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PAPER

An efficient activity ionic liquid-enzyme system for biodiesel production

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The efficient production of biodiesel in hydrophobic ionic liquids using immobilized lipase was demonstrated. The use of ionic liquids containing long alkyl chains on the cation has the important advantage of producing homogeneous systems at the start of the reaction but, when the reaction is complete, a three-phase system is created that allows selective extraction of the products using straightforward separation techniques, while the ionic liquid and the enzyme can be reused. Fifteen ionic liquids based on different alkyl chain length of the methyl imidazolium cation ([C_{10}MIM], [C_{12}MIM], [C_{14}MIM], [C_{16}MIM] and [C_{18}MIM]) combined with [BF_4], [PF_6] or [NTf_2] anions were assayed as reaction media for two immobilized lipases (*Candida antarctica* lipase B and *Pseudomonas fluorescens* lipase AK) for biodiesel production. The highest synthetic activity was obtained in [C_{16}MIM] [NTf_2] using Novozym 435 (immobilized *Candida antarctica* lipase with 245.13 U g^{-1} IME), its activity being more than three times higher than in a solvent-free system. Additionally, in this IL the fatty acid methyl esters production was 90.29% after 3 h, while in the solvent-free system it was 27.3%. The influence of several reaction parameters, such as temperature, methanol-to-oil molar ratio, alkyl-chain length of the alcohols, IL : substrate volume ratios, amount of enzyme, and oils feedstock were studied and optimized.

1. Introduction

The serious reduction of fossil fuel resources and an increasing ecological awareness have led to the search for renewable sources fuel, such as plant biomass. Biodiesel is a low-emissions diesel substitute fuel made from renewable resources or waste lipid. The most common method for biodiesel production is transesterification (alcoholysis) of oil (triglycerides) with methanol in the presence of a catalyst, which gives biodiesel (fatty acid methyl esters, FAMEs) and glycerol (by-product). The transesterification reaction is normally a sequence of three consecutive reversible reactions. In this process, triglyceride (TAG) is converted stepwise into diglyceride (DAG), monoglyceride (MAG), and, finally, glycerol, with 1 mol of alkyl esters being formed in each step (Fig. 1).

The transesterification process can be carried out in different ways, using an alkali catalyst, an acid catalyst or a biocatalyst. Chemical processes are used on an industrial scale, although the reaction has several drawbacks: it is energy intensive, glycerol recovery is difficult, the acidic or alkaline catalyst has to be removed from the product, alkaline waste water requires treatment, and water and free fatty acid cause a partial saponification reaction which produces soap. Biodiesel

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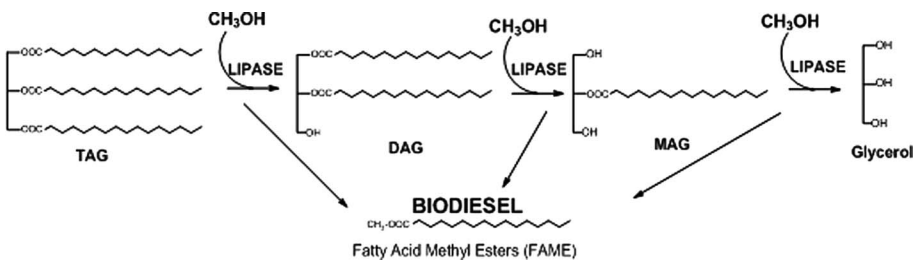

Fig. 1 Biocatalytic production of biodiesel.

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production with a biocatalyst eliminates all these disadvantages, producing biodiesel with a very high degree of purity.[1] However, the process has not been implemented on an industrial scale for several reasons, including the high cost of enzymes, low lipase activity and enzyme inhibition by alcohol.[2]

Enzymatic biodiesel production from raw vegetable oils has been extensively studied by many authors in recent years.[1–4] Some authors have even investigated the use of waste cooking oil to obtain enzymatic biodiesel and to reduce the cost of raw materials.[2,5]

Nevertheless, in lipase-catalyzed biodiesel production, methanol, which is not soluble in oil, has traditionally caused the critical problem of enzyme deactivation. To avoid this problem, a series of conventional organic solvents (petroleum ether, hexane, cyclohexane, *tert*-butanol and *tert*-amyl alcohol) were assayed. *Tert*-amyl alcohol was found to be the optimal reaction medium for enzymatic biodiesel production, presumably due to its good ability to solubilize the co-substrate, methanol, and the by-product, glycerol. Other approaches have been examined, including the stepwise addition of methanol or salt solution.[6] Although these approaches give good results, they involve laborious procedures, including the periodic addition of methanol or the removal of organic solvents from the biodiesel.

