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Enzyme-mediated epoxidation of methyl oleate supported by imidazolium-based ionic liquids

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ABSTRACT

In the present manuscript we describe and discuss the use of hydrophobic and hydrophilic ionic liquids (ILs) as efficient supports to the enzyme-catalyzed epoxidation of biodiesel. The use of nine different lipases in three different ILs (BMI.PF_6, BMI.NTf_2 and BMI.BF_4) gave high biodiesel conversion rates in short reaction times using hydrogen peroxide (30%, v/v) as the epoxidation agent. A drastic behavior change is observed by altering the media from a hydrophobic IL to a hydrophilic IL. For instance, the use of Amano A. lipase (from *Aspergillus niger*) in hydrophilic BMI.BF_4 yielded the epoxidized compound in 89% in the first reaction hour, and in the mean time, hydrophobic BMI.PF_6 yielded the same product in 67%. The use of other lipases resulted in the desired epoxidized derivative and also in the 1,2-diol as a result of a reversible epoxy ring-opening promoted in the reaction media. Conversions and selectivities depended on the nature of the IL, on reaction time and on the selection of the lipase enzyme.

1. Introduction

Due to increasing environmental concerns and petroleum supply security, the use of biomass as raw material for polymers, chemicals, fine chemicals and fuel applications has become an imperative issue in recent years [1]. In this context, one observes widespread interest in the chemistry of fats and oils as well as their derivatives [2], which are now renowned as a wealth of bio-based products and viable alternatives to petroleum-based fuels. As a consequence, demands for renewable feedstocks to obtain industrial products have been granted increasing importance. For example, it has been shown that epoxidation of fats, oils and fatty acid mono-esters is an important way to improve their physical–chemical properties and also to obtain starting materials for a large number of products. For instance, the epoxidation of biodiesel increased its overall oxidative stability and lowered its overall friction coefficient, even when done in the presence of additives, improving its quality [3,4]. Moreover, epoxy derivatives of fats and oils are also applied as lubricants, PVC-plasticizers and stabilizers, reactive diluents for paints, intermediates in the production of polyurethane-polyols, polyester polymers and alkyd resins, as well as components of blended lubricants and adhesives [5,6]. Methyl oleate epoxide, for example, is a key intermediate used in the synthesis of other derivatives such as carbonates [7] and azides [8]. It is worth mentioning that nowadays different products based on epoxidized derivatives of fats and oils are already commercially available.

Besides, the use of environmentally friendly reaction media is highly interesting in the current world's scenery and demands. In this sense, the use of ionic liquids (ILs) as reaction media has become a viable and attractive industrial alternative to organic solvents [9]. Additionally, it has been shown by some of us that imidazolium-based ILs (Fig. 1 1-*n*-butyl-3-methylimidazolium hexafluorophosphate, BMI.PF_6; 1-*n*-butyl-3-methylimidazolium bis(trifluoromethylsulfonyl)imide, BMI.NTf_2; 1-*n*-butyl-3-methylimidazolium tetrafluoroborate, BMI.BF_4) are excellent media that support different enzyme-catalyzed reactions [10]. Enzymes are usually active in ILs that comprise the species BF_4^-, PF_6^- and NTf_2^- [11], possibly due to the lower hydrogen bond basicity of the enzyme-compatible anions [12]. In this sense, the use of enzymes supported in an appropriate IL would be a very attractive system to promote epoxidation reactions of biodiesel (methyl oleate). In addition, we can envisage the use of hydrogen peroxide as the oxidant agent in the reaction, which is an attractive idea from both environmental and economic standpoints [6].

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Furthermore, vegetable oils, especially soybean oil, are relatively inexpensive [13].

