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Article

Biocatalysis at extreme temperatures: enantioselective synthesis of both enantiomers of mandelic acid by transterification catalyzed by a thermophilic lipase in ionic liquids at 120 °C.

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Abstract: The use of biocatalysts in organic chemistry for catalysing chemo-, regio- and stereo-selective transformations has become an usual tool in the last years, both at lab and industrial scale, not only because of their exquisite precision, but also due to the inherent increase in the process sustainability. Nevertheless, most of the interesting industrial reactions involve water-insoluble substrates, so that the use of (generally not green) organic solvents is generally required. Although lipases are perfectly capable of maintaining their catalytic precision working in those solvents, reactions are usually very slow and consequently not very appropriate for industrial purposes. Thus, the use of thermophilic enzymes at high temperatures can help in accelerating reaction rates. In this paper we describe the use of lipase from *Geobacillus thermocatenolatus* as catalyst in the ethanolysis of racemic 2-(butyryloxy)-2-phenylacetic to furnish both enantiomers of mandelic acid, an useful intermediate in the synthesis of many drugs and active products. The catalytic performance at high temperature in a conventional organic solvent (*iso*octane) and four imidazolium-based ionic liquids has been assessed. Best results were obtained using 1-ethyl-3-methyl imidazolium tetrafluoroborate ($EMIMBF_4$) and 1-ethyl-3-methyl imidazolium hexafluorophosphate ($EMIMPF_6$) at temperatures as high as 120°C, observing in both cases very fast and exquisite enantioselective kinetic resolutions, respectively leading exclusively to the (*S*) or to the (*R*)-enantiomer of mandelic acid, depending on the anion component of the ionic liquid.

Keywords: *Geobacillus thermocatenolatus*; lipases; ethanolysis; ionic liquids; kinetic resolution; mandelic acid.

1. Introduction

The employ of biocatalysts in organic chemistry, either alone [1,2] or combined with chemical catalysts [3,4] for developing selective transformations has become an usual tool in the last years [5,6]. This fact is based on the extremely enzymatic precision (chemo-, regio- and stereo-selectivity) acquired when applied in biotransformations not only at lab but also at industrial scale [7-11], being used mainly in pharma industry [12-21]. Moreover, moving from chemical catalysis to biocatalysis leads to an increase in the process sustainability, provided that biocatalysis and green chemistry usually go hand-in-hand [2,18,20,22-24].

In fact, one of the green credentials of biocatalysis derives from the fact that biotransformations can be conducted under very mild reaction conditions, e.g., atmospheric pressure, room temperature or aqueous media. Anyhow, harsh conditions required for many industrial processes, such as high temperature and/or the use of organic (co)solvents may impede the use of some enzymes. For solving these drawbacks, the employ of thermotolerant biocatalysts obtained from thermophilic organisms is an excellent alternative [25-29], as these thermozymes can efficiently work at very high

temperatures [30-32] and are generally very resistant to organic solvents-promoted denaturation [33,34]. Among all the arsenal of enzymes available for being used in biotransformations, lipases (triacylglycerol hydrolases, EC 3.1.1.3) are one of the most frequently applied, as they are easily available, do not need cofactors and display a wide range of substrate recognition [35-39]. Furthermore, their ability for working in almost anhydrous organic solvents allows conducting reactions in the sense of synthesis instead of hydrolysis, therefore favoring the transformation of many organic compounds, which are generally water-insoluble, and thus reverting the original enzymatic selectivity [40-43].

The use of lipases from thermophiles has been frequently reported [29,44,45]; additionally, it is worth to mention that several lipases from mesophiles (such as lipase B from *Candida antarctica* [46,47], lipase from *Bacillus licheniformis* [48], lipase from *Rhizopus oryzae* [49] , ELBn12 lipase (an alkaline thermotolerant lipase, from a mesophilic *Enterobacter* sp. [50]), lipase Lip3 from *Drosophila melanogaster* [51]) have being genetically modified in order to increase their thermal stability. Amongst the lipases from thermophiles, the term "thermoalkaline (TA) lipases" describe some enzymes resistant not only to temperature (70-80°C) but also to the presence of alkaline media (pH values between 8 and 10) [45]. These enzymes possess a peculiar feature in their 3D structure, due to the presence of a relatively large lid domain (around 70 residues) formed by 2 alpha-helices (α6 and α7)[52], so that the opening of this lid domain upon exposing the active site requires a significant conformational change [53]. One of the most representative examples of TA lipases is that one from *Geobacillus* (formerly *Bacillus* [54]) *thermocatenulatus.* From this microorganism, Schmidt-Dannert et al. [55-58] described two lipases, namely BTL1 and BTL2, this last one being crystallized in its open form by Carrasco-Lopez et al. [52,59]. The stereoselectivity of BTL2 towards 29 chiral substrates was initially tested by Liu et al [60], reporting only good results for the acylation of 1-phenylethanol and 1-phenylpropanol with vinyl acetate (as well as for the hydrolysis of the corresponding esters). Later, this enzyme, immobilized on different supports *via* diverse methodologies, has been extensively tested on different substrates, some of them chiral [61-80], generally with moderate results. Additionally, chemical [71,74,81-83] and genetic [72,84-89] modifications of BTL2 lipase for improving its catalytic behavior (typically, to reduce the steric hindrance around the active site) have been also reported. Finally, different papers in recent literature have employed the reported 3D structure of BTL2 for performing molecular simulations aiming to rationalize its catalytic performance and stability [53,90-93]

Anyhow, although TA lipases are very resistant to organic solvents and high temperatures, the boiling point of the solvents clearly limits the maximum operational temperature. In this sense, the use of RTILs (Room-Temperature Ionic Liquids) can be very convenient, as they display very high boiling points and have been proven to be compatible with enzymatic catalysis [94-98].

In this paper we present the results obtained in the kinetic resolution of 2-(butyryloxy)-2-phenylacetic acid via ethanolysis catalysed by BTL2 using different ionic liquids (1-butyl-3-methyl imidazolium tetrafluoroborate ($BMIMBF_4$), 1-butyl-3-methyl imidazolium hexafluorophosphate ($BMIMPF_6$), 1-ethyl-3-methyl imidazolium tetrafluoroborate ($EMIMBF_4$) and 1-ethyl-3-methyl imidazolium hexafluorophosphate ($EMIMPF_6$) at high temperatures (90 and 120ºC), comparing the results with those obtained with a conventional organic solvent (*iso*octane)

2. Results

The kinetic resolution of racemic 2-(butyryloxy)-2-phenylacetic acid (*R*, *S*)-**1** to yield pure enantiomers of mandelic acid (*R*) or (*S*)-**2** was selected as test reaction to check the performance of BTL2, as depicted in Figure 1 **(a)**.