In this context, the use of ionic liquids (ILs) as reaction medium has opened up new opportunities for the enzymatic production of biodiesel. The interest that ionic liquids hold as green solvents resides in their negligible vapor pressure, excellent thermal stability, high ability to dissolve a wide range of organic and inorganic compounds, and their non-flammable nature, which can be used to avoid the problem of the emission of volatile organic solvents to the atmosphere. Moreover, their solvent properties, such as miscibility or immiscibility with water or some organic solvents (*e.g.* hexane), can be tuned by selecting the appropriate cation and anion, which increases their usefulness for recovering products from the reaction mixture.[5] The most interesting biotransformations in ILs have been observed at low water content, or when using nearly anhydrous media, because of the ability of hydrolases to carry out synthetic reactions.[7] Furthermore, it is possible to design two-phase reaction systems that easily allow product recovery.[8] Recently, four studies have reported biodiesel enzymatic production by lipases in ionic liquid with promising results.[9] In all these works hydrophobic ionic liquids were used, and FAME production was higher than that obtained both in solvent free systems and organic solvents.

In the present study, we have investigated biodiesel production in ionic liquids catalyzed by lipase and the straightforward extraction of the products using the properties of appropriate ionic liquids. Fifteen ionic liquids based on different alkyl chain lengths of the methyl imidazolium cation ([C_{10}MIM], [C_{12}MIM], [C_{14}MIM], [C_{16}MIM] and [C_{18}MIM]) combined with [BF_4], [PF_6] or [NTf_2] anions were assayed as reaction media for two immobilized lipases (*Candida antarctica* lipase B and *Pseudomonas fluorescens* lipase AK) for biodiesel production. The C_{16}MIM NTf_2 was seen to be an excellent reaction medium for biodiesel production catalyzed by Novozyme 435. The influence of several reactions parameters, such as temperature, methanol-to-oil molar ratio, alkyl-chain length of the alcohols, IL : substrate volume ratio, amount of enzyme and oil feedstock was studied.

2. Experimental

Free (Novozym® 525 L) and immobilized (Novozym® 435) *Candida antarctica* lipase B (EC 3.1.1.3) were from Novo Spain, and free *Pseudomonas fluorescens* lipase AK was from Amano Enzyme Japan. Ionic liquids at a purity of 99% were from IoLiTec (Germany). Accurel MP 1000 was obtained from Membrane GmbH Accurel Systems, Lewatit VP OC 1600 was obtained from Lanxess Chemical S.L., and Celite 545 was obtained from Merck. Olive and sunflower oils were purchased from a supermarket, waste cooking oil was obtained from a restaurant provenience, and palm oil was purchased from Sigma-Aldrich Chemical Co. Standard fatty acids (oleic, palmitic, linoleic, and stearic), fatty acid esters (methylpalmitoleate, methyllinoleate, methylstearate), monoglycerides (monolinolein, monoolein, monopalmitin, monostearin), diglycerides (1,3-dilinolein, 1,3-diolein, 1,3-dipalmitin and 1,3-distearin) and triglycerides (trilinolein and triolein) were from Sigma–Aldrich Chemical Co. Solvents and other chemicals were purchased from Merck and were of the highest purity available.

Transesterification reactions

Ninety microlitres of methanol (2.21 mmol) and 310 μL of triolein (0.32 mmol) were added to screw-capped vials of 1 mL total volume containing 800 μL of ionic liquid or *tert*-butanol. The mixture was vigorously shaken to obtain a homogeneous system. The reaction was started by adding 28 mg of Novozym 435 (10%, w/w, based on oil weight) and run at 60 °C in a glycerol bath with shaking. At regular time intervals, 30 μL aliquots were withdrawn and suspended in 470 μL of hexane. The biphasic mixture was vigorously shaken for 3 min to extract all the substrates and products into the hexane phase. Then 400 μL of the hexane extracts were added to 100 μL of 250 mM ethyl decanoate (internal standard) solution in hexane and 40 μL of the resultant solution was analyzed by HPLC. The volume of each reaction medium was increased up to 5 times to distinguish the phases at the end of the enzymatic reaction. For every study, the following test conditions were optimized: temperature, methanol-to-oil molar ratio, alkyl-chain length of the alcohols, IL : substrate volume ratio, amount of enzyme, and oil feedstock.

Immobilization method

Lipase AK (30 mg) was dissolved in 2 mL of water and mixed with 1 g of support. The mixture was shaken for 1 h at room temperature. The suspension was centrifuged and washed twice with 3 mL of water. The amounts of lipase adsorbed by the different supports were quantified by the Lowry method. The immobilized enzyme was immediately frozen at –20 °C and lyophilized for 48 h. Novozym 525 L was purified by ultrafiltration as reported previously,[10a] resulting in a *Candida antarctica* lipase B solution of 18.156 mg mL^{-1} and a purification degree of 1.5-fold. The immobilization process was the same as for lipase AK.