The current industrial process for obtaining these epoxides is based on the Prileschajew reaction [14], which uses short chain percarboxylic acids. For all reasons mentioned above, it is necessary to develop novel, cheap, efficient and environmentally friendly methodologies to perform the epoxidation of fats and oils and their derivatives, such us methyl oleate [15]. Recently, different research groups have studied heterogeneous catalytic systems based on aluminum oxide and bi-metal oxides and hydrogen peroxide, which have lower activity compared to the industrial process but are easy to recover and reuse [6,16]. It is worth mentioning that several deactivation issues were described referring to the presence of both Brönsted [17] and Lewis [16] acid sites, which are probably related to the acid-catalyzed decomposition of hydrogen peroxide. On the other hand, it was recently shown that *CALB* enzyme catalyzes the epoxidation of methyl oleate in homogeneous media in the presence or absence of acyl donors, achieving good activities and selectivities [18a]. In fact, the use of enzymes to perform epoxidation reaction is not new and some articles are found in the literature [18b–d]. However, to the best of our knowledge, the potential of lipase enzymes to promote epoxidation reactions of bio-based compounds in ILs was not exploited. Based on our interest in bio-based fuels [19] and polymers [20] and in our experience using enzyme-catalyzed reactions in ILs [10,21a], we disclose herein that the use of some lipases, from different microorganisms, supported in hydrophobic (BMI.PF_6, BMI.NTf_2) or hydrophilic (BMI.BF_4) ILs results in a heterogeneous catalytic system for the synthesis of epoxidized methyl oleate derivatives with high yield and selectivity in short reaction times under an eco-friendly process.

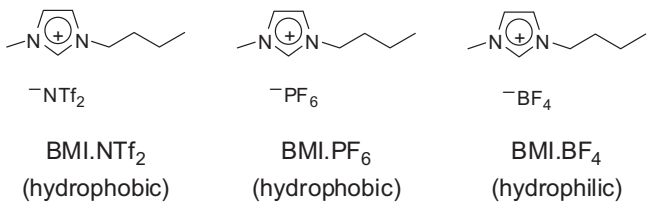


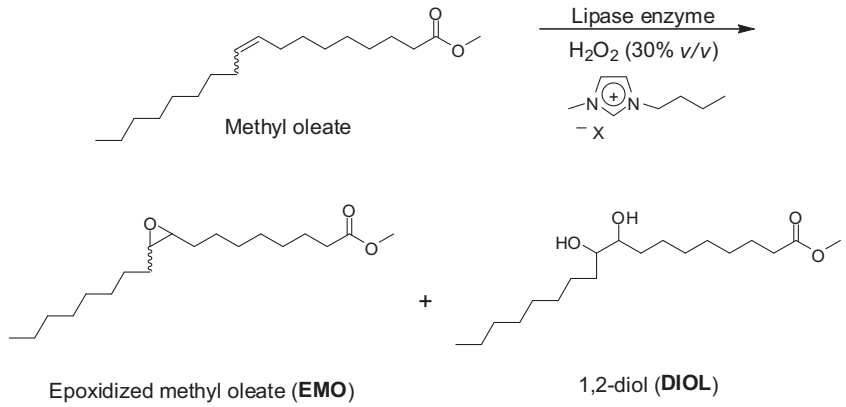
Fig. 1. Hydrophobic and hydrophilic imidazolium-based ionic liquids (ILs).

2. Results and discussion

An initial screening was performed using nine different commercially available lipases to promote the epoxide formation (**EMO**) and epoxy ring-opening (**DIOL**) reactions (Scheme 1) in a hydrophobic IL (BMI.NTf_2). The results are summarized in Table 1.

It is interesting to highlight that all reactions carried out in the presence of acyl donor (acetic acid 10 mol%) gave worse results, probably due to enzyme instabilization. For this reason we decided not to use any acyl donor in further reactions under the tested conditions.

As shown in Table 1, despite the hydrophobicity of the tested IL, all lipases were successfully supported in the ionic media and



Scheme 1. Enzyme- (lipase-) catalyzed epoxidation (**EMO**) and epoxy ring-opening (**DIOL**) reaction using methyl oleate as the substrate and hydrogen peroxide (30%, v/v) in different imidazolium-based ILs at 30 °C.

Table 1
Epoxidation and epoxy ring-opening using hydrophobic BMI.NTf_2 and hydrogen peroxide (30%, v/v) at 30 °C.