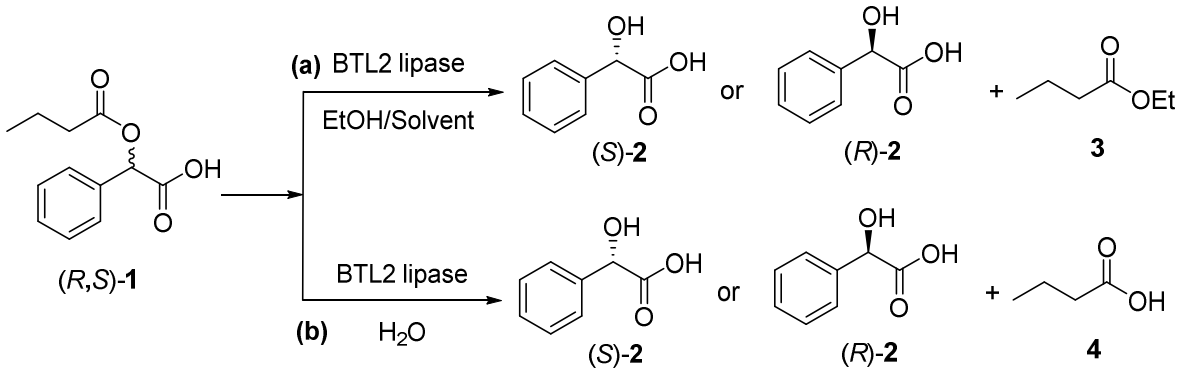

Figure 1. Kinetic resolution of 2-(butyryloxy)-2-phenylacetic acid (*R, S*)-**1** via BTL2-catalyzed ethanolysis **(a)** or BTL2-catalyzed hydrolysis **(b)**.

(*R*)-mandelic acid and analogues are key synthons in the preparation of several drugs or biologically active compounds, being in the core of semi-synthetic antibiotics (cephalosporins as cefamandole [99] or penicillins as MA-6-APA II [100]) or anti-cholinergic drugs (such as oxybutynin [101] and homatropine [102]). Moreover, derivatives of (*R*)-mandelic acid have been also used as chiral synthons in the preparation of some drugs with different therapeutical activities: platelet/anti-thrombotic agents (clopidogrel [103]), vasodilator (cyclandelate [104]), anti-tumor (complex of *cis*-[Pt{2-(α-hydroxy)benzylbenzimidazole))2Cl2] [105], antiobesity [106,107] or CNS-stimulant dopaminergic agents ((*R*)-pemoline [108]). Conversely, (*S*)-mandelic acid is used for the production of non-steroidal anti-inflammatory drugs such as deracoxib and celecoxib [109]

Selection of (*R, S*)-**1** as model substrate was based in many previous studies in which BTL2 (mainly immobilized) had been tested for catalyzing the stereoselective hydrolysis (Figure 1**(b)**) [61,65,67,72,74]. Thus, Palomo *et al* [61] reported the hydrolysis of (*R, S*)-**1** using several preparations of BTL2 immobilized on different supports (octadecyl agarose, glyoxyl agarose or polyethylenimine-modified (PEI) sepabeds [63]), at different pH values (5, 7 and 9) and different temperature (4, 25 and 37°C). These authors carried out the kinetic resolution of (*R, S*)-**1** at low conversion values (15%, no reaction time reported), obtaining the best results (ee > 99%, E >100) at low temperatures (4°C) using octadecyl or glyoxyl-agarose, being (*R*)-**2** the major enantiomer; anyhow, resolution at higher temperatures (25 or 37°C) was only moderate. Interestingly, the use of PEI-sepabeads caused and inversion on the enzymatic stereobias, leading to the (*S*)-**2** antipode, although with low or moderate enantioselection. A similar behavior was reported by Fernandez-Lorente *et al* [64] with BTL2 immobilized on octylagarose; these same authors described the use of immobilized chemically-aminated BTL2 derivatives covalently linked to CNBr-agarose or adsorbed on glyoxylagarose, and in this case (*S*)-**2** (with only one exception) was the major enantiomer [65]. On the other hand, Godoy *et al*. [72], using wild-type BTL2 as well as some mutants produced by introducing a unique cysteine into different positions of the protein surface, reported a low enantioselectivity (24% ee of the corresponding (*R*)-**2** acid for the soluble wild-type enzyme and ranging from 19-38% for the different mutants) in the hydrolysis of (*R, S*)-**1** at pH=7 and 25°C. Remakably, the direct immobilization of BTL2 and its mutants on disulfide supports slightly increased the enantioselectivity (ee fluctuating from 20 to 50%), while the use of aldehyde-disulfide supports dramatically increased the enantiomeric discrimination up to ee values higher than 99% [72].

2.1. Ethanolysis of (R, S)-***1*** *in* isooctane *at different temperatures.*

Nevertheless, hydrolysis is not a good alternative for checking the real thermotolerance of a lipase, as the stability of these enzymes is much higher when they are working on organic solvents [33,41,94]. Thus, we decided to use a water-free reaction media, selecting EtOH as nucleophile instead of water and a water-insoluble organic solvent (Figure 1**(a)**). Opting for EtOH was based on our previous studies on esterification of phtalic acids with BTL2 [110], and it has been very recently corroborated by Shehata *et al*. [53]; indeed, these authors have published a molecular dynamics (MD)

simulation of the effect of different polar and non-polar solvents on the thermostability and lid-opening of BTL2, reporting that the open (active) conformation of BLT2 is more stable in EtOH than in MeOH and even water. Additionally, this same study revealed that the overall lipase structure became more stable in non-polar organic solvents, while it was destabilized in polar solvents except EtOH. Thus, it seems reasonable to use EtOH as nucleophile. On the other hand, *iso*octane (2,2,4-trimethylpentane) was the organic solvent selected, as we had previously described its excellent behavior in lipase-promoted catalysis [111-116].

Thus, following the experimental procedure described in Section 4.3, the ethanolysis of (*R*, *S*)-**1** was tested at three different temperatures (40, 70 and 90°C). The progress curves are depicted in Figures 1 to 3.

