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Selective esterification of phthalic acids in two ionic liquids at high temperatures using a thermostable lipase of *Bacillus thermocatenulatus*: A comparative study

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Abstract

The aims of the present paper were to obtain the best conditions for improving the selectivity of the esterification and amidation reactions catalysed by *Bacillus thermocatenulatus* lipase (BTL) at 90 and 120 °C in two ionic liquids (IL), 1-butyl-3-methyl imidazolium tetrafluoroborate (Bmim BF_4) and 1-butyl-3-methyl imidazolium hexafluorophosphate (Bmim PF_6), changing: (i) substrates (isomeric phthalic acids and 4-aminobenzoic acid), (ii) nucleophiles (alcohols) and (iii) water activity.

When the substrate was changed, best selectivity towards the formation of the monoester (74% at 1 h) over the diester (0% at 1 h) was obtained in Bmim BF_4 at 120 °C when isophthalic acid was employed. Nevertheless, in Bmim PF_6 though a higher yielding was reached, no selectivity was obtained with any substrate. Ethanol was selected as the best nucleophile, while the increase of length, steric hindrance and rigidity of the alcohol diminished the selectivity of the process. Reaction yield increased when setting a 0.4 water activity of the reaction media value at 30 °C, when the esterification of isophthalic acid with ethanol catalysed by BTL in Bmim BF_4 was performed at 90 °C (88% at 0.5 h).

Keywords: Ionic liquids; *Bacillus thermocatenulatus* lipase; Water activity; Selectivity

1. Introduction

Lipases are a versatile group of biocatalysts. Their natural function is the hydrolysis of triglycerides to partial glycerides and fatty acids during digestion. These enzymes are widely used in organic chemistry because of their high specifity and selectivity. However, many lipases are only moderately stable at high temperatures at which the majority of the industrial processes are performed. This can be solved by using lipases from thermophilic microorganisms, whose drastic conditions resistance has been developed by nature [1,2]. The majority of purified and characterized thermophilic lipases are obtained from *Bacillus* spp. In the present paper we have selected *Bacillus thermocatenulatus* lipase (BTL) due to its high hydrophobicity and thermostability, which makes it a promising biocatalyst for many industrial processes [3].

Nevertheless, at these extremely high temperatures conventional organic solvents are usually volatiles [4]. Furthermore, there is an urgent need to develop alternative solvents and technologies due to the governments and other regulatory organisms pressure to protect the environment. Although enzymes are environmentally friendly catalysts, some enzyme-catalysed reactions require harmful organic solvents [5–7]. And finally, the use of reaction media engineering can certainly improve the performance of lipases for a given application [8]. The most recent achievement was the discovery that enzyme-catalysed reactions can be performed in ionic liquids (IL). The first report of the successful use of these solvents found the performance of *Candida antarctica* B lipase to be similar to that of conventional organic solvents in alcoholysis, ammoniolysis and perhydrolysis [9].

ILs are salts that below 100 °C or close to room temperature are liquids. Moreover, physical properties of ILs (density,

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viscosity, melting points, polarity, miscibility, etc.) can be finely tuned by the appropriate selection of anions and/or cations [10–12]. Due to this fact, several enzymes and substrates (e.g. carbohydrates) that cannot be dissolved in organic solvents could be directly solubilized in ILs [13–15]. Furthermore, ILs are generally very good solvents to carry out nucleophilic substitution reactions [16–18] and different enzymes such as hydrolases (proteases and lipases) and oxidoreductases (peroxidases and dehydrogenases) maintain their activity when they are suspended in ILs.

In the present paper, 1-butyl-3-methyl imidazolium tetrafluoroborate (Bmim BF_4) and 1-butyl-3-methyl imidazolium hexafluorophosphate (Bmim PF_6) were selected due to their different viscosity (330 cP at 20 °C for Bmim PF_6 and 154 cP at 20 °C for Bmim BF_4) [19]. Furthermore, these two ILs show a very high polarity that is in the range of a medium chain alcohol, such as 1-hexanol or 1-octanol, with marked contributions from the anion as well as the cation, e.g.: for Bmim BF_4 0.673 and Bmim PF_6 0.666 on the Reichardt's polarity scale (0 for non-polar tetramethylsilane and 1 for polar water) [20]. Moreover, Bmim BF_4 is miscible with water, while Bmim PF_6 is inmiscible.