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HPLC analysis

Reaction products were determined by a Shimadzu HPLC equipped with a multi-channel pump (mod LC-20AD) and a DAD detector (mod SPD-M20A), using a LiChroCart Lichrospher RP-18 column form Merck (25 cm length, 4.6 μm internal diameter and 5 μm particle size). Three mobile phases were employed; phase A, composed of acetronitrile and water (80 : 20, v/v), phase B of acetronitrile only, and phase C, composed of isopropanol and hexane (55 : 45, v/v). The flow rate was 1.2 mL min^{-1} and the injection volume 40 μL. The protocol employed for the mobile phase involved a linear gradient of 100% (v/v) A, decreasing to 0% v/v A in 5 min, while phase B was increased up to 100%, and maintained for 2 min, before decreasing to 50% in 16 min. Phase C increased up to 50%, and the final mixture (50 : 50, v/v B/C) was held for 10 min. Finally, the system was restored to its initial condition for 6 min. Elution profiles were monitored at 210 nm. Peaks in the chromatograms were identified by comparing retention times with the appropriate corresponding standards. The conversion to products was calculated using the following:

$$G_o = MAG + DAG + TAG$$

$$MAG\ (\%) = (MAG_t/G_o) \times 100$$

$$DAG\ (\%) = (DAG_t/G_o) \times 100$$

$$TAG\ (\%) = (TAG_t/G_o) \times 100$$

$$FAMEs\ (\%) = (FAMEs_t/G_o) \times 100$$

where G_o is the glyceride content at time t = 0, MAG_t, DAG_t and TAGt is the total sum of glycerides (mono, di and triglycerides) at time t = t and $FAMEs_t$ is the total sum of all fatty acid methyl esters, at a time t = t. The areas of substrates and products were normalized with respect to the internal standard area. One unit of specific activity was defined as the amount of enzyme that produced 1 μmol FAME per min.

Novozym 435 is a commercial lipase and consists of 10%-by-wt lipase B physically adsorbed within 90% by wt Lewatit VPOC 1600 support,[10b] so, 28 mg of Novozyme 435 contains 2.8 mg of lipase B.

Differential scanning calorimetry of ILs

A differential scanning calorimeter DSC 2920 (TA Instruments, New Castle, DE) was used to measure the melting point of ionic liquids. Samples were hermetically sealed in pressure vessel. The DSC temperature program was as follows: 25 °C of initial temperature held for 10 min, and then a gradient of 1 °C min^{-1} up to 100 °C.

3. Results and discussion

The enzymatic methanolysis of lipase was tested for the synthesis of fatty acid methyl esters (FAMEs) in fifteen different ionic liquids at 60 °C. The ionic liquids tested in this work are not capable of carrying out the transesterification reaction without the presence of lipase. The catalytic efficiency of the immobilized enzyme (Novozym 435) on the methanolysis reaction was then checked in media containing methyl imidazolium ionic liquids (MIM) of different cation chain lengths (C_{10}, C_{12}, C_{14}, C_{16} and C_{18}) and in combination with three different anions ([BF_4], [PF_6] and [NTf_2]). Ionic liquids were dried in a vacuum oven at 60 °C at least for 24 h before reaction to remove all water, so that the final water content was lower than 0.10–0.15%, depending on the on hygroscopic properties of ILs. The melting point (T_m) was determined for all the ionic liquids used by differential scanning calorimetry (Table 1). It has been pointed out that the T_m of methyl imidazolium type ionic liquids is governed by van der Waals forces rather than by the electrostatic forces, and for such cations, the change of phase transition temperature depends of the anions more than on cations.[11] Moreover, the T_m increases with the alkyl chain length of the cation for the same anion. These results are in agreement with those of other authors.[11,12] When using IL with a T_m higher than 60 °C was used first liquefied at 80 °C and then kept liquid at 60 °C while substrates (methanol and oil) were added to carry out the enzymatic transesterification process.

Table 1 Melting point (T_m) for ILs by DSC (°C)

Cation	Anion		
	[NTf_2]	[PF_6]	[BF_4]
[C_{10}MIM]	–28.9[a]	34.8	–4.2[a]
[C_{12}MIM]	16.7	56.5	34.0
[C_{14}MIM]	34.6	73.0	38.0
[C_{16}MIM]	42.6	77.0	49.6
[C_{18}MIM]	51.3	80.3	66.8

[a] Data from Zhang *et al.*, 2006.

Fig. 2 shows that triglyceride consumption and the production of methyl esters was almost complete after about 6 h. The presence of intermediate products (MAG and DAG) was detected by HPLC, and the total consumption of MAG required a slightly longer time of about 12 h.