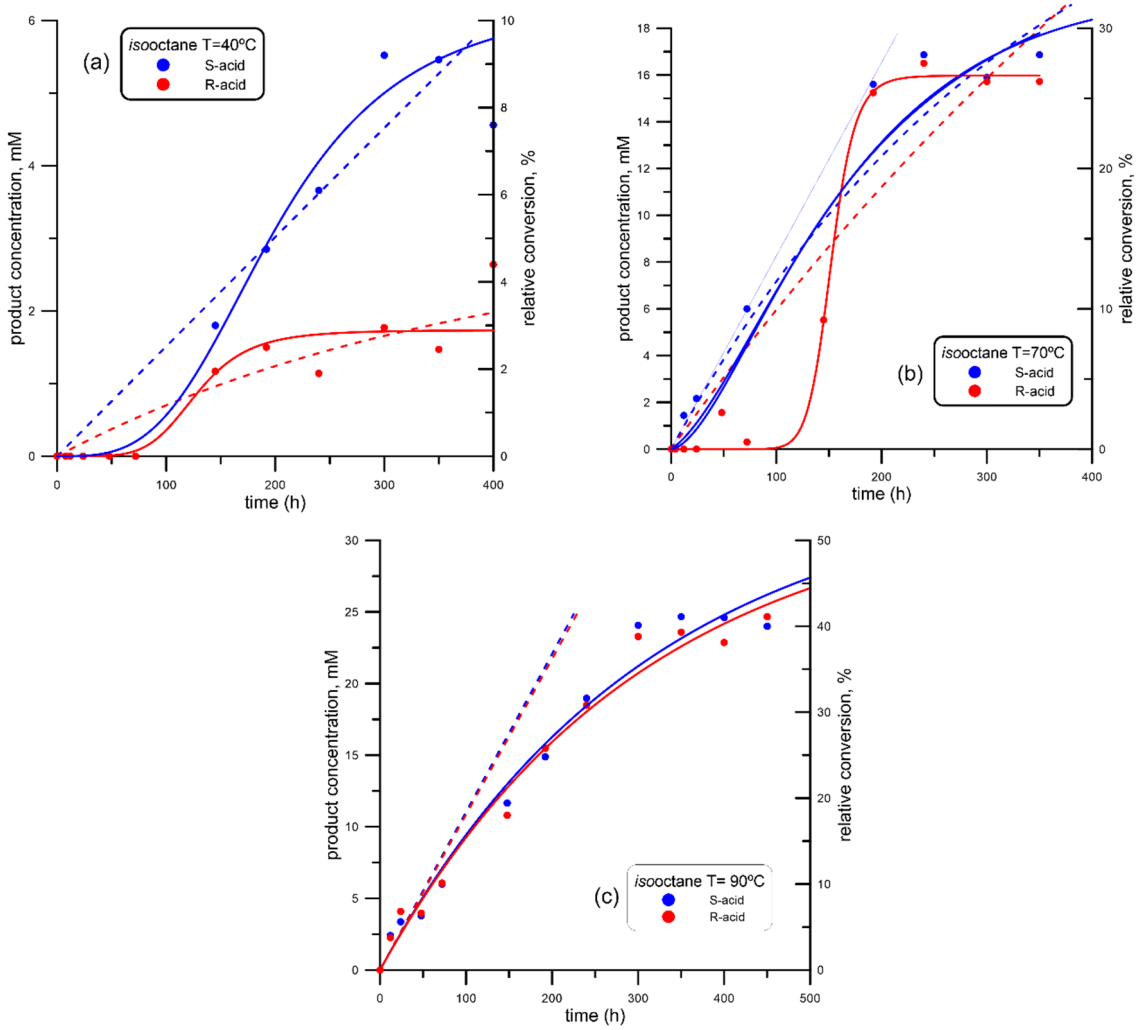

Figure 1. Progress curve of the BTL2-catalyzed production of both enantiomers of mandelic acid ((*R*)-acid, in red; (*S*)-acid, in blue) *via* ethanolysis of racemic 2-(butyryloxy)-2-phenylacetic acid (*R*, *S*)-**1**, using isooctane as organic solvent at different temperatures: (**a**) 40°C; (**b**) 70°C; (**c**) 90°C.

As can be seen from Fig.1 1, the reaction at 40°C (Figure 1a) proceeds very slowly, reaching a global conversion of around 10% for (*S*)-**2** after 400 h and 3-4% for the (*R*)-**2** counterpart. Also, a very strong lag-time is observed for both enantiomers, not detecting any trace of ethanolysis of (*R*, *S*)-**1** in the first 75 hours. A similar behavior can be found for the generation of (*R*)-**2** at 70°C (Figure 1b), while for (*S*)-**2** at that temperature and for both enantiomers at 90°C (Figure 1c), no lag-time was observed, following a typical exponential grow. Thus, all the progress curves were adjusted using the program INRATE implemented inside SIMFIT fitting package (version 7.6, Release 9), a very powerful (and toll-free) Open Source software for simulation, curve fitting, statistics, and plotting, using a library of models or user-defined equations [117] (accessible at https://simfit.org.uk/simfit.html). Using this program, data were fitted either to lag-time kinetics or to standard single exponential growing model. Form these mathematical fitting, several parameters were calculated (shown in Table 1) and used to quantify the activity and enantioselectivity of BTL2 in the kinetic resolution of (*R*, *S*)-**1** *via* ethanolysis.

Table 1. Quantitaive assessment of the ethanolysis of racemic 2-(butyryloxy)-2-phenylacetic acid (*R*, *S*)-**1** catalyzed by BTL2 using *iso*octane as organic solvent at different temperatures.

	Medium	T (°C)	V_S[1]	V_R[1]	V_S/V_R	t_{MAX}[4]	[C] max[5]	P[6]	[(*S*)-2][7]	[(*R*)-2][7]	E[7]
#1	*iso*octane	40	0.015[2] 0.027[3]	0.008[2] 0.014[3]	1.9[2] 1.9[3]				0	0	nd
#2	*iso*octane	70	0.083[2]	0.063[2] 0.332[3]	1.32[2]	24	2.26	0.09	1.44	0	>200
#3	*iso*octane	90	0.110[2]	0.108[2]	1.02[2]				4.0	3.75	nd

[1]Initial rate (mM/h). [2]Single exponential model. [3]Lag-time model. [4]Higher reaction time (h) at which only one enantiomer is detected. [5]Concentration (mM) of the only isomer detected at that higher reaction time. [6]Productivity (mM acid/h) at the higher reaction time. [7]Enantiomeric ratio, calculated at 12 h

As commented before, reaction was extremely slow at 40°C; increasing the temperature to 70°C (Figure 1b), the reaction rate increased very markedly, and the behavior for both enantiomers was clearly different: while the generation (*S*)-**2** is detected from the early reaction stages, and follows a single exponential model, it is not until 100 h when (*R*)-**2** is clearly detected, quickly growing after that point to reach similar conversion values than those observed for (*S*)-**2** after 200 h. When the temperature was increased up to 90°C (Figure 1c), both enantiomers were produced by a similar pattern, at the same initial rate (Table 1, entry #3) and reaching similar conversion degrees (around 40%) after 500h, with no enantioselectivity at all. Higher temperatures were not tested as it would mean approaching the boiling point of i*so*octane (99.6°C)

Overall, best results are those obtained at 70°C at short reaction times. In fact, inside the time interval from 0 to 75h, the enantioselectivity is almost perfect, although at the expenses of a low overall conversions (around 10%, Figure 1b). Nevertheless, as these results are quite unsatisfactory, the use of room-temperature ionic liquids (RTILs) as reaction media was tested.

