

Article

The Use of Ion Liquids as a Trojan Horse Strategy in Enzyme-Catalyzed Biotransformation of (*R,S*)-Atenolol

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Abstract: The enzymatic method was used for the direct biotransformation of racemic atenolol. The catalytic activities of commercially available lipases from *Candida rugosa* were tested for the kinetic resolution of (*R,S*)-atenolol by enantioselective acetylation in various two-phase reaction media containing ionic liquids. The composed catalytic system gave the possibility to easily separate substrates and products of the conducted enantioselective reaction and after specific procedure to reuse utilized enzymes in another catalytic cycle.

Keywords: kinetic resolution; (*R,S*)-atenolol; lipase; ionic liquids; enzyme-catalyzed biotransformation

1. Introduction

Recently, the growing demand of optically pure compounds for pharmaceutical, chemical, and cosmetic industries is fully understood by scientists, which is confirmed by the increasing number of papers published in this area. β -blockers are one of the classes of drugs, which are playing a relevant role in the treatment of various human diseases. Atenolol is included to the compounds of an amino-alcohols and is frequently used in treatment of hypertension, angina pectoris, and arrhythmia [1–5]. Due to the fact, all β -blockers possess chiral carbon atom within their moiety, they are presented as enantiomers. Although, it was described in many research studies, that the (*S*)-enantiomers of abovementioned APIs are responsible for the desired therapeutic effects, β -blockers are still widely commercially available and administered to patients as racemates. While, the replacement of racemic drugs into optically pure compounds would cause less side effects. In order to achieve this effect, there are many various strategies as well as chemical synthesis, which allow to obtain enantiomerically pure compounds, including (*S*)-enantiomers of β -blockers [6–9].

Enzymes are profoundly effective and particular biocatalysts, which are present in the living creatures. They exist in huge assortments as far as the kinds of responses catalyzed by them for example oxidation, hydrolysis, isomerization, ligation, bond cleavage, and bond arrangement. In addition, compound based catalyses are performed with a lot higher constancy, under mellow response conditions and are exceptionally effective as far as number of steps, giving them an edge over their synthetic partners. The one of a kind attributes of catalysts makes them exceptionally material for a number of synthetic change responses in pharmaceutical businesses, for example, particular acylation and deacylation, specific hydrolysis, deracemization, esterification, transesterification, and numerous others [10–13]. Therefore, these days, the utilization of enzymes in biotransformation has become a fascinating territory for researchers. Among the entirety of catalysts, the hydrolases are the most as often as possible utilized, due to the expansive substrate range and impressive strength. Lipases delegated

hydrolyses are universal proteins which catalyze the enantioselective biotransformation, for example esterification, transesterification, and hydrolysis permitting one to get optically unadulterated mixes, which was widely revealed in various papers [14–26]. Also, lipases do not require any cofactors, and keep up their operational steadiness and synergist action both in fluid and additionally natural media. These compounds are industrially accessible in free or immobilized structures. In any case, the utilization of local chemicals as a rule is related with detachment issues from response blend. Moreover, the free compound is progressively touchy to response media just as temperature [27–29]. Along these lines, the use of local compounds in industry is restricted. All things considered, so as to make a catalyst taking care of in biotechnological process progressively advantageous, different strategies are utilized, and immobilization or direct addition of ionic liquids are one of them [30–38]. One of the best bits of leeway of this procedure is the chance of decreasing the absolute expense of biotransformation by simply evacuation and move of the immobilized catalyst starting with one response blend then onto the next. Besides, the limited proteins for the most part are described by expanded reactant action [39–43].

In this study, enantioselective transesterification of racemic atenolol with the use of two commercially available lipases from *Candida rugosa* (MY and OF) in various catalytic systems containing ionic liquids was investigated. Additionally, the effect of trojan horse strategy of ionic liquids accepted as reusability of utilized biocatalysts was studied, and the high catalytic activity as well as operational stability and enantioselectivity of the enzyme after five reaction cycles was confirmed.