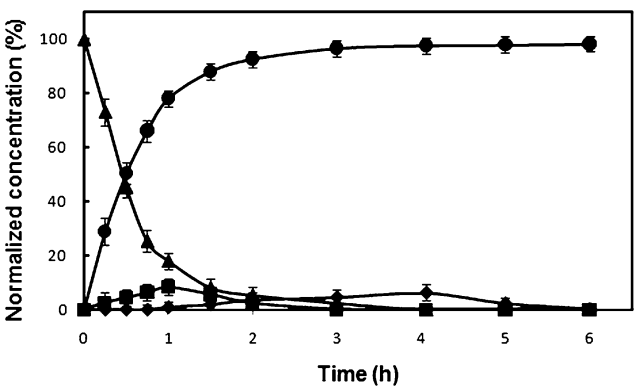

Fig. 2 Time course for methanolysis of triolein by Novozyme 435 in [C_{16}MIM] [NTf_2], at 60 °C.: (●) FAME, (▲) triglycerides (■) diglycerides, and (◆) monoglycerides.

The time course for methanolysis catalyzed by the enzyme was charted for all ILs, and the initial rate of the reaction for each case was determined from the slope of the best-fit of the product concentration *versus* time curve. For all the assayed ILs, both the synthetic activity and product yield at 24 h by Novozym 435 are shown in Fig. 3.

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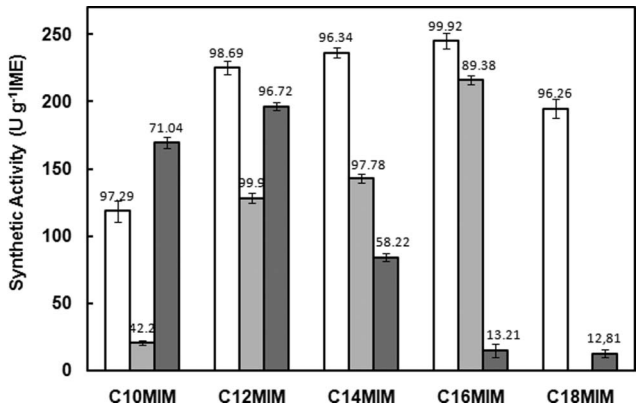

Fig. 3 Synthetic activity of FAMEs in fifteen ionic liquids based on different alkyl chain length of methyl imidazolium cation ([C_{10}MIM], [C_{12}MIM], [C_{14}MIM], [C_{16}MIM] and [C_{18}MIM]) combined with [BF_4] (dark grey), [PF_6] (light grey) or [NTf_2] (white). The numbers are the yield after 24 h of reaction time.

In all cases, an increase in alkyl chain length of the corresponding imidazolium cation improved synthetic activity up to a maximum, after which it decreased, which agrees with previous results obtained for Novozym 435 when catalyzing the synthesis of citronellyl esters,[7c] or in the synthesis of butyl propionate.[13] This can be partially explained by the higher solubility of the oil substrate in the more hydrophobic ILs, since the hydrophobic character of the IL increases with the alkyl chain length of the cation for the same anion.[13,14] On the other hand, viscosity increases with the increasing length of the alkyl chain substituent on the imidazolium ring increases, although it also depends on the anion type. For the [CnMIM] cations, the viscosity increased in this order with the anion type: [PF_6] > [BF_4] > [NTf_2].[8b] Increasing viscosity negatively affects the operating process through increased mass transfer resistance. Among the ionic liquids tested for biodiesel production, as seen in the literature, only methyltrioctylamonium trifluoroacetate (TOMA TFA) has a long alkyl chain length (C8)[9c] However, this ionic liquid has never been used in its completely pure state, and free traces of TFA have been demonstrated to act as acid catalyst or the synthesis of FAMEs by transesterification, so that the results obtained, in this work may not only be due solely to the enzymatic activity.[9c]

Tert-butanol was employed as a model system to examine the effects of ILs on enzyme activity. This organic solvent improves the enzymatic process because it dissolves both methanol and triglyceride, and decreases mass transfer resistances. Although an alcohol, *tert*-butanol is not a substrate for the lipase, because this enzyme does not show activity on tertiary alcohols. The synthetic activity in *tert*-butanol was 239.04 U g^{-1} IME and the product yield on FAMEs was 98.20% after 24 h, results that reflect those of authors.[3,7a,b] However, it should be noted that when a solvent is used, the processing cost is higher due to the extra processing equipment required for the separation of the co-solvent and the cost of the co-solvent itself.