2.2. Ethanolysis of (R, S)-***1*** *in RTILs at different temperatures.*

As commented in the Introduction, RTILs have been described to be compatible with enzymatic catalysis [94-98]. RTILs (organic salts consisting of an organic cation and a polyatomic inorganic anion, liquid under 100°C), are broadly regarded as green solvents [118,119], as they have extremely high enthalpies of vaporization (making them effectively nonvolatile), as well as high chemical and thermal stabilities and remarkable solvating power. Anyhow, the large number of steps required for their synthesis, sometimes demanding the use of nonrenewable crude oil sources and some toxic intermediates, may alter their consideration of eco-friendly solvents [120].

Anyhow, the most popular RTILs are those based on imidazolium cations [121-124], being 1-butyl-3-methyl imidazolium tetrafluoroborate ($BMIMBF_4$), 1-butyl-3-methyl imidazolium hexafluorophosphate ($BMIMPF_6$), 1-ethyl-3-methyl imidazolium tetrafluoroborate ($EMIMBF_4$) and 1-ethyl-3-methyl imidazolium hexafluorophosphate ($EMIMPF_6$) probably the first ones to be broadly commercialized. Their properties have been profusely described, especially including their complete miscibility with EtOH [125-134], the other main component of the reaction medium, used both as cosolvent and nucleophile. As these RTILs possess a very high boiling point, the use of their mixtures with EtOH allows the possibility of employing these binary mixtures at high reaction temperatures,

without any noticeable EtOH evaporation. Thus, the ethanolysis of (*R*, *S*)-**1** was tested at two different temperatures, 90°C (similar to the maximum tested with *iso*octane) and 120°C, a temperature higher than the boiling point of the organic solvent. The results are depicted in Figures 2 to 5, while Table 2 summarizes the parameters obtained from the fitting of the corresponding progress curves.

2.2.1. Ethanolysis of (*R*, *S*)-**1** using RTILs based on 1-butyl-3-methyl imidazolium (BMIM) as solvent

When the tetrafluoroborate ($BMIMBF_4$) solvent is used (Figure 2), it can be observed how the employ of the lower reaction temperature (90°C, Figure 2a) leads to a different kinetic behavior in the generation of both enantiomers of mandelic acid. In fact, as the (*S*)-acid (in blue) is detected from the earlier reaction stages, the correspondent (*R*)-acid (in red) is not produced until a lag-time of around 12 h has been overpassed, experimenting a rapid increase in its production leading to an overall sigmoid curve (Figure 2a, red solid line); remarkably, a similar initial rate (V_S =3.85 mM/h, Table 2) was calculated considering a single exponential model (Figure 2a, red dotted line) or the sigmoid lag-time model, red solid line). In this case, (*S*)-acid (in blue) is the best-recognized enantiomer, as also observed using *iso*octane (Figure 1), although for $BMIMBF_4$ the initial rate is 35 times higher than that observed for isooctane (V_S =0.11 mM/h, Table 1). Furthermore, the reaction in this RTIL is not only faster but also more enantioselective than in *iso*octane, as the lag-time observed for the generation of the (*R*)-acid allows the production of exclusively (*S*)-**2** at the first stages of the reaction (t_{MAX} 10h, [(*S*)-**2**]$_{MAX}$ 13.6%, corresponding to 22.7% conversion).

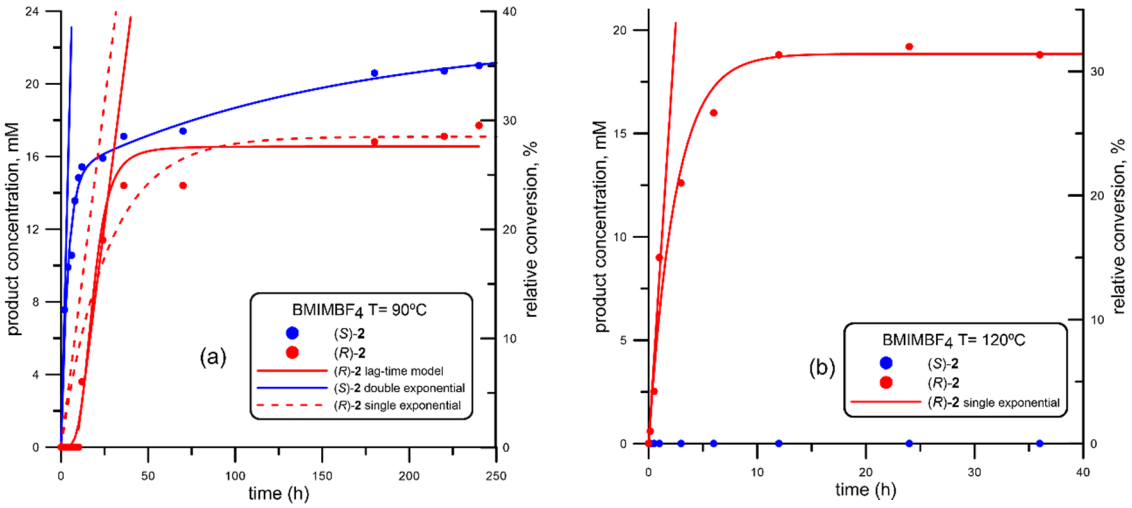

Figure 2. Progress curve of the BTL2-catalyzed production of both enantiomers of mandelic acid ((*R*)-acid, in red; (*S*)-acid, in blue) *via* ethanolysis of racemic 2-(butyryloxy)-2-phenylacetic acid (*R*, *S*)-**1**, using **BMIMBF$_4$** at different temperatures: (**a**) 90°C; (**b**) 120°C. Fitting parameters shown in Table 2, corresponding to entries #**4** ($BMIMBF_4$ 90°C) and #**5** ($BMIMBF_4$ 120°C)

Another interesting aspect to be taken into account is that, while the generation (*R*)-**2** remains constant after a certain reaction time (around 30% after 50 h, according to the sigmoid fitting), the production of (*S*)-**2** is slowly increasing after that time, although at a lower reaction rate than that observed at the early stages; that is the reason why the overall (*S*)-**2** production follows a double exponential fitting. This slower second reaction rate could be caused by an inhibition promoted by the increasing amounts of (*R*)-**2** present in the reaction media, as the slope change in the (*S*)-**2** production is observed only after a certain accumulation of (*R*)-**2.**

Table 2. Quantitaive assessment of the ethanolysis of racemic 2-(butyryloxy)-2-phenylacetic acid (*R*, *S*)-**1** catalyzed by BTL2 using different RTILs at different temperatures.

