table 1
Characterisation of commercial lipases

Lipase	Microbial source	Protein content (wt%)	Activity (tributyrin assay) (kLU/g commercial lipase)	Activity (triolein assay) (kLU/g commercial lipase)	Specific activity (tributyrin assay) (LU/mg protein)	Specific activity (triolein assay) (LU/mg protein)
M	*Mucor javanicus*	5.6 ± 0.1	7.4 ± 0.2	5 ± 1	132 ± 4	89 ± 18
G	*Penicillium camembertii*	0.80 ± 0.02	0.14 ± 0.02	0.14 ± 0.04	17 ± 2	17 ± 4
F	*Rhizopus oryzae*	4.3 ± 0.3	22 ± 3	28 ± 7	512 ± 70	651 ± 160
AY	*Candida rugosa*	1.1 ± 0.2	5.9 ± 0.9	4.7 ± 0.8	536 ± 82	427 ± 73
R	*Penicillum roqueforti*	0.64 ± 0.08	6.5 ± 0.6	0.4 ± 0.1	1016 ± 94	63 ± 16
AK	*Pseudomonas fluorescens*	2.0 ± 0.2	24 ± 2	4 ± 1	1200 ± 100	200 ± 50
PS	*Pseudomonas cepacia*	0.55 ± 0.06	9.4 ± 0.2	1.8 ± 0.5	1709 ± 36	327 ± 90
A	*Aspergillus niger*	3.31 ± 0.03	0.69 ± 0.03	0.05 ± 0.01	21 ± 1	1.5 ± 0.3

The activity was determined measuring the initial rate of hydrolysis of tributyrin and triolein, catalyzed by a measured volume of the commercial lipase solutions having a concentration of 5 $mg_{commercial\ lipase}/mL_{solution}$. The activity thus determined was expressed in terms of LU/g (μmol of butyric or oleic acid formed per minute and per gram of commercial lipase). The use of two different substrates gives information regarding the preference of the lipases toward long chain or short chain fatty acids. Lipase AK was more effective toward tributyrin (24 kLU/g) rather than triolein (4 kLU/g), similarly behaved lipase R, lipase A, lipase PS, and lipase M as reported in Table 1. Some lipases, G and AY, gave almost the same activity showing no substrate preference. Only lipase F seemed to show a slight preference toward triolein.

Specific activity was then calculated as the activity (μmol/min or LU) per milligram of protein. This parameter is more interesting than the previous one since, due to the low purity of the commercial lipases, specific activity allows a real and effective comparison among enzyme activities toward a particular substrate. The results, reported in the last two columns of Table 1, show that specific activity follows the series lipase PS > lipase AK > lipase R > lipase AY > lipase F > lipase M > lipase A > lipase G, if tributyrin is used as the substrate. The series is different if triolein is used as the substrate. In that case: lipase F > lipase AY > lipase PS > lipase AK > lipase M > lipase R > lipase G > lipase A.

3.2. Immobilisation of lipases on macroporous polypropylene

The eight commercial lipases were immobilised onto polypropylene through physical adsorption. The support used is a highly hydrophobic polypropylene powder, whose characterisation was previously reported [27]. The pore size distribution, determined by mercury intrusion porosimetry, shows a maximum centered at about 3 μm, the surface area is 79 m^2/g and the cumulative pore volume is about 2 cm^3/g.

The adsorption of lipases on hydrophobic polymeric supports was reported in several works [25–28]. The success of hydrophobic materials in lipase adsorption depends on the structural features of "typical" lipases [26]. The natural function of lipases is the hydrolysis of triglycerides. Their catalytic activity sharply increases when the substrate forms a separate phase at which lipases are adsorbed. This phenomenon, called "interfacial activation", was firstly observed by Sarda and Desnuelle [32]. As a result of X-rays based knowledge of the structure of several lipases, the greatest part of them seemed to have a recurrent structural domain – an amphiphilic amino acidic chain, called "lid" or "flap", that buries the active site [33,34] – that is related to the affinity of lipases toward hydrophobic interfaces [33,35]. Indeed, most lipases exist in two conformations, that is, an open and a closed conformation. The closed conformation dominates in water solution, while the open one occurs in the presence of a hydrophobic interface. In the closed conformation the flap exposes the hydrophilic side toward the water and the hydrophobic one toward the catalytic site. In the presence of a hydrophobic interface the lipase changes its conformation and exposes the catalytic site to the hydrophobic phase constituted by the substrate (triglyceride). It is likely that during the adsorption process the lipase changes its conformation in the presence of the hydrophobic support surface and is thus adsorbed in the open conformation.