When methanolysis in a solvent free system without ionic liquid was analyzed, a synthetic activity of 11.92 Ug^{-1} IME was calculated and a production yield of 45.46% after 24 h was obtained. Such a decrease in synthetic activity may be due to the above mentioned enzyme deactivation by methanol or to the low solubility of methanol in triglycerides. To avoid these effects, the addition of methanol was conducted in three stages, 1/3 molar equivalent methanol (30 μL) every eight hours, which increased the synthetic activity to 76.55 Ug^{-1} IME and of the product yield after 24 h to 87.21%.

On the other hand, for IL containing NTf_2 and PF_6 anions, the maximum synthetic activity was obtained for [C_{16}MIM] cation, while the activity was higher for IL containing the NTf_2 anion than for the PF_6 anion (see Fig. 3). The highest synthetic activity was obtained in [C_{16}MIM] [NTf_2] (245.13 Ug^{-1} IME, a value more than three times that obtained in the free solvent system). Additionally, the FAME production in [C_{16}MIM] [NTf_2] was 90.29% after 3 h while in the solvent-free system it was only 27.3%. However, for ILs based on the BF_4 anion the observed behaviour of the enzyme cannot be explained by the properties of the IL containing this anion. The highest synthetic activity was obtained for the [C_{12}MIM] cation, although the synthetic activity decreased as the alkyl chain of the imidazolium cation increased. This observation is in agreement with others authors who have described similar behaviours for ILs based on the BF_4 anion. All the immobilized lipases decreased their activity as the alkyl chain of the imidazolium cation increased in different synthetic reactions.[16] These observations may be explained by the different enzyme microenvironments of each assayed biocatalyst in their respective interaction with IL.[13]

In most of the ionic liquids tested in this work, the reaction mixture was homogeneous, but, when the reaction finished, a triphasic system was created through the appearance of a FAME phase (upper layer), a glycerol phase (middle layer), and an ionic liquid phase containing the enzyme (the lower layer). Additionally, a decrease in the reaction temperature (brought about with ice) results in the solidification of many ionic liquids (see Table 1), which facilitates the extraction of the biodiesel product. These observations demonstrate the suitability of using the ionic liquids tested for the production of biodiesel.

Novozym 525-L is a commercially available soluble lipase. Novozym 435 is a heterogeneous biocatalyst based on lipase B from *Candida antarctica* (CALB, Novozym 525-L) immobilized within a macroporous resin of poly-(methylmethacrylate) (Lewatit VP OC 1600), and containing roughly 10% by weight of CALB physically adsorbed within the support.[17] These two biocatalysts are among the most widely used enzymes in Biotechnology because of their versatility, especially in non-aqueous media. However, *Pseudomonas fluorescens* lipase seems to be a better choice for biodiesel synthesis because it is methanol-resistant.[18] In this sense, the two soluble lipases (Novozym 525-L and the *Pseudomonas fluorescens* lipase) were immobilized onto four supports, Lewatit VP OC 1600, celite, silica and Accurel MP1000 (a macroporous polypropylene support) to study their biodiesel synthetic activity in [C_{16}MIM][NTf_2] at 60 °C. The chosen supports have been used by other authors for lipase immobilization and have also been used for biodiesel production both in organic solvents and in free solvents systems.[18,19] In all cases, the lipases immobilization on the supports was by physical adsorption.

The synthetic activity for the different immobilization derivatives are depicted in Fig. 4, as can be seen, each immobilized lipase showed a different synthetic activity level on each one of the assayed immobilization supports. The synthetic activity of

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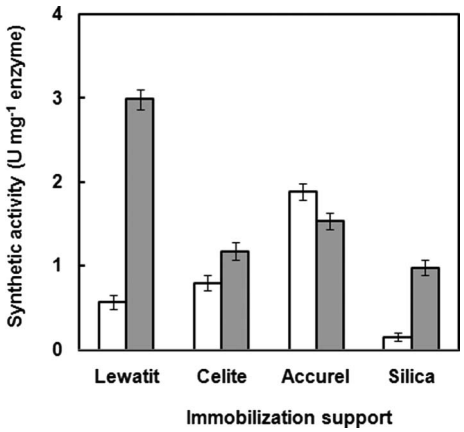

Fig. 4 Synthetic activity of FAMEs catalyzed by lipase AK (white) and lipase B (grey) immobilized on different supports.

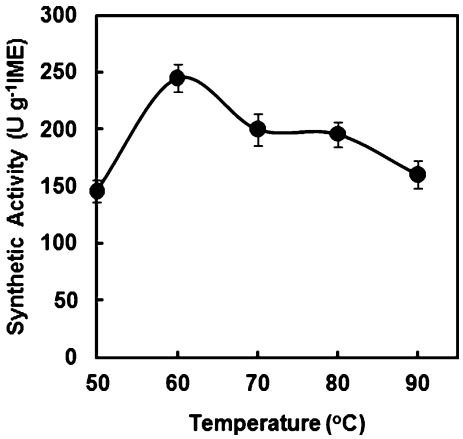

Fig. 5 Effect of temperature on the synthetic activity in $[C_{16}MIM][NTf_2]$, at the fixed conditions of alcohol to oil molar ratio (6 : 1) and Novozym 435 (10% w/w, based on oil weight).