	RTIL	T (°C)	V_S[1]	V_R[1]	V_{MAX}/V_{min}[2]	t_{MAX}[3]	[C] max[4]	P[5]	[(*S*)-2][6]	[(*R*)-2][6]	E[6]
#4	BMIM BF_4	90	3.85	0.76	5.1	10	13.6	1.36	15.42	13.6	1.1
#5	BMIM BF_4	120	----	8.14	----	36	18.8	0.52	0	18.8	>200
#6	BMIM-PF_6	90	2.0	0.31	6.4	24	17.0	0.71	14.0	0	>200
#7	BMIM-PF_6	120	0.04[b]	0.96	24	8	6.3	0.79	0	3.06	>200
#8	EMIM-BF_4	90	4.08	0.52	7.8	12	18.2	3.03	18.2	0	>200
#9	EMIM-BF_4	120	53.4	0	----	2.5	29.2	11.7	28.1	0	>200
#10	EMIM-PF_6	90	1.86	----	----	240	16.2	0.81	2.28	0.06	51.3
#11	EMIM-PF_6	120	1.3		----	54	9.8	0.18	8.52	0	>200

[1]Initial rate (mM/h). [2] V_S/V_R in all cases except for entries #5 and #7, when it should be V_R/V_S. [3]Higher reaction time (h) at which only one enantiomer is detected. [4]Concentration (mM) of the only isomer detected at that higher reaction time. [5]Productivity (mM acid/h) at the higher reaction time. [6]Enantiomeric ratio, calculated at 12 h

Anyhow, when performing the ethanolysis at 120°C (Figure 2b), the observed reaction pattern is radically different to that obtained at 90°C, as now only the (*R*) enantiomer of mandelic acid is produced through a fast and almost perfect single exponential fit, displaying an initial rate (V_R =8,14 mM/h, Table 2) twice that one obtained for the preferentially-recognized (*S*)-**2** at 90°C. Indeed, the reaction did not progress after 40 h, and no traces of (*S*)-**2** were detected under these conditions, so that the enantioselectivity is absolutely perfect, leading to an overall conversion of around 30%.

An inversion in the stereobias of BTL2 in the recognition of both enantiomers of mandelic acid had been previously reported, although in the hydrolysis of (*R*,*S*)-**1** and associated to the different methodology and support used for the immobilization of this lipase [61,63-65], as already mentioned in the Introduction. A simple explanation of this modification in the enantiodiscrimination of BTL2 upon increasing the temperature would demand a detailed computational study, which is out of the scope of this manuscript.

In Figure 3, the results of the ethanolysis of (*R*,*S*)-**1** using BMIMPF_6, keeping constant the cationic component (1-butyl-3-methyl imidazolium) but changing the anion from BF_4 to PF_6 are shown.

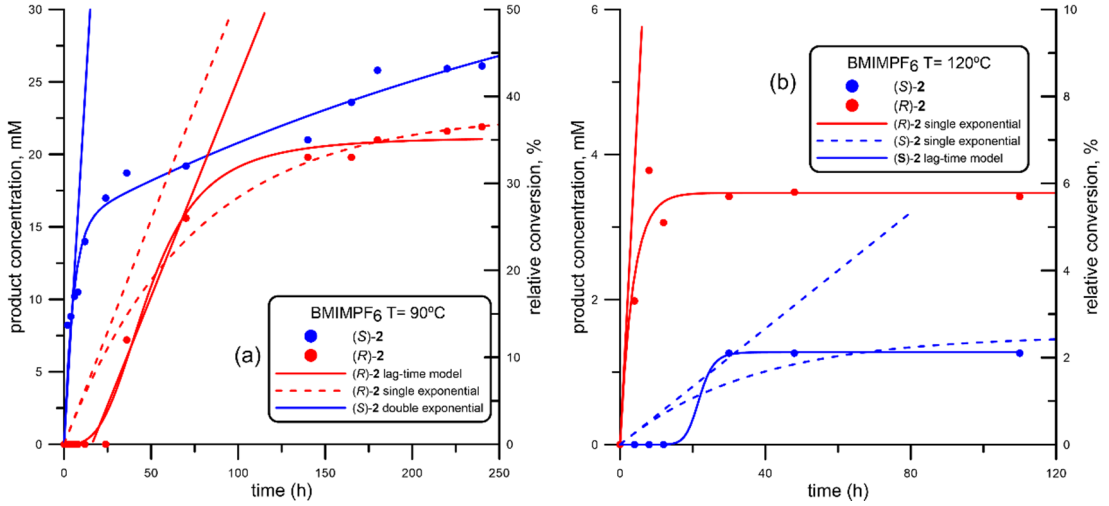

Figure 3. Progress curve of the BTL2-catalyzed production of both enantiomers of mandelic acid ((*R*)-acid, in red; (*S*)-acid, in blue) *via* ethanolysis of racemic 2-(butyryloxy)-2-phenylacetic acid (*R*, *S*)-**1**, using **BMIMPF$_6$** at different temperatures: (**a**) 90°C; (**b**) 120°C. Fitting parameters shown in Table 2, corresponding to entries #**6** (BMIMPF$_6$ 90°C) and #**7** (BMIMPF$_6$ 120°C)

As can be seen, the behavior is somehow similar to that observed using BMIMBF$_4$, hence, when performing the ethanolysis at 90°C (Figure 3a), the (*S*) enantiomer of mandelic acid is more quickly produced, once again following a double exponential fit, and displaying a smaller initial rate than that one obtained using BMIMBF$_4$., but also higher if compared to that calculated using *iso*octane (Table 1). Similarly, the inversion in the stereobias at 120°C is also detected, but now the reaction proceeded very poorly compared to that depicted in Figure 2b, as only a maximum of 6% conversion is detected for (*R*)-**2**.

2.2.2. Ethanolysis of (*R*, *S*)-**1** using RTILs based on 1-ethyl-3-methyl imidazolium (EMIM) as solvent

The results obtained using RTILs in which 1-ethyl-3-methyl imidazolium (EMIM) is the cation are shown in Figures 4 and 5. More concretely, Figure 4 shows the reaction progress curves when using EMIMBF$_4$ at 90°C (Figure 4a) and 120°C (Figure 4b).