Fig. 1 reports the loading of the eight commercial lipases. The same amount of all the commercial lipase powders was weighed and dissolved in the same volume of phosphate buffer 20 mM pH 6. The adsorption process was carried out by putting in contact the lipase solution with a weighed amount of the polypropylene powder. The enzymatic loading, calculated according to the equation reported in Section 2.4, follows the series: lipase F > lipase AK > lipase PS > lipase AY > lipase M > lipase R > lipase A > lipase G.

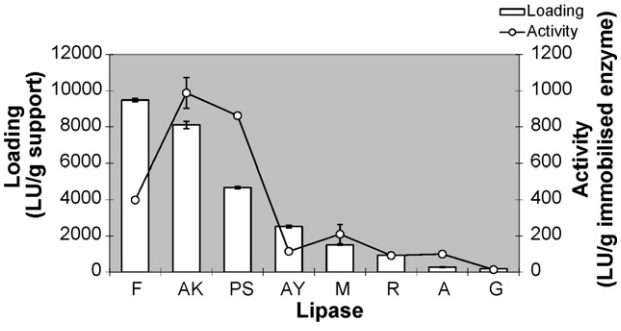

Fig. 1. Loading and activity of commercial lipases immobilised onto macroporous polypropylene.

Table 2
Catalytic efficiencies (activity/loading) of commercial lipases adsorbed onto macroporous polypropylene

Lipase source	Catalytic efficiency
Mucor javanicus	0.14
Penicillium camembertii	0.07
Rhizopus oryzae	0.04
Candida rugosa	0.045
Penicillium roqueforti	0.10
Pseudomonas fluorescens	0.12
Pseudomonas cepacia	0.18
Aspergillus niger	0.37

The catalytic activity of the immobilised preparations was then checked through the tributyrin assay. The activity of the immobilised preparations, reported in the second *y* axes of Fig. 1, follows the series: lipase AK > lipase PS > lipase F > lipase M > lipase AY ≃ lipase A ≃ lipase R > lipase G. The immobilised lipase F, that reached the highest loading, was less active than lipases AK and PS. Similarly behaved lipase AY (*C. rugosa*) that was less active than lipase M although reached a higher loading.

Loading and activity of the immobilised biocatalyst are in first approximation correlated. The higher the loading the higher the activity. In truth, the immobilisation process may lead to the inactivation of a certain fraction of enzyme molecules. This may be due to the distortion of the tertiary structure of the enzyme caused by the enzyme–support interaction that leads to structural rearrangements [25]. In addition also the orientation of the adsorbed lipase may play a role; indeed, due to the hydrophobic nature of the support, it is likely that a certain fraction of lipase molecules interacts with the support through the hydrophobic surface surrounding the active site. This may hinder the substrate reaching of the active site. Nevertheless this interaction with a hydrophobic surface should stabilise the enzyme in its open (active) conformation [36]. All these effects may affect at a different extent each immobilised lipase.

Catalytic efficiency, defined as the ratio between activity and loading, gives the hint of the adaptation level of the immobilised lipase. The higher the ratio the lower the amount of lipase inactivated by the adsorption process. The catalytic efficiencies of the immobilised lipases are reported in Table 2. The following series was found: lipase A > lipase PS > lipase M > lipase AK > lipase R > lipase G > lipase AY > lipase F.