CALB lipase (Novozym 525-L) immobilized on Lewatit in the laboratory (2.98 U mg^{-1} enzyme) was similar to that shown by their commercially available counterpart (2.45 U mg^{-1} enzyme).

The lipase AK seemed to be a worse catalyst than CALB for the proposed reaction, except in the case of Accurel, when the advantage of using lipase AK was slight. The results shown in Fig. 4 could be explained by taking into account the active role of the immobilization support in the microenvironment and its interaction with ILs.[7d] Thus, adsorption onto a macroporous polyacrylic resin (Lewatit) takes place largely through presumed hydrophobic interactions. In contrast, the silica support may interact electrostatically with positive groups on the enzymes surface, while the macroporous polypropylene support (Accurel) is hydrophobic, and previous studies have shown that, at low loading, the hydrophobic interactions between lipases and polypropylene are strong enough to cause inactivation of a significant fraction of the enzyme molecules.[18] Furthermore, in water free systems the hydration level of the enzyme is one of the fundamental parameters to be considered, since it strongly affects enzyme activity. In the case of immobilized enzymes, the partitioning of water among the components of the system (enzyme, support and reagent mixture) strongly depends on the hydrophilic/hydrophobic balance of the support.[20] On the basis of the obtained results, Novozym 435 was used in the subsequent steps of works.

In order to improve the efficiency of the lipase-catalyzed synthesis of FAMEs, the influence of temperatures ranging from 50 to 90 °C was also studied in $[C_{16}MIM][NTf_2]$ (Fig. 5).

As can be seen, the synthetic activity of Novozyme shows a curve with a maximum level of activity (245.13 U g^{-1} IME) at 60 °C. The synthetic activity in the range of 60 to 90 degrees is greater than at 50 °C, because IL viscosity decreased when the temperature increased.[15,21] In all cases, the FAME production after 24 h was close to 100%, except at 90 °C (93.87%).

The molar ratio of methanol to oil has been reported to be one of the most important variables affecting the yield of fatty acid methyl ester.[4] The stoichiometric ratio for the transesterification reaction requires only 3 moles of alcohol and 1 mole of triglyceride to yield 3 moles of fatty acid methyl ester and 1 mole of glycerol, although a higher molar ratio resulted in a larger ester conversion rate in a shorter time. Furthermore, this value depends on the properties of oil, the type of catalyst, and the reaction media used.[2,3,9]

The effect of the substrate molar ratio (methanol:triolein) on synthetic activity in $[C_{16}MIM]$ $[NTf_2]$ is shown in Fig. 6. The results indicated that the highest activity was achieved at a 6 : 1 ratio, and that methanolysis decreased when the molar ratio was higher than 6 : 1, because the high methanol concentration resulted in the inactivation of Novozym 435,[9b] and because, under such conditions, the triolein was not completely dissolved in methanol. However, in a free solvent system, the immobilized lipase B was partially deactivated by a methanol : oil ratio of 6 : 1 (11.92 U g^{-1} IME). These results led to the conclusion that the presence of the ionic liquid mitigates the inactivation of the biocatalyst. These results are in concordance with those of other authors.[9]

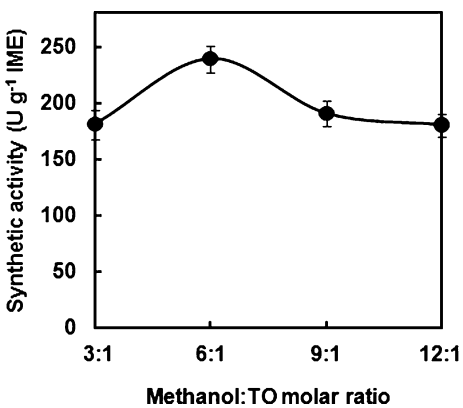

Fig. 6 Influence of methanol : triolein molar ratio on the synthetic activity in $[C_{16}MIM][NTf_2]$, at the fixed conditions of temperature (60 °C) and Novozym 435 (10% w/w, based on oil weight).

The effect of the ionic liquid-to-substrate volumetric ratio on synthetic activity in $[C_{16}MIM]$ $[NTf_2]$ is shown in Fig. 7, in the fixed conditions of 60 °C, an alcohol to triolein molar ratio of 6 : 1 and 10%w/w Novozym 435 (based on oil weight).