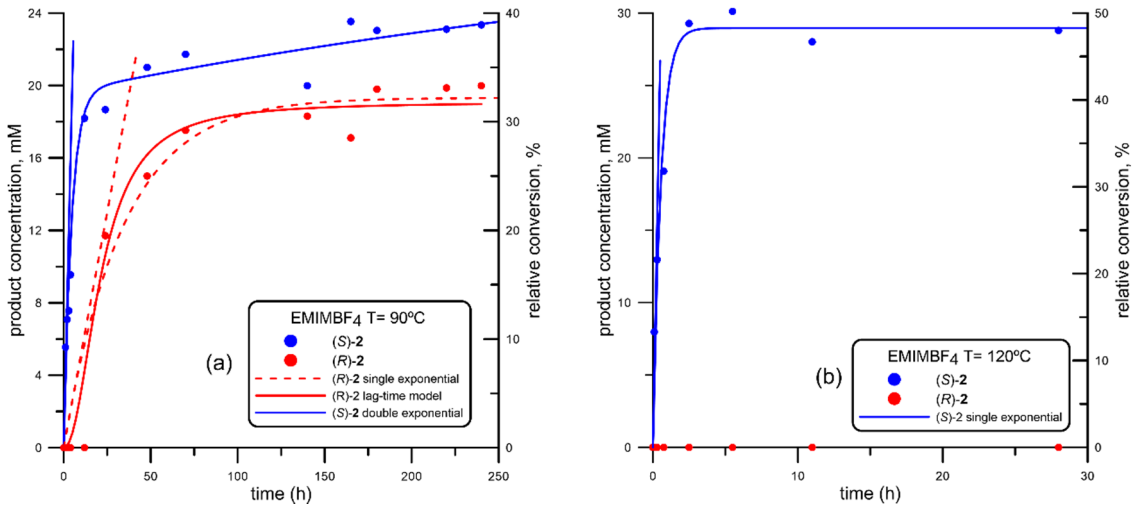

Figure 4. Progress curve of the BTL2-catalyzed production of both enantiomers of mandelic acid ((*R*)-acid, in red; (*S*)-acid, in blue) *via* ethanolysis of racemic 2-(butyryloxy)-2-phenylacetic acid (*R*, *S*)-**1**, using **EMIMBF$_4$** at different temperatures: (**a**) 90°C; (**b**) 120°C. Fitting parameters shown in Table 2, corresponding to entries #**8** (EMIMBF$_4$ 90°C) and #**9** (EMIMBF$_4$ 120°C)

Comparing Figure 4a (EMIMBF$_4$) with Figure 2a (BMIMBF$_4$), a similar behavior is observed.As depicted in Figure 4a, the lag-time for the detection of (*R*)-**2** is slightly higher than that observed using BMIMPF$_4$, and also the initial rate is somewhat higher (4,08 *vs* 3.85 mM h^{-1}, Table 2), so that it was possible to detect only (*S*)-**2** in the first 12 hours, reaching a concentration of 18.2 mM (around 30% conversion) with a perfect enantioselectivity Once again, as the reaction proceeded and the (*R*)-**2** enantiomer is being produced (lag-time and single exponential models almost similar), the rate of production of (*S*)-**2** was reduced, so that once again the overall behavior for (*R*)-**2** can be fitted to a double exponential curve.

When the reaction temperature is increased up to 120°C (Figure 4b), a fantastic and fast kinetic resolution can be observed. In fact, the initial rate in the generation of (*S*)-**2** (V_S =53.4 mM h^{-1}, Table 2) is one order of magnitude higher than that obtained at 90°C; moreover, no traces of (*R*)-**2** were detected during the whole reaction time, so that the shape of the progress curve fits to a single exponential plot leading to around 50% conversion (the maximum for a kinetic resolution) in only 5 hours.

Results obtained using EMIMPF$_6$ as solvents are depicted in Figure 5. As can be seen, at both temperatures only small traces of (*R*)-**2** were detected, while the generation of (*S*)-**2** follows single exponential kinetics. At 90°C, the kinetic resolution observed using this solvent (Figure 5a) is slower (V_S around one half, See Table 2, entries #8 vs #10) than that obtained with EMIMBF$_4$ (Figure 4a), although this fact is compensated with an absence of production of (*R*)-**2**, so that the kinetic resolution is considerably better in terms of enantioselectivity, allowing around a 30% conversion after 250 h. When increasing the temperature to 120°C (Figure 5b), the kinetic resolution was slightly slower and the maxinum conversion was half that obtained at 90°C.

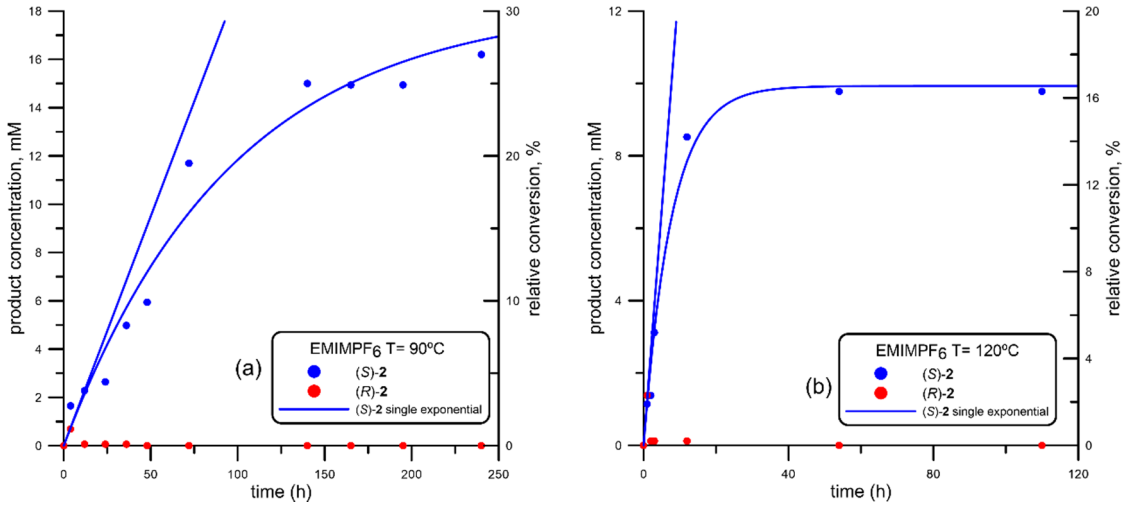

Figure 5. Progress curve of the BTL2-catalyzed production of both enantiomers of mandelic acid ((*R*)-acid, in red; (*S*)-acid, in blue) *via* ethanolysis of racemic 2-(butyryloxy)-2-phenylacetic acid (*R*, *S*)-**1**, using **EMIMPF$_6$** at different temperatures: (**a**) 90°C; (**b**) 120°C. Fitting parameters shown in Table 2, corresponding to entries #**9** (EMIMPF$_6$ 90°C) and #**10** (EMIMPF$_6$ 120°C).

3. Discussion

It is generally accepted that enzymes in RTILs are more active and stable as the hydrophobicity of the RTIL increases (see the review from Liu and coworkers [94], as well as the references cited therein). This fact is commonly related to the higher preservation of the essential water layer

surrounding the enzyme structure, resulting in a decrease of the protein-ion interactions with a concomitant reduction of enzyme denaturation [95]. Therefore, the use of water-inmiscible RTILs, more hydrophobic than the corresponding water-soluble ones, has been recommended [94]. Anyhow, these assumptions have been described for pure RTILs. In fact, mixtures of RTILs and organic solvents have been reported to increase the catalytic activity, stability and enantioselectivity of enzymes compared to the single RTIL (probably by reducing the viscosity of the RTIL and diminishing mass-transfer limitations [94]); on the other hand, the proportion and the nature of the organic cosolvent is usually crutial to reach good activity and selectivities (see [94] and references cited therein). Varela *et al.* [134] collected some data on theoretical studies of mixtures of RTILs and alcohols, concluding that molecular dynamics simulations of the solvation of alcohols in RTILs may resemble water behavior. Thus, the different regions of the bulk RTIL can interact with the analogous (polar or apolar) parts of the solute molecules, and this fact is pivotal for understanding both the mesomorphic structure of the mixtures as well as their dynamics, following a pattern termed "nanostructured solvation" [134]. Anyhow, theoretical studies describing the effect of RTIL/organic solvent mixtures on the structure of enzymes are still missing.