Ent.	Enzyme	Time (h) and yield (%)					Prod.
		1 h	2 h	3 h	4 h	5 h	
1	*From Aspergillus niger* (Amano A.)	64	45	48	56	58	**EMO**
		18	27	26	12	14	**DIOL**
2	*From Candida antarctica B* (Novozyme 435)	62	62	54	58	34	**EMO**
		14	18	15	13	48	**DIOL**
3	*From Candida rugosa* (Amano AY)	69	55	58	52	45	**EMO**
		14	20	13	21	29	**DIOL**
4	*From Penicillium camembertii* (Amano G 50)	54	45	34	66	47	**EMO**
		12	23	20	16	25	**DIOL**
5	*From Penicillium roqueforti* (R Amano K)	67	46	47	47	48	**EMO**
		11	27	28	23	25	**DIOL**
6	*From Pseudomonas cepacia* (PS-D Amano I)	62	58	49	45	59	**EMO**
		12	15	32	25	16	**DIOL**
7	*From Pseudomonas fluorescens* (Amano lipase AK)	54	55	49	52	56	**EMO**
		12	15	24	22	15	**DIOL**
8	*From Porcine pancreas* (type II, Aldrich)	53	50	33	47	57	**EMO**
		12	25	45	26	24	**DIOL**
9	*From Lipase type II* (Calf tongue roof)	78	58	61	68	47	**EMO**
		9	14	20	10	23	**DIOL**

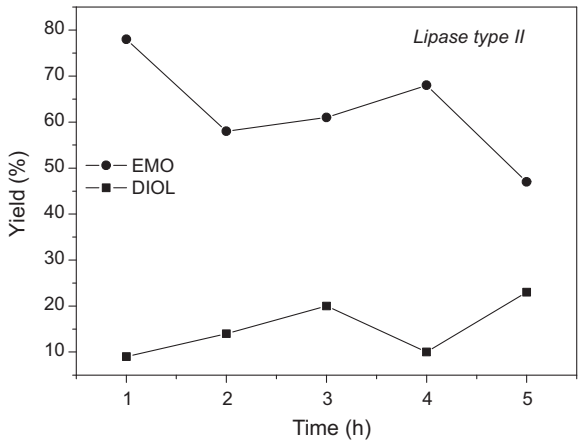


Fig. 2. Observed behavior of **EMO** and **DIOL** formation catalyzed by *Lipase Type II* (Calf tongue roof) enzyme in BMI.NTf_2 at 30 °C. Note that the observed behavior is similar for all tested lipase enzymes (see Table 1).

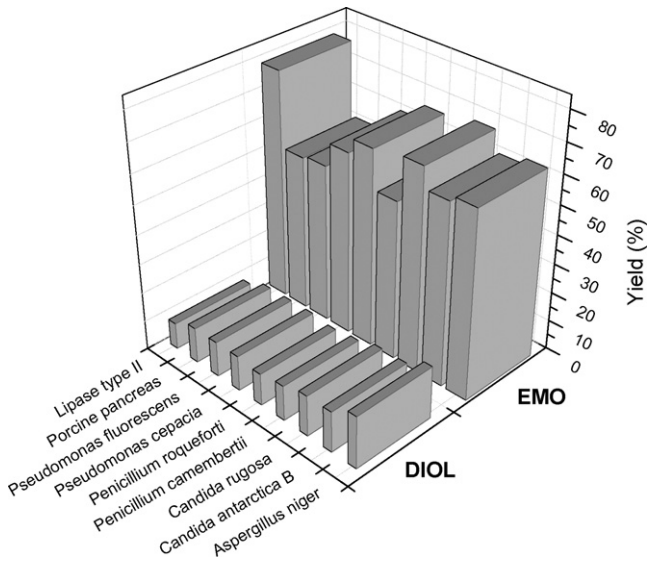


Fig. 3. Obtained yields of **EMO** and **DIOL** in the first reaction hour under the studied conditions using 'wet' hydrophobic IL BMI.NTf_2 at 30 °C.

showed good activity. This means that all enzymes showed a comparable behavior. Indeed, the use of this 'wet' hydrophobic ionic liquid gave very interesting results. In the first reaction hour, in all cases, a high yield of **EMO** formation and low yield of **DIOL** are observed. The results obtained for **EMO** conversion in the reaction performed using *Lipase type II* (Table 1, Entry 9) are even more expressive. It was possible to close the oxirane ring in 78%, whilst the **DIOL** formation was accomplished in mere 9% yield. Additionally, the methyl oleate conversion (**EMO** + **DIOL**) ranged from good to excellent in all cases. Moreover, the requested reactions time are very short. In Fig. 2 (for *Lipase type II*) we see a representative graphic of the reaction during a period of five hours.