3.3. Screening of immobilised lipases toward biodiesel synthesis

The comparison among the eight immobilised lipases onto polypropylene toward the methanolysis of soybean oil was performed. According to previous works [15,30], the reactions were carried out at 40 °C, with a stoichiometric ratio alcohol:oil = 8:1. The results are shown in Fig. 2. Six of the eight lipases (those from *M. javanicus*, *P. camembertii*, *R. oryzae*, *C. rugosa*, *P. roqueforti*, and *A. niger*) did not display any activity (at least at the investigated loading). Only the lipases from *P. fluorescens* (lipase AK) and from *P. cepacia* (lipase PS) were able to give

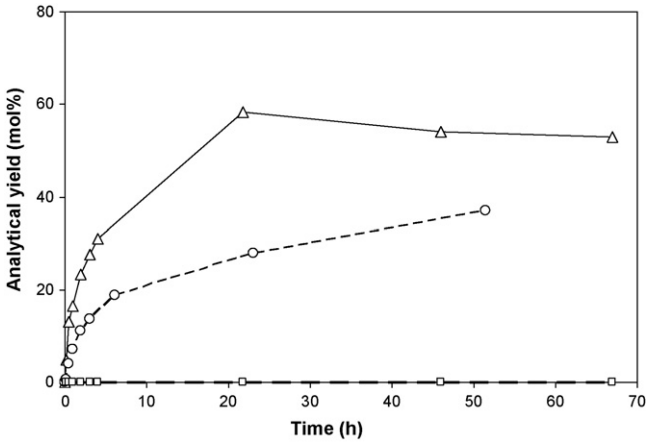

Fig. 2. Screening of immobilised lipases on macroporous polypropylene toward biodiesel synthesis. Soybean oil (2 g) and methanol were mixed in the stoichiometric ratio 1:8. Immobilised lipase (125 mg) was added to the substrates mixture at 40 °C with a constant shaking (100 oscillations/min). *Pseudomonas fluorescens* lipase (△); *Pseudomonas cepacia* lipase (◯); *Mucor javanicus* lipase, *Penicillium camembertii* lipase, *Rhizopus oryzae* lipase, *Candida rugosa* lipase, *Penicillium roqueforti* lipase, and *Aspergillus niger* lipase (□).

ester yield of 58 mol% (after 22 h) and 37 mol% (after 51.5 h), respectively.

This result does not mean that the other lipases are absolutely inactive, but that this is their behaviour toward this reaction whether immobilised on polypropylene. A partial explanation may be due to the different loading and hydrolytic activity of the different immobilised lipases. More likely, also a inhibiting effect due to methanol could be invoked [5].

Previous screenings of commercial lipases showed that, lipase PS was more active than lipase AK toward the methanolysis of vegetable oil [14,15,37]. In all these works the lipase powders were suspended in the reaction medium. They were commercial preparations [14] or at maximum 'pH-tuned' preparations [15]. In the present work the enzyme screening was carried out after the immobilisation; thus, data reported in Fig. 2 are the result of two different effects. Firstly, they depend on lipase activity toward the specific reaction (vegetable oil methanolysis); this is tuned by the effect of the support in increasing/decreasing enzyme activity and stability. The catalytic efficiency gives a measure of the capacity of an enzyme to adapt to the support surface saving its catalytic activity once immobilised. Thus, the fact that lipase PS has a catalytic efficiency equal to 0.18 whereas that of lipase AK is 0.12 (Table 2) would mean that the first lipase is better adapted than the latter to polypropylene. Nevertheless, in opposition to this last consideration and to that found for free enzyme powders in biodiesel synthesis [14], results in Fig. 2 shows that the immobilised lipase from *P. fluorescens* is the most active biocatalyst. In truth, the catalytic efficiency was obtained from hydrolytic activity measurements, hence they are not necessarily significant regarding the enzyme behaviour in non-aqueous media. Thus, from the previous considerations we can hypothesize that the polypropylene support protects *P. fluorescens* lipase by the inhibiting action of methanol better than what done by other previously reported supports [14,15].

Table 3
Fatty acid composition of vegetable oils

	Sunflower oil (mol%)	Soybean oil (mol%)	Fried oil (mol%)
Palmitic acid	6.8	10.4	7.9
Stearic acid	3.9	3.7	4.0
Oleic acid	23.6	28.1	29.8
Linoleic acid	65.7	52.8	58.3
Linolenic acid	–	5.1	–
Average molecular weight ($g\,mol^{-1}$)	876.4	873.7	876.0

On the basis of the obtained results, the immobilised lipase from *P. fluorescens* was used in the following steps of the work.