2. Results and Discussion

2.1. Enantioselective Acetylation of (*R,S*)-Atenolol with the Use of Ionic Liquids and Lipase from *Candida rugosa*

The enantioselective biotransformation of (*R,S*)-atenolol was investigated with the use of commercially available lipase from *Candida rugosa* OF and MY (Figure 1) in various two-phase reaction media. The solubility of atenolol in organic solvents is seriously limited. Therefore, the conducted studies were focused on testing various reaction systems to omit the solubility issue of racemic compound. The composed and tested catalytic systems differed from each other in kind of an ionic liquid as well as utilized isoform of the enzyme. It directly resulted in obtaining various products' quality for a specific catalytic system. Nevertheless, among all of the tested reaction systems, some showed acceptable parameters of performed kinetic resolution (Table 1). During the studies, it was observed that, in all cases, the value of conversion was increasing with a time increase. However, among all the tested catalytic systems, the best results were obtained by using lipase from *Candida rugosa* OF. After 240 h of incubation, the (*S*)-atenolol acetate was obtained, with the highest value of enantiomeric excesses of product which equalled $ee_p = 95.23\%$, whereas the enantioselectivity was $E = 56.07$. The application of *Candida rugosa* lipase MY resulted in obtaining acceptable results in specific reaction system, however, the purity of the achieved products was lower compared to the results obtained with the use of the other tested isoform of *Candida rugosa* lipase. Finally, the use of lipase from *Candida rugosa* MY allowed one to obtain the (*S*)-atenolol acetate with the $ee_p = 95.10\%$, whereas the $E = 43.15$, after 240 h of incubating the reaction mixture.

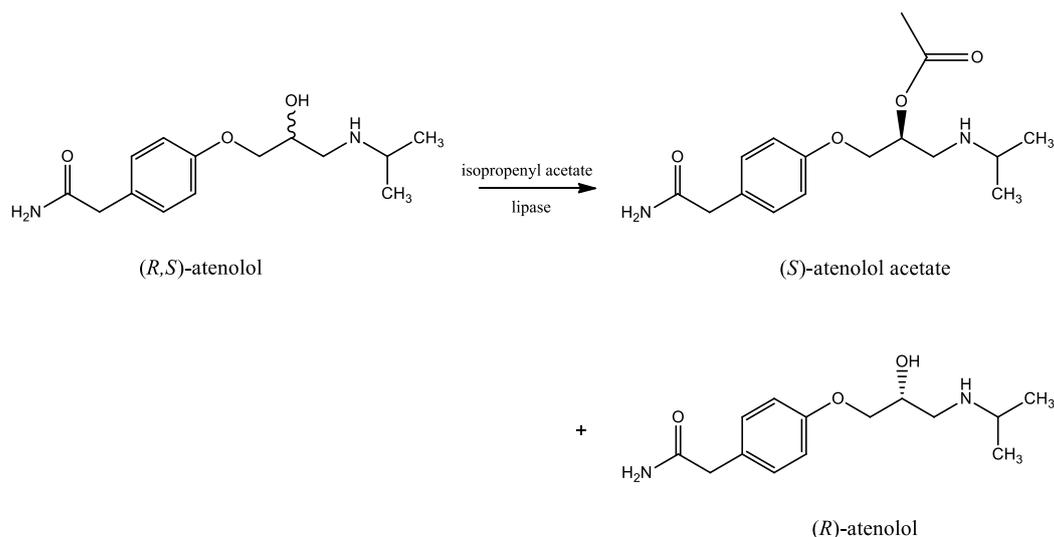


Figure 1. Enantioselective transesterification of (R,S)-atenolol with the use of lipase from *Candida rugosa* as biocatalyst.

Table 1. Enzymatic parameters of performed kinetic resolution including: enantiomeric excesses of substrates (ee_s) and products (ee_p), conversion (c), and enantioselectivity (E) of different reaction systems screened for the enantioselective transesterification of (R,S)-atenolol after 240 h of incubation.