Another often-studied parameter for lipase-catalysed reactions in organic media is the water activity (a_w). High product yields can be obtained when water activity is subject to careful control and fixed at an optimum value [21].

The influence of ILs on the stereoselectivity [22] and the regioselectivity towards sugars [23] of lipases has been previously reported. Nevertheless, in the present paper, for the first time the selective esterification and amidation of phthalic acids by using a thermostable BTL in IL has been studied. The aims of the present paper were to obtain the best conditions for improving the selectivity of esterification and amidation reactions catalysed by BTL at 90 and 120 °C in two ILs Bmim BF_4 and Bmim PF_6 by changing: (i) substrates (isomeric phthalic acids and 4-aminobenzoic acid), (ii) nucleophiles (alcohols) and (iii) water activity.

2. Experimental

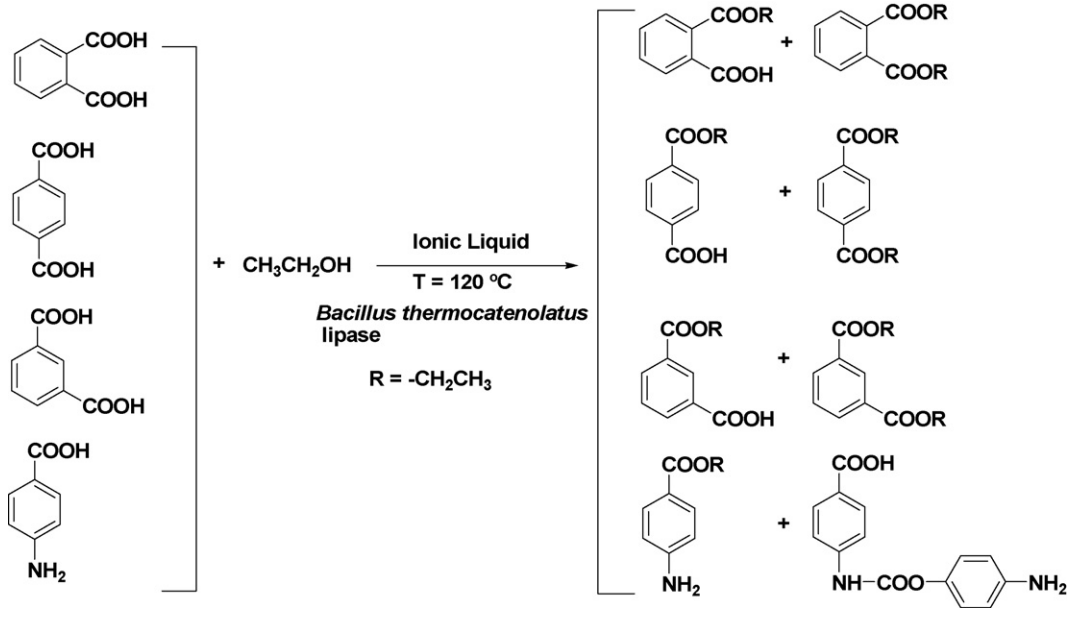
2.1. Materials

A non-commercial lipase from *B. thermocatenulatus* isolated as previously described [24] was a gift from the Biochemistry, Genetics and Immunology Department of the Universidad de Vigo.

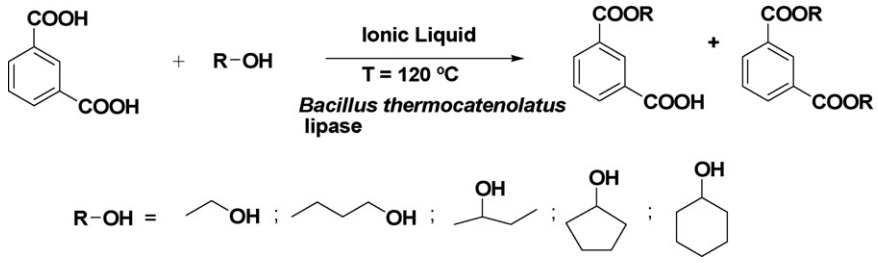
All solvents of the highest purity commercially available and used without purification were purchased from Merck Chemical (Darmstadt, Germany). All other chemicals: alcohols used as nucleophiles (ethanol, 1-butanol, 2-butanol, cyclopentanol and cyclohexanol), carboxylic acids employed as substrates (phthalic acid, isophthalic acid, terephthalic acid and 4-amino benzoic acid), standards for a_w calibration ($LiCl{\cdot}H_2O$ ($a_w = 0.11$), $MgCl_2{\cdot}6H_2O$ ($a_w = 0.32$), $Mg(NO_3)_2{\cdot}6H_2O$ ($a_w = 0.52$) and $NaCl{\cdot}H_2O$ ($a_w = 0.72$)) and ILs (Bmim BF_4 and Bmim PF_6), were purchased from Sigma–Aldrich (Barcelona, Spain).