3.4. Comparison among virgin and waste vegetable oils for enzymatic biodiesel synthesis

The most used vegetable oils in the biocatalytic biodiesel synthesis are those obtained by soybean seeds [38–41], sunflower seeds [20,29,42,43], and rapeseed [38]. Also other oleaginous species as *Jatropha* [44], palm and coconut kernel [45], rice-bran oil [46,47], castor oil [48], etc. have been considered. Attention has also been paid to the use of low-value triglycerides such as those from restaurant grease [49], waste edible oil [8], and animal fats, i.e. tallow [38]. In principle all the triglyceride sources should be considered equivalent as lipase substrates, but some substances, i.e. phospholipids, even present in very low amounts, can negatively affect enzyme activity [5].

In the present work three different vegetable oils – namely soybean oil, sunflower oil and a mixture of waste fried oils – were used. The fatty acid composition of these oils, as determined by GC analysis, is reported in Table 3. All oils contained a high percentage of unsaturated fatty acids, linoleic acid (52.8–65.7 mol%), and oleic acid (23.6–29.8 mol%). The content in saturated fatty acid was lower being in the range 6.8–10.4 mol% for palmitic acid and 3.7–4.0 mol% for stearic acid. Finally, only soybean oil contained about 5% of the polyunsaturated linolenic acid (C18:3; *n*-3).

The knowledge of the fatty acid composition allowed to calculate the average molecular weights of the vegetable oils. These were very similar for the three samples, being 876.1 g/mol (sunflower oil), 873.7 g/mol (soybean oil), and 876.0 g/mol (waste fried oil).

The three oils were used for biodiesel synthesis. All reactions were performed at 40 °C by using a stoichiometric ratio methanol:oil 8:1, and the immobilised lipase AK as the biocatalyst. Fig. 3 shows the ester yield in the first 4 h of reaction obtained by the three different vegetable oils. After 4 h of reaction the yield obtained by soybean oil was 31 mol%, whereas those obtained by sunflower oil and fried waste were 29 mol% and 27 mol%, respectively. Although, the difference among the yields is not big, a slight higher yield is obtained at all the investigated times for soybean oil. Thus, it was used in the following steps.

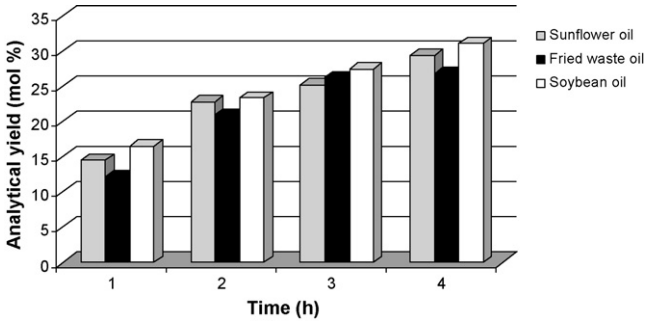

Fig. 3. Effect of triglyceride feedstock on initial analytical ester yield of FAME. Vegetable oil (2 g) and methanol were mixed in the stoichiometric ratio 1:8. Immobilised lipase AK (125 mg) was added to the substrates mixture incubated at 40 °C with a constant shaking (100 oscillations/min).

3.5. Effect of the temperature on the alcoholysis reaction

The effect of the temperature on the analytical yield of FAME obtained by the methanolysis of soybean oil catalyzed by the immobilised lipase AK is shown in Fig. 4. Enzyme activity usually displays a bell shape trend with temperature. Initially, the reaction rate, and thus product yield at a fixed time, of an enzyme catalysed reaction increases by increasing the temperature till a maximum is reached. After that value, further increase of the temperature results in an enzyme inactivation. Iso et al. found that the conversion of triolein and propanol to propyl oleate catalysed by lipase AK was affected by the temperature. Almost the same conversion ratio was obtained at 50 and 60 °C while at 70 °C the lipase was strongly inactivated [12]. Noureddini et al. investigated a temperature range 25–60 °C obtaining a slight change in transesterification activity of immobilised lipase PS. A temperature of 35 °C gave the highest FAME content (63 mol%) [14].