In fact, for BTL2 it has been reported, based on molecular dynamics simulation, how the use of an apolar organic solvent (toluene) made the lipase structure more rigid, even in simulations carried out at 450°K (176.85°C) [90], without observing any tendency for the lid to open, and claiming that either inserting a thin layer of water around the enzyme or promoting a single point mutation (G116P) would be required for retaining activity in acyl-transfer processes [90]. Additional, another very recent study confirmed the reduced flexibility of BTL2 in non-polar organic solvents, using molecular dynamics on toluene and cyclohexane, and thus confirming the enhancement of thermostability of BTL2 in the presence of this type of solvents [53]. These theoretical studies would support our results obtained using *iso*octane as solvent in the BTL2-catalyzed ethanolysis of 2-(butyryloxy)-2-phenylacetic acid (*R*, *S*)-**1** (Figure 1 and Table 1), where we described how increasing the reaction temperature up to 90°C promoted a moderate rise in the reaction rate, but with no enantioselectivity, as the lid should not open (the only water present in the medium would be that one retained in the enzymatic liophilizate, not enough to reach a proper concentration), and therefore not upholding the lid flexibility required for the proper enzymatic enantiodiscrimination [135,136].

It has been also described that polar solvents (water and short-chain alcohols) lead to enhanced fluctuation of BTL2's lid at low temperatures, but surprisingly the open conformation turned out to be more stable in EtOH than in water or MeOH [53]. Thus, considering the benefitial effect of EtOH on BTL2, we decide to check the catalytic behavior in mixtures between different RTILs and EtOH. As pointed out in Section 4.3, EtOH was used in a high molar excess compared to the starting substrate (3.42 M EtOH versus 60 mM for (*R*,*S*)-**1**), but if we consider the molar fraction of EtOH and the RTIL, the situation is different, as indicated in Table 3.

Table 3. Composition of the different reaction mixtures EtOH/RTIL (1mL/4mL)

RTIL	MW [1] (g/mol)	Density [1] (g/mL)	[RTIL], M	[EtOH], M	Molar fraction X_{RTIL}	Molar fraction X_{EtOH}
$BMIMBF_4$	226.02	1.21	4.28	3.42	0.56	0.44
$BMIMPF_6$	284.19	1.38	3.89	3.42	0.53	0.47
$EMIMBF_4$	197.97	1.29	5.22 [2]	3.42	0.60	0.40
$EMIMPF_6$	256.13	1.48	4.62	3.42	0,57	0.43

[1] Data taken from SciFinder Database

As can be seen from Table 3, the molar composition of the reaction mixture is not exactly the same, because of the differences in the molecular weight and density of the four RTILs, although average values of (0.56±0.04) and (0.44±0.04) for X_{RTIL} and X_{EtOH} can be considered.

The most hydrophobic RTIL, water-insoluble $BMIMPF_6$, is definitively not the best option for the ethanolysis of (*R*,*S*)-**1**, as shown in Figure 3, neither at 90 nor at 120°C; in fact, the initial rate in the generation of (*S*)-**2** at 90°C is not the highest, although enantioselectivity is perfect (E>200) up to 24 h. At 120°C, an inversion in the stereobias at 120°C was observed, but only a maximum of 6% conversion is detected for (*R*)-**2**. Looking at the literature, (*R*)-**1** is the recognized enantiomer in the hydrolysis of (*R*,*S*)-**1** by wild-type BTL2 [72], as well as by some immobilized preparation of this enzyme [61], so that the "canonical" recognition, as predicted by the well-known Kazlauskas' rule (Figure 6a), based on the relative size of substituents around the stereocentre [137] would be that one depicted in Figure 6b.

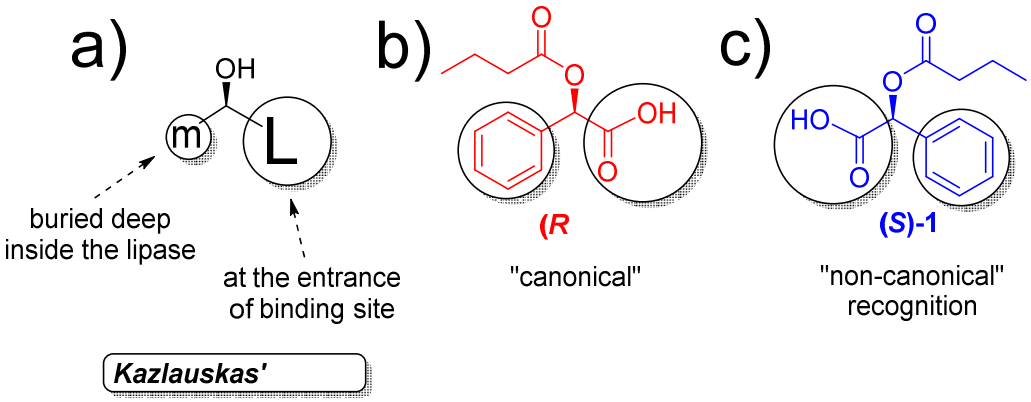

Figure 6. (a) The Kazlausskas' rule; (b) "canonical" recognition of (*R*)-**1** enantiomer; (c) "non-canonical" recognition of (*S*)-**1** enantiomer

Actually, the large (L) binding pockets is located at the entrance of binding site, while the other medium (m) pocket is buried deep inside the lipase; thus, this would mean that the phenyl ring of the (*R*)-substrate would be the one interacting with the cavity inside the 3D structure of BTL2 in the canonical recognition pattern. This interaction maybe could be attributed to a stacking of the phenyl moiety of (*R*)-**1** with Phe17, a residue which changes its conformation in the open structure and allows the access of the substrate to the catalytic Ser [52]; a similar interaction has been proposed for other aromatic substrates with lipases [138,139]. Anyhow, using $BMIMPF_6$ at 90°C, the non-Kazlauskas rcognition (Figure 6c) is majoritarian, while increasing temperature up to 120°C, an alteration of the enantioselectivity is observed, but this could be attributed to an enzyme inactivation, as long as the activity dropped dramatically.