2.2. Selectivity assays

Selectivity studies of esterification catalysed by BTL at the same experimental conditions were carried out (Scheme 1) with several acids (phthalic acid, isophthalic acid, terephthalic acid and 4-amino benzoic acid) and ethanol, in Bmim BF_4 and Bmim PF_6 at 120 °C. The reactions were performed in a closed glass reactor at 120 °C coupled to a thermostatised oil bath for several days. As a consequence of the use of high temperatures, the reactor was rapidly cooled down previously to the opening to avoid the vaporization of the alcohols. The reaction mixture included: an organic solvent (IL, 4 mL), an



Scheme 1.



Scheme 2.

organic acid (30 mM) and an alcohol (1 mL), assuring that the quantity of alcohol was in excess with respect to the organic acid. Then, the enzyme (4 mg solid/mL) was added. In order to ensure that reaction is due only to the lipase, a reaction test without lipase was carried out. Samples of 100 μL were taken at different time and analyzed by HPLC. As ILs cannot be directly injected into the HPLC, the samples were extracted with 1 mL of diethyl ether and the solvent evaporated at room temperature. Remaining samples were re-diluted with 400 μL hexane/2-propanol (1:1) (v/v) and filtered with syringe filters of 0.2 μm (Teknokroma, Barcelona, Spain). HPLC was performed using a Chiracel column OD (20 μm, 250 mm × 4.6 mm) for phthalic acid, isophthalic acid and terephthalic acid using a mobile phase of hexane:2-propanol:trifluoroacetic acid (90:9:1), a 0.8 mL/min flux, and a wavelength of 254 nm were used; and for 4-amino benzoic acid, the analytical conditions were hexane:2-propanol:trifluoroacetic acid (95:5), 0.6–0.8 mL/min flux and at 254 nm.

2.3. Water activity assays

The a_w measurement were performed with a Rotronic-Hygroscop instrument previously calibrated using standard salts with fixed a_w values. Samples of lipase and IL were previously pre-dried at vacuum in the presence of anhydrous P_2O_5. Experiments were carried at a_w values 0.4, 0.6 and 0.8 (measured at 30 °C), so that these a_w values must be considered as an orientative value of the real a_w value at 90 and 120 °C, the temperatures at which selective reactions were performed. In a standard experiment, different amounts of water were added to the reaction media (bulk reaction) placed in the cell until reaching the equilibrium.

2.4. Selectivity studies at controlled water activity

The selectivity studies at controlled a_w were followed by monitoring esterification reactions between different acids with several alcohols using a thermostable BTL at 0.4, 0.6 and 0.8, selected as low, medium and high a_w values. The experimental conditions were the same than selectivity assays in the absence of water (Section 2.2).

3. Results and discussion

3.1. Selectivity of the esterification reactions of different organic acids in Bmim BF_4 and Bmim PF_6 catalysed by B. thermocatenulatus lipase

The esterification reactions could be produced in the absence of the biocatalyst due to the use of IL that are highly polar media at high temperatures, and because of the imidazolium cation that could catalyse the reaction by acid catalysis. Nevertheless, no esterification traces were observed without the addition of lipase.

Conversion data of the different organic acids are shown in Table 1. Phthalic acid showed low selectivity with both ILs, and the formation of monoester and diester occurred simultaneously during the whole esterification process (data not shown). Nevertheless, isophthalic and terephthalic acids in Bmim BF_4 showed a very high selectivity, the monoester being synthesized faster than of the diester, 79% at 1 h and 44% at 0.5 h, respectively.

Table 1
Esterification of phthalic acids with ethanol at 120 °C using *Bacillus thermocatenulatus* lipase in Bmim BF_4 and Bmim PF_6

Acid	Ionic liquid	Time (h)	Yield (%)[a]	Monoester (%)	Diester/amide (%)
Phthalic acid	Bmim BF_4	3	79	49	30[b]
	Bmim PF_6	6	100	67	33[b]
Isophthalic acid	Bmim BF_4	1	74	74	0[b]
	Bmim PF_6	0.3	62	38	24[b]
Terephthalic acid	Bmim BF_4	0.5	44	44	0[b]
	Bmim PF_6	12	89	39	51[b]
4-Amino benzoic acid	Bmim BF_4	0.5	89	57	32[c]
	Bmim PF_6	0.3	92	38	54[c]

[a] Yield at time of maximum conversion.
[b] Diester product.
[c] Amide product.