If we look at the ester yield after 3 h of reaction, we see that the immobilised lipase AK is inactivated by the highest temperature (60 °C), indeed only a 3 mol% yield is obtained. At 50 °C a ester yield of 10 mol% was obtained. The highest yields were obtained at 40 °C (27.5 mol%) and 30 °C (26.4 mol%).

Hence in the investigated temperature range, the commonly observed effect of reaction rate increasing with temperature is overcome by its inactivating effect on the immobilised lipase.

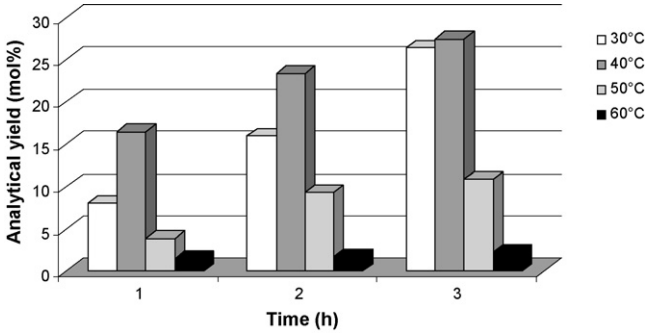

Fig. 4. Effect of temperature on initial analytical yield of FAME. Soybean oil (2 g) and methanol were mixed in the stoichiometric ratio 1:8. Immobilised lipase AK (125 mg) was added to the substrates mixture incubated at a controlled temperature (30, 40, 50, and 60 °C) with a constant shaking (100 oscillations/min).

The fact that a low temperature, such as 30 °C, permits a good catalyst performance is an advantage from the point of view of process energy saving. Thus this temperature was used in the last steps of the work.

3.6. Effect of water content

The effect of water content on enzyme activity was then determined. Although water is not involved as a reagent or a product in a transesterification reaction, its content is important since it favours the expression of the full enzymatic activity. Water acts as a 'lubricant' of polypeptide chains, thus conferring to the enzyme the necessary mobility to explicate the catalytic action [50]. Since our reaction system is composed by different phases – the substrates (triglycerides and methanol), the enzyme and the support – water is partitioned at a different extent among the components.

In order to study the effect on enzymatic activity a weighed amount of water was added to the system and the resulting enzyme activity toward triglyceride methanolysis was measured. The results in Fig. 5 show that the lipase from *P. fluorescens* immobilised on polypropylene is inactive when the reaction medium is kept dry, but its activity sharply increases when about 0.5 mg water/mg of biocatalyst are added to the system. Further addition of water brings to a slight but constant decrease of enzymatic activity.

3.7. Effect of enzyme loading

Six solutions at different concentration (in the range 0.125–7.50 mg/mL) of the commercial lipase AK were prepared. The same amount of polypropylene powder was suspended in each solution to obtain six different immobilised preparations at different loadings, namely: 10, 50, 100, 200, 400 and 600 mg of commercial lipase/g of support. The initial and the residual activities were determined at the beginning and at the end of the immobilisation process through the tributyrin assay.

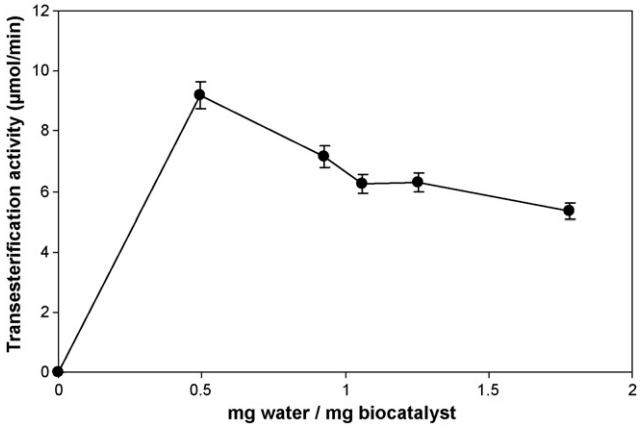

Fig. 5. Effect of water content on transesterification activity lipase AK immobilised on macroporous polypropylene. Soybean oil (2 g) and methanol were mixed in the stoichiometric ratio 1:8. Immobilised lipase (125 mg) was added to the substrates mixture incubated at 30 °C with a constant shaking (100 oscillations/min).