It is interesting to point out that the reaction conducted in the absence of hydrogen peroxide does not result in **DIOL** formation independently of the IL tested. This showed that closing the ring is required prior to the **DIOL** formation from the oxirane ring (**EMO**) catalyzed in the ionic media. Somehow, it is expected and logical. As can be depicted from Fig. 2 and the data in Table 1, we could observe that the epoxy ring opening is a consequence of the **EMO** formation, as shown in Scheme 2.

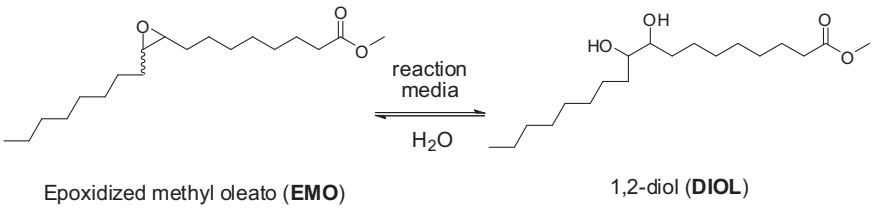
It has earlier been stated that lipases do not perform epoxide ring opening and that these reactions can be mediated by *Epoxide hydrolases* [21b]. As a consequence, it can be attributed to the ionic liquid effect. Indeed, the use of pure **EMO** and H_2O_2 in BMI.NTf_2 results in **DIOL** formation in poor yields.

Based on an elegant study of the diffusion coefficients of metal compounds in "wet" and "dry" ILs [22], it was pointed out that "wet" ILs (as the current case) are nanostructured materials which allow polar neutral molecules to undergo fast diffusion in polar or 'wet regions' [23]. Additionally, it was explained that ILs must be understood as nanostructured media when supporting enzymes [23]. Moreover, some researchers have very recently described that in the present context, enzymes in water-immiscible ILs should be also considered as included into hydrophilic gaps of the network [24]. Therefore, the observed stabilization of enzymes could be attributed to the maintenance of this strong network around the protein, displaying exceptional synthetic activity and operational stability [25a].

It is worth noting that the obtained epoxide yield is among the highest ever published for methyl oleates. Moreover, the reaction time is very short, rendering this methodology one of the fastest ever described. Note that higher yields (almost quantitative) but requiring longer reaction times have already been published ear-

Table 2
Epoxidation and epoxy ring opening using 'wet' BMI.PF_6 and hydrogen peroxide (30%, v/v) at 30 °C.

Ent	Enzyme	Time (h) and yield (%)					Prod.
		1 h	2 h	3 h	4 h	5 h	
1	*From Aspergillus niger* (Amano A.)	67	72	68	70	77	**EMO**
		19	11	19	25	16	**DIOL**
2	*From Candida antarctica B* (Novozyme 435)	66	72	68	67	70	**EMO**
		15	15	8	14	18	**DIOL**
3	*From Candida rugosa* (Amano AY)	69	65	62	68	69	**EMO**
		20	19	19	18	20	**DIOL**
4	*From Penicillium camembertii* (Amano G 50)	60	65	73	61	64	**EMO**
		29	22	15	31	27	**DIOL**
5	*From Penicillium roqueforti* (R Amano K)	73	78	75	70	82	**EMO**
		22	16	18	21	14	**DIOL**
6	*From Pseudomonas cepacia* (PS-D Amano I)	77	66	63	67	73	**EMO**
		15	19	22	20	19	**DIOL**
7	*From Pseudomonas fluorescens* (Amano lipase AK)	67	68	66	72	64	**EMO**
		20	21	20	11	21	**DIOL**
8	*From Porcine pancreas* (type II, Aldrich)	80	76	71	74	81	**EMO**
		11	16	12	18	16	**DIOL**
9	*From Lipase type II* (Calf tongue roof)	18	57	73	77	79	**EMO**
		32	30	16	15	14	**DIOL**