Lipase	Reaction Medium	ee_s	ee_p	c	E
<i>Candida rugosa</i> OF + MY	Toluene [EMIM][BF ₄]	18.29%	95.34%	16.10%	50.14
<i>Candida rugosa</i> OF		32.21%	95.23%	25.27%	56.07
<i>Candida rugosa</i> MY		8.13%	95.10%	7.87%	43.15
<i>Candida rugosa</i> OF + MY	Toluene [EMIM][OTf]	24.96%	83.07%	23.11%	13.78
<i>Candida rugosa</i> OF		31.29%	84.21%	27.09%	15.81
<i>Candida rugosa</i> MY		3.44%	77.52%	4.25%	8.17
<i>Candida rugosa</i> OF + MY	Toluene [EMIM][EtSO ₄]	1.79%	5.67%	23.99%	1.14
<i>Candida rugosa</i> OF		2.14%	5.91%	26.58%	1.15
<i>Candida rugosa</i> MY		2.10%	3.31%	38.82%	1.09

2.2. Effect of Reaction Time

Among all tested influencing factors on enzyme-catalyzed biotransformations, it was observed that the incubation time of the reaction mixture is one of the most critical parts of the kinetic resolution of racemic compounds. According to the other studies it was widely described that in case of too long incubation of reaction medium, the enantioselectivity and enantiomeric excess of both products and substrates decrease rapidly. It is caused, due to the fact that the conversion value could be higher than 50%, which results that the reaction can no longer be considered as enantioselective due to a lack of substrate.

The tested reaction systems consisted of commercially available lipases from *Candida rugosa*, isopropenyl acetate (2 μ L) as acetylating agent, (R,S)-atenolol (3.0 mg) and ionic liquid (500 μ L) as well as toluene (10 mL) as the reaction medium. The performed biotransformations were carried out for 240 h at 30 °C. As shown in Figure 2, the conversion, enantiomeric excess of substrate, and enantiomeric ratio were increasing along with reaction time. The enantiomeric excess of the product decreased very slowly over the same period of time. After 240 h of reaction, the value of conversion was the highest (Figure 2) and differed depending on the kind of catalytic system (Table 1).