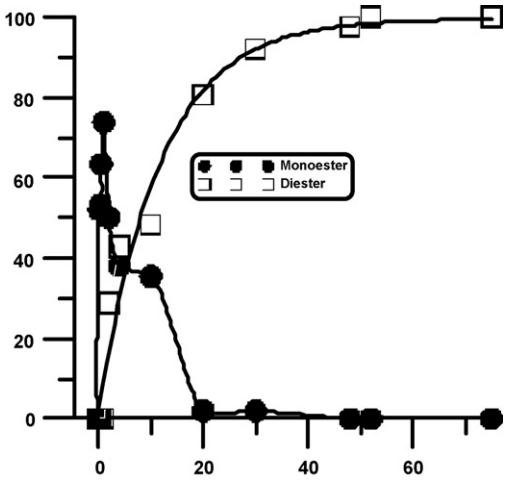


Fig. 1. Selective esterification of isophthalic acid with ethanol at 120 °C using *Bacillus thermocatenulatus* lipase in Bmim BF_4.

Furthermore, it must be underlined that when isophthalic acid was used the enzyme was stable during the whole process, and the formation of the diester from the monoester started at 1 h (Fig. 1). No selectivity was observed for any organic acid in Bmim PF_6. These results are not in agreement with previous studies with these two IL and other enzymatic process, which reported good results in Bmim PF_6 using Novozym 435 lipase [25]. Other authors have concluded that selectivity improved due to the ability of IL to dissolve the substrate [13]. Our results could be caused by the distinct ILs anions nature, which could interact in a different manner with the enzyme affecting differently conformational stability of the active site. Furthermore, the smaller viscosity of Bmim PF_6 in comparison with Bmim BF_4, could also explain present results, as viscosity of the reaction medium has been known to control enzyme activity affecting mass transfer limitation [26]. Therefore a lower reaction rate would be expected in an IL with a higher viscosity.

Another relevant aspect is the different water-miscibility of both ILs; Bmim BF_4 is water-miscible but Bmim PF_6 is not. It has been previously concluded [27] that thermostable lipases display a high operational stability in the absence of water, so we should hypothesize that in Bmim PF_6 best results will be obtained with BTL. Nevertheless, as esterification leads to a parallel formation of water, this fact could inhibit the thermophilic enzyme; thus, in the presence of a miscible liquid like Bmim BF_4 which could solve water molecules, the enzyme selectivity and activity were improved.

For the 4-amino benzoic acid, selectivity could be related to the possible formation of the corresponding ester or amide depending on the IL employed. In Bmim BF_4 (Table 1) the production of the ester predominated over the synthesis of the amide, but in Bmim PF_6 the amidation product was the most abundant. Furthermore the reaction yields in both IL were very similar (Table 1).

To conclude, best results of selectivity were obtained when isophthalic acid was employed as the organic acid and Bmim BF_4 as solvent (although Bmim PF_6 reached higher yielding). Thus, further studies with isophthalic acid were carried out with different nucleophiles acids to describe in more detail the selectivity of BTL.

Table 2
Esterification of isophthalic acid with different alcohols using *Bacillus thermocatenulatus* lipase at 120 °C in Bmim BF_4

Alcohol	Time (h)	Yield (%)*	Monoester (%)	Diester (%)
Ethanol	1	74	74	0
1-Butanol	3	14	14	0
2-Butanol	0.3	80	34	46
Cyclopentanol	48	5	5	0
Cyclohexanol	48	7	7	0

* Yield at time of maximum conversion.

3.2. Esterification of isophthalic acid with several alcohols in Bmim BF_4 at 120 °C

Different alcohols: *n*-alkanols (with different length of chain, primary and secondary alcohols) and cycloalkanols as rigid structures, to study the preference of BTL for these substrates were tested (Scheme 2). As observed in Tables 1 and 2 none of the tested alcohols improved the selectivity versus ethanol. In general terms, it was observed that the selectivity and the reaction yields diminished inversely with the length of chain, the steric hindrance and the rigidity of alcohols (Table 2). Thereby we can conclude that the BTL shows higher affinity for short chain primary and secondary alcohols than for cyclic alcohols. Interestingly, an unusual high reaction yield was obtained with 2-butanol although its high steric hindrance. Thus we can hypothesize that 2-butanol enhances lipases activity by favouring the opening of the lid that covers the lipase active sites as we have previously reported for 2-propanol [28].