Scheme 2. Lipase-catalyzed ring opening (**DIOL**) from **EMO** in ILs.

lier [25b]. Since we could not avoid the epoxy ring opening under the studied conditions, it is crucial for our main goal that **EMO** be obtained within the first reaction hour, since the best results were acquired in this period of time. In Fig. 3 we visualize the best yields of **EMO** and **DIOL** in the first reaction hour for all tested enzymes.

Enzymes such as *Candida rugosa* (Table 1, Entry 3) and from *Penicillium roqueforti* (Table 1, Entry 5) afforded **EMO** in good yields as well (69% and 67%, respectively). The best **DIOL** yield was achieved using the lipase from *Candida antarctica B* (Table 1, Entry 2) in five hours (48%). The obtained results indicate that, under the tested conditions, all studied lipases preferentially promote methyl oleate conversion into **EMO** instead of oxirane ring opening to give the **DIOL** product.

To verify the efficiency of hydrophobic ILs in the epoxidation reaction and epoxy ring opening, we decided to perform the same reaction using 'wet' hydrophobic BMI.PF_6 as the reaction medium. All results are summarized in Table 2.

It is very clear from the data in Table 2 that the anion effect plays a role in the enzyme-catalyzed epoxidation of methyl oleate. As expected, the anion effect over enzymatic reactions can be very drastic [26]. Considering the first hour of reaction in BMI.PF_6, the best **EMO** yield (80%) was obtained when a lipase from *Porcine pancreas* was used (Table 2, Entry 8), whilst in the mean time the **DIOL** yield was only 11%. Selecting *Lipase type II* (Table 2, Entry 9), only 18% of **EMO** and 32% of the **DIOL** were produced using the same media. It is worth mentioning that, when utilizing similar reaction conditions, however with BMI.NTf_2 as reaction media, the yield was 78% and 9% (for *Lipase type II*), respectively, as shown in Table 1 (Entry 9). The present case exemplifies the importance of anion selection and screening when using different enzymes.

Another important issue to be considered is the reaction time. We noted that in BMI.PF_6 at the settled temperature (30 °C), *Lipase type II* (Table 2, Entry 9), lipase from *Penicillium roqueforti* (Table 2, Entry 5) and from *Porcine pancreas* (Table 2, Entry 8) gave the best **EMO** yields (79%, 82% and 81%, respectively). Nevertheless, the required reaction time was 5 h. During the course of the reaction, the yield increased for *Lipase type II* (Table 2, Entry 9) and oscillated by using the lipase from *Penicillium roqueforti* (Table 2, Entry 5) as well as that from *Porcine pancreas* (Table 2, Entry 8), as seen in Fig. 4.

As may clearly be depicted from Fig. 4, *Lipase Type II* displays a different behavior. Nevertheless, it also shows a high **EMO** yield and low **DIOL** yield. Moreover, all enzymes tested in BMI.PF_6 showed good conversion rates, affording **EMO** in very good yields either within the first or fifth reaction hours, depending on the enzyme. A better visualization is provided by Fig. 5 for the first and for the fifth reaction hours.

In the case of the lipase from *Candida antarctica B* in BMI.PF_6 (Table 2, Entry 2), the highest yield was detected in the second hour (72%). Lipase from *Penicillium camembertii* (Table 2, Entry 4) afforded **EMO** in 73% in the third reaction hour and from *Pseudomonas fluorescens* (Table 2, Entry 7) displayed the highest yield in the fourth hour (72%). For all other lipases tested in BMI.PF_6 as the reaction media, the maximum yield was reached either in the first or in the last hour of the established reaction time. The best