The ionic liquid : substrate volume ratio shown in Fig. 7, 1 : 0.5, 1 : 1, 1 : 5 and 1 : 10, corresponds to ionic liquid : triolein volume ratio as follows, 1 : 0.38, 1 : 0.77, 1 : 3.87, 1 : 7.75, respectively. In all cases, the initial system for the transterification

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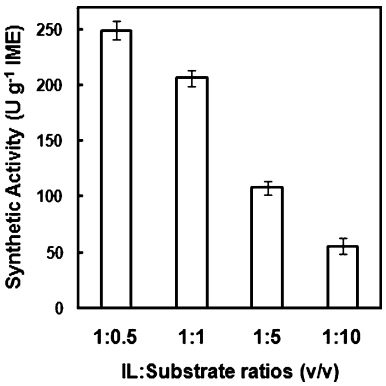

Fig. 7 The effect of ionic liquid : substrate ratio (based on v/v) on the synthetic activity in [C_{16}MIM] [NTf_2] at the fixed conditions of temperature (60 °C), alcohol to oil molar ratio (6 : 1) and Novozym 435 (10% w/w, based on oil weight).

reaction was a homogenous medium. For the case of [C_{16}MIM] [NTf_2], the synthetic activity of FAMEs decreased when the IL : substrate ratio increased, because the higher methanol concentration present in the reaction media the greater the enzyme deactivation.

None of the recent works by other authors using ionic liquids as a medium for the enzymatic production of biodiesel has analyzed this effect, since all used an ionic liquid : oil volume ratio of 1 : 1,[9] except Miyawaki and Tatsuno[9c] who demonstrated that the addition of 80% of methyltrioctylamonium trifluoroacetate accelerated the butanolysis of triolein, since traces of free TFA can act as acid catalyst for the synthesis of FAMEs by transesterification.

In order to analyze the performance of the acyl acceptor on the synthetic activity of biodiesel in [C_{16}MIM][NTf_2] medium, four primary alcohols (methanol, ethanol, *n*-propanol and *n*-butanol) was compared in the fixed conditions of temperature (60 °C), alcohol to triolein molar ratio (6 : 1) and Novozym 435, 10% w/w (based on oil weight). (Fig. 8).

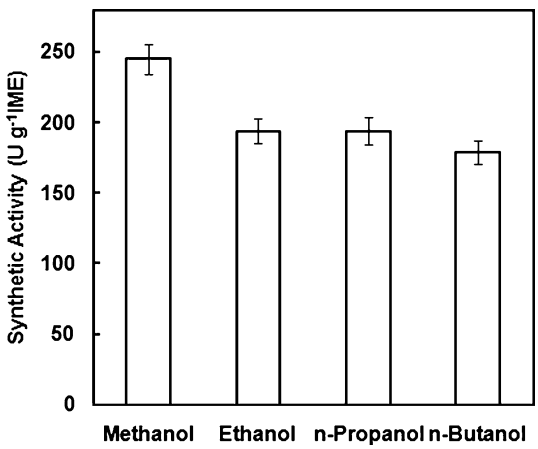

Fig. 8 Effect of different acyl acceptors on the synthetic activity in [C_{16}MIM] [NTf_2] at the fixed conditions of temperature (60 °C), alcohol-to-oil molar ratio (6 : 1) and Novozym 435 (10% w/w, based on oil weight).

The highest synthetic activity was achieved with methanol, with enzymatic alcoholysis decreasing with increasing alcohol alkyl chain length. Furthermore, alcohols with higher molecular mass showed higher density and greater mass transfer limitations.[4] Among these alcohols, methanol and ethanol are the cheapest which is one of the reasons why used for industrial biodiesel production.

To optimize the process biodiesel production, commercial grade and analytical reagent methanol have been used for enzymatic methanolysis, it being found that both the synthetic activity and the yield were the same. As economical considerations are a major issue in biodiesel production, the cheaper grade methanol is more frequent.

The high cost of lipase is one of the main problems for the application of enzymatic processes to the industrial production of biodiesel fuel from vegetable oil.[11] For this reason, the effect of the quantity of enzyme was studied on the methanolysis reaction in [C_{10}MIM][NTf_2] at 60 °C (Fig. 9).

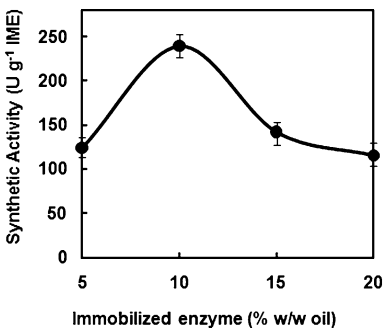

Fig. 9 Influence of immobilized lipase B quantity on synthetic activity for the synthesis of FAMEs in [C_{16}MIM][NTf_2] (based on olein weight) at the fixed conditions of temperature (60 °C) and alcohol-to-oil molar ratio (6 : 1).