Thermophiles are reported to contain proteins which are thermostable and resistant to denaturizing and proteolysis [29]. Furthermore, their cellular membrane is composed by saturated fatty acids that provide the demanded hydrophobic environment and rigidity for the cells needed to live at high temperatures [30]. Thus, it could be hypothesized that the low activity and selectivity obtained when cyclic alcohols were used could be due to the rigid nature of the lipase active site which makes it resistant to high temperatures but little versatile in respect to the type of substrates that could bound.

3.3. Influence of water activity in the esterification and selectivity of the lipase of B. thermocatenulatus

Activity of solid biocatalysts is affected by the initial and the remaining amount of water in the reaction medium [31,32]. In organic media, it is well known that the amount of water associated with the enzyme, rather than the total water content in the reaction system, is the key determinant of the enzymatic activity [33]. The quantity of water depends on the nature of the biocatalyst, the solvent and the solid biocatalyst [34,35]. Thermodynamic a_w has generally been considered the right parameter to quantify the hydration level of the enzyme. The influence of water content in mesophilic lipases for catalysing reactions in slightly hydrated organic solvents is well

Table 3
Esterification of isophthalic acid with ethanol catalysed by *Bacillus thermocatenulatus* lipase at different a_w values and 90 and 120 °C in Bmim BF_4

Acid	Alcohol	Temperature (°C)	a_w	Time (h)	Yield (%)[a]	Monoester (%)	Diester (%)
Isophthalic acid	Ethanol	90	–[b]	20	40	6	34
			0.4[c]	0.5	88	84	4
			0.6[c]	1	51	24	57
			0.8[c]	0.5	49	18	31
Isophthalic acid	Ethanol	120	–[b]	1	74	74	0
			0.4[c]	2	80	80	0
			0.6[c]	0.3	56	47	9
			0.8[c]	0.3	29	18	11

[a] Yield at time of maximum conversion.
[b] Without adding water.
[c] Water activity of the reaction media measured at 30 °C.

documented [35,36]. However, the influence of a_w in reactions catalysed by thermophilic lipases in IL has not yet been studied. Moreover, when comparing enzyme selectivity and activity in different solvents, it is very important to distinguish the effects of the solvent properties from other effects. So, the control of a_w is necessary to dilucidate which are the effects of the solvent from differences in enzyme hydration.

Table 3 shows the obtained results at different water activities values (0.4, 0.6 and 0.8) and temperatures (90 and 120 °C) using isophthalic acid and ethanol as substrates in Bmim BF_4 for the esterification reaction catalysed by BTL. When no water was added to the reaction media ($T = 90$ °C), a 40% yield was obtained at 48 h without selectivity for the monoester formation. Nevertheless, at 120 °C a 74% yield was obtained at only 1 h, reaching to 74% of the monoester (Table 3). Thus, temperature improves selectivity of the process.

As can be seen from Table 3, at both temperatures when increasing a_w, no improvements in the selectivity and the yield were obtained. Thus, at both temperatures the best a_w value was 0.4. These results agree with previous reported studies, describing that the addition of water to the medium in esterification processes provokes a decreasing of the equilibrium yield, as well as a diminution of the enzyme activity [37]. Moreover, accumulated water has also been shown to adversely affect the long-term stability of the enzyme [37] because most of the covalent processes involved in irreversible or reversible inactivation of proteins such as deamidation, peptide hydrolysis and cysteine decomposition require water, and are less frequent in non-aqueous media [27]. Thus, increasing the water amount would favour these processes, inactivating BTL.

Furthermore, Bmim BF_4 is extremely polar (0.673 Reichardt's scale) [20], therefore enhancing the interaction with water molecules indispensable for the acquisition and the maintenance of the catalytic conformation of the enzyme. Thus, the IL could strip off the water molecules tightly bound to the enzyme into the IL, resulting in either alteration and/or distortion of the catalytic conformation, and consequently deactivating the enzyme. Furthermore these extremely polar solvents could interact with the secondary structure of the functional protein via multiple hydrogen bonds or other strong interactions, and could lead to the unfolding of the enzyme and its consequent deactivation.

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