Changing from $BMIMPF_6$ (Figure 3) to $BMIMBF_4$ (Figure 2), the resolution become faster at 90°C, (Table 2) but even better at 120°C, when the inversion of the canonical recognition is absolute, up to the point that no (*S*)-**2** is detected at all, and the (S)-selection is maintained until the reaction ends (after 12 h, Figure 2b). Filice et al. [140] described that the tetrafluoroborate anion, in aqueous media, does not cause negative effects on BTL2, as it happens with other lipases. Anyhow, the alteration in the canonical (Figure 6b) recognition to the non-Kazlauskas pattern (Figure 6c) upon heating at 120°C can be caused by many possible factors. Indeed, a different hydrogen bonding arrangement is one of the reported reason for changing the enantioselection of lipases [141,142]), and it is well known that the anionic component of a RTIL is the main responsible of establishing the hydrogen–bonding network with the enzyme [94]; anyhow, this assumption would demand a molecular dynamics study, out of the scope of this paper.

On the other hand, the use of more hydrophilic 1-ethyl-3-methyl imidazolium (EMIM) cation has been recommended for BTL2 [140]. Similarly, for other lipases it has been also shown how the shorter the alkyl chain in the cationic imidazolium, the higher the activity [143], while Filice et al.

recommended the use of hexafluorophosphate (PF_6^-) combined with EMIM, as generally RTILs containing had a very negative effect on the enzyme activity. [140]. In fact, by looking at the progress curves obtained in the ethanolysis of (*R*,*S*)-**1** using EtOH/EMIMPF$_6$, and depicted in Figures 5a (90°C) and 5b (120°C), the kinetic resolutions are quite perfect, although better at 90°C, both in terms of a higher reaction rate (Table 2, entry #10 vs entry #11) and activity, as for 120°C the reaction did not progress to a conversion higher than 20% after 120 h, while at a lower temperature the maximum value detected was around 30% and still growing, according to the curve shape. In any case, it is noteworthy to observe how BTL2 is exquisitely enatioselective under these reaction conditions, as no traces of the "canonical" (*R*)-**2** isomer were detected.

When using EtOH/EMIMBF$_4$ at 90°C (Figure 4a), the situation is quite similar to that obtained with BMIMBF$_4$ (Figure 2a) or BMIMPF$_6$ (Figure 3a); nevertheless, the kinetic resolution is surprisingly perfect when using EtOH/EMIMBF$_4$ at 120° (Figure 4b), as in only 2.5 h a perfect 50% conversion in (*S*)-**2** is obtained, and again no traces of the (*R*)-antipode are formed. Thus, the best results, both in terms of reaction rate and enantioselectivity, are those obtained using this RTIL formed by the most hydrophilic cation and the most hydrophilic anion, at 120°C. Once again, we cannot propose a certain reason to explain this behavior, as that was not the purpose of this manuscript. According to the theoretical studies from Shehata et al. [53], the presence of EtOH in the reaction medium would allow the fluctuation of the lid to allow the active site to get exposed, and is very compatible with BTL2 stability, as for sure EMIMBF$_4$ has also proven to be.

4.1. Materials

Lipase from *Geobacillus thermocatenulatus* was a kind gift of the Biochemistry, Genetics and Inmunology Department of the Universidad de Vigo. All solvents of the highest purity commercially available and used without purification were purchased from Fluka. All other chemicals and RTILs (1-butyl-3-methyl imidazolium tetrafluoroborate (BMIMBF$_4$), 1-butyl-3-methyl imidazolium hexafluorophosphate (BMIMPF$_6$), 1-ethyl-3-methyl imidazolium tetrafluoroborate (EMIMBF$_4$) and 1-ethyl-3-methyl imidazolium hexafluorophosphate (EMIMPF$_6$) were purchased from Sigma-Aldrich (Barcelona, Spain).

4.2 Synthesis of (R,S) 2-(butyryloxy)-2-phenylacetic acid.-

20 mmol of mandelic acid acid in 200 ml of diethyl ether were added to 2.88 ml triethylamine (20 mmol). Subsequently, a solution of 2.131 ml (20 mmol) butyryl chloride in 100 ml ethyl ether was dropped. The reaction was carried out in a flask at 25 ° C for approximately 4 hours, giving a yield of 50%. Reaction progress was followed by HPLC, ^{1}H-NMR and ^{13}C-NMR (Bruker AC-250 (^{1}H), 63 MHz (^{13}C)).

Simply by adding water unreacted acid could be separated from the ester, which remained in the organic phase. Acid was removed performing successive washig with water. Subsequently, organic phase was dried with anhydrous sodium sulphate and the remaining ether was removed using rotary evaporator. Successive extraction with diethyl ether, allowed the isolation of 7 grams of yellowish oil as a final product ((*R, S*) 2-butanoiloxy phenyl acetic). Spectroscopic data were according to those previously reported in literature [144]

4.3 Resolution of 2-(butyryloxy)-2-phenylacetic acid by alcoholysis reaction

Reactions were carried out in closed glass vials and the temperature of the experiments varied between 40 and 120 ° C. In the case of *iso*octane, temperatures of 40, 70 and 90 ºC were used, whereas ionic liquids were tested at 90 and 120 ºC. To maintain the temperature fixed, a thermostatized bath oil was used for several days. The reaction mixture included: an organic solvent or RTIL (4 mL), 2-(butyryloxy)-2-phenylacetic acid (60 mM) and ethanol (1 mL), assuring a molar excess of alcohol *versus* 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. As a consequence of the employment of high temperatures, the vials were rapidly cooled down for sampling previously to the opening to

avoid any evaporation of the alcohol; thus, aliquots of 100 µL were taken at different times. As RTILs cannot be directly injected into the HPLC, the samples were extracted with 1 mL of diethyl ether and the solvent evaporated at room temperature; subsequently, they were re-diluted with 400 µL hexane/2-propanol (1:1) (v/v) and filtered using syringe filters (Millex-GV (PVDF), 0.22 µm pore size). HPLC analysis was performed using a Chiracel OD (20 µm (250 x 4.6 mm) chiral column, a mobile phase composed by *n*-hexane/2-propanol/trifluoroacetic acid (90/9/1), a 0.8 ml/min flux, and a wavelength of 254 nm. Peak assignation was determined using pure compounds as standards.

5. Conclusions

Binary mixtures of EtOH and Room Temperature Ionic Liquids (RTILs) have proven to be an excellent reaction media for the enantioselective kinetic resolution (KR) of racemic 2-(butyryloxy)-2-phenylacetic acid via ethanolysis catalyzed by lipase form *Geobacillus thermocatenulatus* (BTL2) at very high reaction temperatures. Thus, the KR carried out using an EtOH/$BMIMBF_4$ at 120°C furnished (*R*)-mandelic acid as the only reaction product (E>200), while by changing the composition of the cationic moiety of the RTIL, then using EtOH/$EMIMBF_4$ at the same temperature of 120°C, a fast and perfect KR led to enantipure (*S*)-mandelic acid. Some hypothesis to explain the enzyme behavior are presented, related to the described positive effect of EtOH on the enzymatic activity of BTL2, although a more detailed theoretical study would be demanded to rationalize the enzymatic stereobias.

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