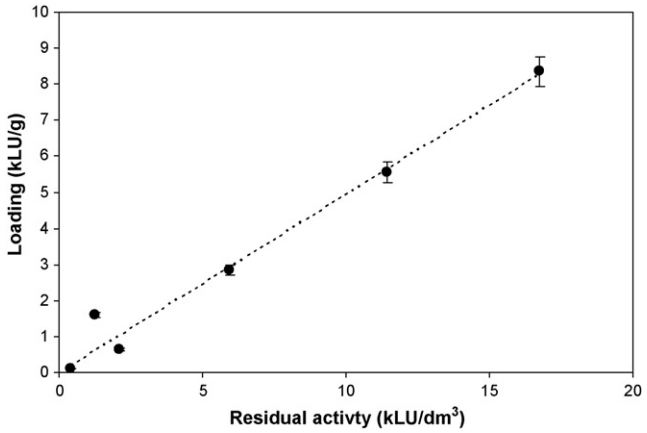

Fig. 6. Adsorption isotherm of lipase AK on macroporous polypropylene at 25 °C.

Fig. 6 reports the adsorption isotherm, a graph reporting the loading (LU/g) versus the lipase concentration in the immobilising solution (LU/dm^3). The loading increases linearly without reaching a saturation value. We cannot exclude that this might be reached, according to a Langmuir type isotherm, at higher loadings. Alternatively an always increasing curve might be explained by the Freundlich model [51].

The immobilised lipases having different loadings were tested toward the methanolysis of soybean oil. The curves reported in Fig. 7 shows that the maximal ester yield increases with the biocatalyst loading. The immobilised lipase having a loading of 600 mg/g (8.35 kLU/g) reaches a ester yield of 96 mol% after 49 h and 98 mol% after 70 h.

The slopes of the initial part of the curves are proportional to the catalytic activity of the immobilised lipases. The slopes increase with loading, but for the two curves at the highest loading (400 and 600 mg/g), the slopes are very similar. This means that the catalytic efficiency, that time measured through the transesterification reaction, is higher for the biocatalyst at the loading 400 mg/g rather than that at 600 mg/g.

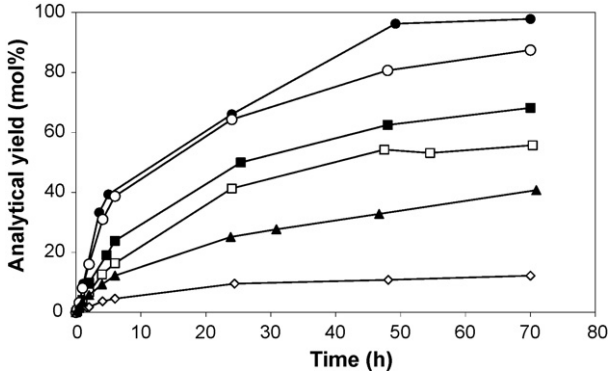

Fig. 7. Effect of lipase loading on analytical yield of FAME. Loading: 10 mg/g (◊); 50 mg/g (▲);100 mg/g (□); 200 mg/g (■); 400 mg/g (○); 600 mg/g (●). Soybean oil (2 g) and methanol were mixed in the stoichiometric ratio 1:8. Immobilised lipase AK (125 mg) was added to the substrates mixture incubated at a controlled temperature 30 °C with a constant shaking (100 oscillations/min).

4. Conclusions

Macroporous polypropylene was used as the support for lipase immobilisation. The purpose was the obtainment of an immobilised biocatalyst for biodiesel synthesis in solvent-free conditions. Of the eight immobilised lipases only those from *Pseudomonas* species were active despite the inactivating effect of methanol. In particular *P. fluorescens* lipase reached the highest ester yields (58 mol%). This biocatalyst was used in the following steps involved in the determination of the optimal reaction conditions. These were obtained using soybean oil as feedstock, a temperature of 30 °C, a water content of 0.5 mg water/mg of biocatalyst, and a loading of 600 mg commercial lipase/g support. The use of these experimental conditions allowed to reach a FAME analytical yield equal to 96 mol% after a reaction time of 49 h.

Acknowledgments

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