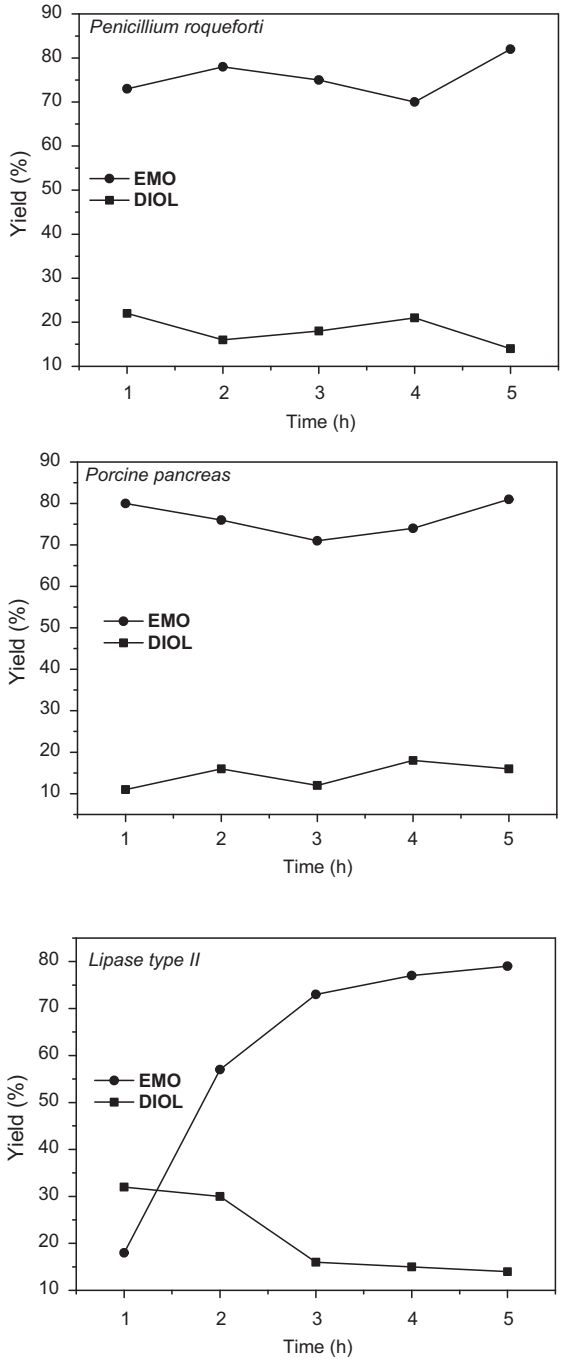


Fig. 4. Observed behavior of **EMO** and **DIOL** formation catalyzed by lipases from *Penicillium roqueforti*, *Porcine pancreas* and *Lipase type II* in BMI.PF_6 at 30 °C.

Table 3
Epoxidation and epoxy ring opening using hydrophilic BMI.BF_4 and hydrogen peroxide (30%, v/v) at 30 °C.

Ent	Enzyme	Time (h) and yield (%)					Prod.
		1 h	2 h	3 h	4 h	5 h	
1	*From Aspergillus niger* (Amano A.)	89	69	24	53	21	**EMO**
		–	25	60	39	54	**DIOL**
2	*From Candida antarctica B* (Novozyme 435)	27	31	29	34	59	**EMO**
		19	47	27	37	39	**DIOL**
3	*From Candida rugosa* (Amano AY)	41	33	52	25	27	**EMO**
		20	36	44	28	22	**DIOL**
4	*From Penicillium camembertii* (Amano G 50)	67	27	29	33	37	**EMO**
		18	39	52	25	22	**DIOL**
5	*From Penicillium roqueforti* (R Amano K)	26	28	52	42	30	**EMO**
		19	33	20	26	18	**DIOL**
6	*From Pseudomonas cepacia* (PS-D Amano I)	45	46	57	55	49	**EMO**
		47	31	3	2	17	**DIOL**
7	*From Pseudomonas fluorescens* (Amano lipase AK)	41	39	37	31	35	**EMO**
		22	39	38	27	24	**DIOL**
8	*From Porcine pancreas* (type II, Aldrich)	34	32	29	26	45	**EMO**
		28	32	42	21	28	**DIOL**
9	*From Lipase type II* (Calf tongue roof)	31	28	35	47	31	**EMO**
		37	40	41	22	30	**DIOL**

DIOL yield was achieved using lipase from *Penicillium camembertii* (Table 2, Entry 4) in the fourth hour (31%).