The maximal synthetic activity was obtained for 28 mg of immobilized enzyme, which represents a 10% w/w (based on oil weight) while higher concentrations led to lows yields. Visual observations during the assays using enzyme concentration higher than 10% indicated that the liquid phase was not of sufficient volume to completely suspend the solid catalyst. In these circumstances, external mass transfer resistance becomes the limiting step for oil transesterification. This behaviour is in accordance with that obtained other researchers.[2] The cost of any enzyme used in biodiesel production is of primary important.

The most used vegetable oils in biocatalysed biodiesel production are those obtained from soybean seeds, sunflower seeds, and rape seeds in many European countries. Countries use different types of oil feedstock depending on abundance or availability; for example, soybean oil in the USA and coconut and palm oil in tropical countries.

The effect of triglyceride feedstock on the initial yield of FAMEs was analyzed in [C_{16}MIM][NTf_2]. In the present work four different vegetable oils were used (olive oil, sunflower oil, palm oil, and waste cooking oil) in addition to triolein. The fatty acid composition of these oils, as determined by HPLC analysis, is reported in Table 2 and is in concordance with those reported by other authors.[18] FAME content (μmoles) profiles for all the oils are shown in Fig. 10 by Novozyme 435 at 60 °C. The reaction rate (μmoles h^{-1}) and production yields (%) after 24 h

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Table 2 Fatty acid composition of the oils (%)

Oil Type	Olive oil	Sunflower oil	Palm oil	Waste oil
Palmitic acid	12.37	6.59	38.67	6.90
Stearic acid	2.98	3.46	3.32	3.89
Oleic acid	80.46	23.43	46.14	30.52
Linoleic acid	4.19	66.52	11.87	58.69

Table 3 Influence of oil type on catalytic efficiency and production yield after 24 h

Oil Type	Rate (μmoles h^{-1})	Yield after 24 h (%)
Triolein	478.08	99.39
Olive	345.71	93.38
Sunflower	407.55	92.78
Palm	428.07	94.11
Cooking waste	440.35	96.91

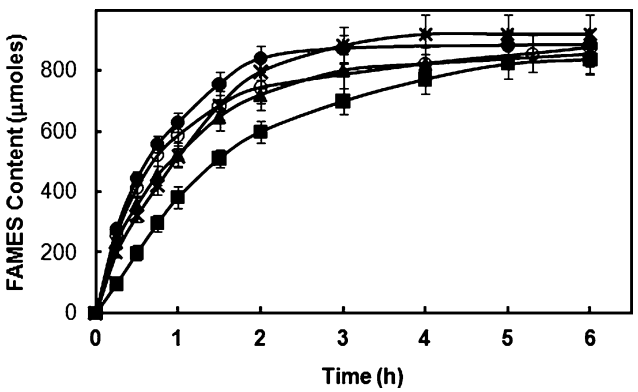

Fig. 10 Profiles of FAME content (μmoles) for every one of the oils: (●) triolein, (■) olive oil, (▲) sunflower oil, (○) palm oil and (□) waste cooking oil.

are shown in Table 3. There were no significant differences in the two parameters for the different oils, the yield after 24 h rouging from 93 to 97%, except in the case of triolein, when it was slightly higher.

Chen *et al.*[5a] showed that waste cooking oil can be used as feedstock for biodiesel production and that most of the properties of the resulting biodiesel are better than those of diesel in China and of biodiesel from Germany and USA. FAME type and degree of unsaturation are important factors for the quality of biodiesel, because they influence cetane number, viscosity, iodine value, *etc.*

Finally, the triglyceride feedstock had no significant effect on the enzyme catalytic efficiency. It seems, then that the use of waste cooking oil could greatly reduce the cost of biodiesel.

Conclusions

The suitability of ionic liquids containing long alkyl chains in cations as reaction media for the enzymatic production biodiesel has been demonstrated. The highest FAME production after 24 h was obtained in C_{16}MIM NTf_2. Several reaction parameters, such as temperature, methanol : oil molar ratio, alkyl-chain length of the alcohols, IL : substrate volume ratios, quantity of enzyme, and oils feedstock, were optimized. Furthermore, when C_{16}MIM NTf_2 is used the reaction mixture is homogeneous, and when the reaction is complete a triphasic system is created through the appearance of a FAMEs phase (upper layer), a glycerol phase (middle layer) and a lower layer with the ionic liquid containing the enzyme, which can be solidified by decreasing the media reaction temperature, thus facilitating the extraction of the biodiesel product. So, the use of ionic liquids containing long alkyl chains on the cations as well as hydrophobic anions ($[PF_6]$ or $[NTf_2]$) allows the selective extraction of the products using straightforward separation techniques.

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