Despite the quality of the obtained results in the two 'wet' hydrophobic ILs, it was necessary to conduct the same study in a hydrophilic IL. Generally, considering imidazolium-based ILs, both cations and anions play a major role in stabilizing enzymes in the presence of water [27]. Since it is nowadays well accepted, we could expect a very different behavior using BMI.BF_4 as a medium to support the lipases. All reactions were carried out under the same conditions (at 30 °C and hydrogen peroxide 30%, v/v). The results are summarized in Table 3.

All reactions performed in BMI.BF_4 afforded **EMO** and **DIOL** in distinctive proportions. Nonetheless, it is important to highlight that reactions carried out in this hydrophilic medium significantly increased the **DIOL** yields for all tested lipases. Somehow, this should be expected, especially because water is now available in much higher concentrations than those detected when using hydrophobic ILs. For instance, we observed 52% of **DIOL** in the third reaction hour using lipase from *Penicillium camembertii* (Table 3, Entry 4).

In a general way, **EMO** formation ranged from reasonable to good yields in all cases described in Table 3. Overall, the use of hydrophilic IL gave poor results when compared to those obtained using hydrophobic ILs (compare Tables 1–3). Nevertheless, the best result is obtained when the lipase from *Aspergillus niger* is employed (Table 3, Entry 1). In the first reaction hour, using BMI.BF_4 as reaction medium, we noted the highest yield of **EMO** in all cases (89%, Table 3, Entry 1), even considering the results obtained with hydrophobic BMI.NTf_2 or BMI.PF_6. The reaction was highly selective and very fast. Additionally, no **DIOL** formation could be noted during this period, indicating the high selectivity of the reaction under the tested condition. Curiously, the best yield of **DIOL** formation (among all) was equally obtained using the same enzyme (Table 3, Entry 1), but in the third reaction hour (60%).

It is worth noting that some authors have previously reported that the lipase from *Aspergillus niger* is not efficient at all to perform transesterification reactions in ILs (no biodiesel formation noted) [10]. However, it is the best lipase among all tested herein to perform the epoxidation reaction in a hydrophilic IL. Since BMI.BF_4 is a hydrophilic IL, water is readily available in the presence of the enzyme to promote other side reaction, that means ester hydrolyze. However, the ester hydrolysis is very slow when compared to the epoxidation reaction. After 6 h of reaction, the only enzyme that promoted the presence of a significant content of free fatty acid in the reaction bulk is that from *Pseudomonas fluorescens*, which displays a 33% of acid content. Less than 10% of free fatty acid was noted after 6 h of reaction in the presence of all other enzymes.

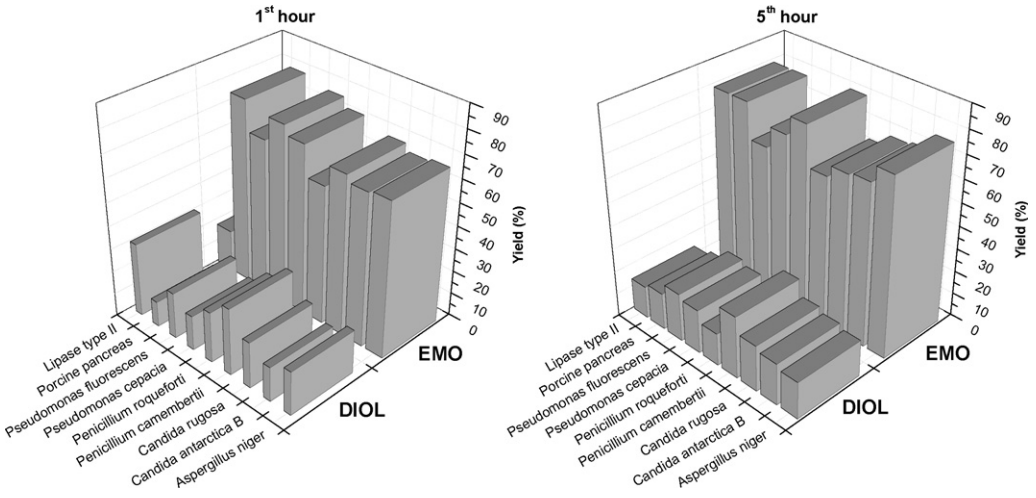


Fig. 5. Obtained yield of **EMO** and **DIOL** in the first and in the fifth reaction hours under the studied conditions using BMI.PF_6 at 30 °C.

Finally, for all tested cases, enzymes and ILs could be recovered and reused without any loss of activity. Enzyme was filtered, washed with acetone and reused without any loss of activity. The ILs were easily recovered after enzyme filtration and reused several times after removal of solvent.

3. Conclusions

Overall, the use of hydrophobic ILs (BMI.NTf_2 and BMI.PF_6) allow the **EMO** formation in high yields and within a few hours and, in many cases, in just one hour of reaction. It is preferred to carry out the **EMO** formation under the tested conditions, with low yields of **DIOL**. The use of a hydrophilic IL (BMI.BF_4) has shown that **DIOL** formation is viable and competes directly with the **EMO** formation. However, the highest yield of **EMO** (89%) was obtained using the enzyme from *Aspergillus niger* in this medium. The reaction takes place in just one hour. Moreover, the **DIOL** yield can be increased in hydrophilic ILs. This result is one of the best ever published considering both selectivity and reaction time. Moreover, the use of a hydrophobic IL favors the competition reaction of epoxidation and hydrolysis, but epoxidation is much faster. And free acid content is less than 10% after 6 h of reaction. It is also noteworthy that no other product could be detected during the reaction, indicating the high selectivity of the catalytic system described herein.

In summary, we have described that ILs are great media to promote the epoxidation of methyl oleates in short reaction times with high yields and high selectivity. Actually, the obtained results are among the best ever reported so far. Additionally, the described methodology has all the advantages of using an eco-friendly system combining the advantages of ILs and the efficiency of enzyme catalysis. Moreover the epoxidizing agent is hydrogen peroxide, which forms water as a byproduct, rendering the entire process extremely "green" and eco-friendly.

4. Experimental

General: Enzymes and all other chemicals were purchased from commercial sources (Amano, Acros or Aldrich) and used without further purification. We used standard and commonly used procedures to the synthesis and purification of ILs. Authentic samples of **EMO**, **DIOL** and methyl oleate were used to determine the HPLC method [28]. HPLC analyses were performed on a Shimadzu LC-20A Prominence liquid chromatograph equipped with an SPD-M20A diode-array detector and a four-solvent delivery system. The solvents were filtered through a 0.45-mm Millipore filter prior to use and degassed by continuous stripping with nitrogen. Injection volumes of 1 mL and a flow rate of 1 mL min^{-1} were used in all analyses. All samples were dissolved in propan-2-ol-*n*-hexane (5:4, v/v). All solvents were HPLC-grade and were used as purchased, without further purification. A Shim-pack VP-ODS (particle size 4.60 mm, 250P 4.6 mm I.D.) column was obtained from Shimadzu. HPLC method: reservoir A contained water, reservoir B contained acetonitrile, and reservoir C contained propan-2-ol-*n*-hexane (5:4, v/v). A 50-min ternary gradient with two linear gradient steps was employed: 30% A + 70% B in 0 min, 100% B in 15 min, 50% B + 50% C in 30 min, followed by isocratic elution with 50% B + 50% C for the last 20 min. Epoxidation reaction. In a thermo-controlled bath ($30 \pm 0.1\ ^{\circ}C$) enzyme lipase (100 mg), IL (1 mL), methyl oleate (2 mL) and hydrogen peroxide (30%, v/v, 1 mL) were added to a Schelenk flask. The mixture was stirred for five hours and the product removed and directed analyzed by HPLC. Enzyme and IL reuse. All enzymes could be efficiently recovered and reused. Enzyme can be filtered, washed with acetone and reused without loss of activity. The ILs can be easily recovered after filtered the enzyme and could be reused several times after solvent remove (vacuum).

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