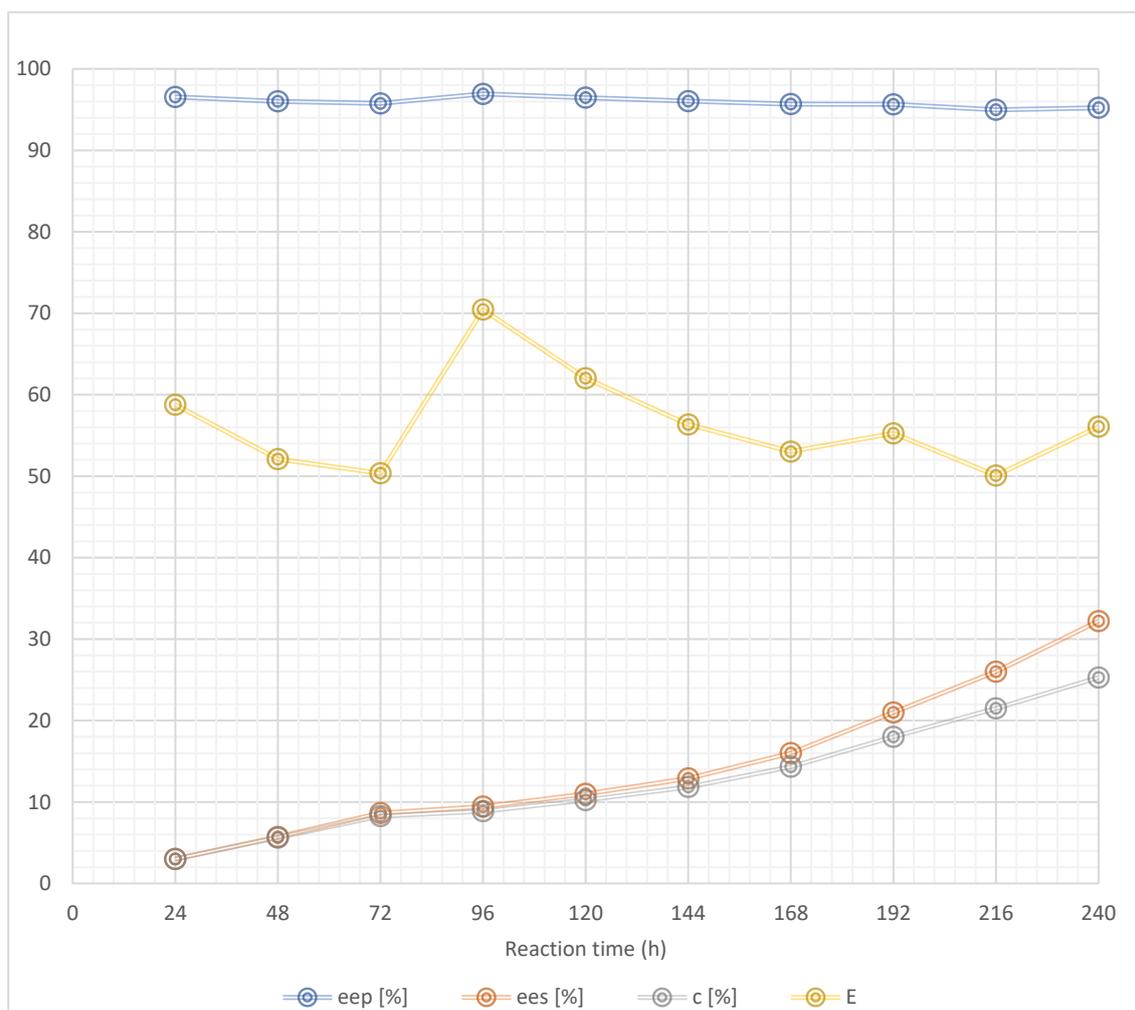


Figure 2. Effect of reaction time on the enzymatic parameters of performed kinetic resolution of (*R,S*)-atenolol including values of both enantiomeric excesses of substrates (ees) and products (eep) as well as conversion (c) and enantioselectivity (E).

2.3. Effect of Lipase

Candida rugosa (OF and MY) lipases in native forms were utilized in the enzyme-catalyzed biotransformation of racemic atenolol with the use of isopropenyl acetate as an acetylating agent in various two-phase reaction medium and investigated for their catalytic and enantioselective properties. As it is shown in Table 1, among all tested catalytic systems, the most satisfactory enantioselectivity were obtained by using lipases from *Candida rugosa* OF. It should be noted that also the enantiomeric ratio and enantiomeric excess of product were higher in reactions using lipase OF than using lipase MY. The observed results were similar for both lipases in terms of its sensitivity in reference to the reaction medium, cause only in one tested reaction medium both enzymes could be stated as enantioselective. During the presented studies it was also decided to investigate the hybrid system composed by two different isoforms of *Candida rugosa*. The study protocol relied on completing reaction system composed of equal mass mixture (5 mg) of lipases from *Candida rugosa* OF and MY. Although the tested system was efficient in certain reaction medium, the use of *Candida rugosa* OF as the only catalyst resulted in obtaining better enzymatic parameters in kinetic resolution of (*R,S*)-atenolol.

2.4. Effect of Reaction Medium

It is reported by numerous scientists, that organic solvents utilized as a reaction medium of enzymatic catalysis are more frequently used than aqueous-based biocatalysis reactions [44,45].

However, because the solubility of (*R,S*)-atenolol in numerous organic solvents is seriously limited, it was decided to conduct the studies in two-phase system containing toluene and various ionic liquids, which additionally gives the possibility of easy separation of the substrates and products of the reaction from the catalytic system. It is possible, because both atenolol and its acetylated form remained only in one phase—ionic liquid. Based on previously conducted studies, the catalytic properties of enzymes including lipases are highly affected by the hydrophobicity of the reaction medium [17]. That is the reason, that the optimal choice of reaction medium is one of the most important parts of optimizing reaction conditions allowing to obtain better enantioselectivity. Among all tested ionic liquids, i.e., [EMIM][BF₄], [EMIM][OTf], and [EMIM][EtSO₄], only [EMIM][BF₄] was suitable for the enantioselective acetylation of racemic atenolol, as shown in Table 1. Even though the solubility of racemic atenolol was sufficient in [EMIM][OTf] and [EMIM][EtSO₄], the reaction mediums composed by these ionic liquids are not effective for the enantioselective biotransformation of racemic atenolol, hence the enantiomeric excess of product and enantioselectivity are significantly lower. Therefore, the use of [EMIM][BF₄] and toluene as the reaction medium seems to be optimal reaction medium and its use resulted in better enantiomeric excess of product, with values higher than 95% (Figure 3). Additionally, the use of this catalytic system made it possible to obtain a high value of enantioselectivity (Figure 4). The E-values were in all tested systems with [EMIM][BF₄] greater than 40, and thus the reactions conducted only in this reaction medium could be found as enantioselective.

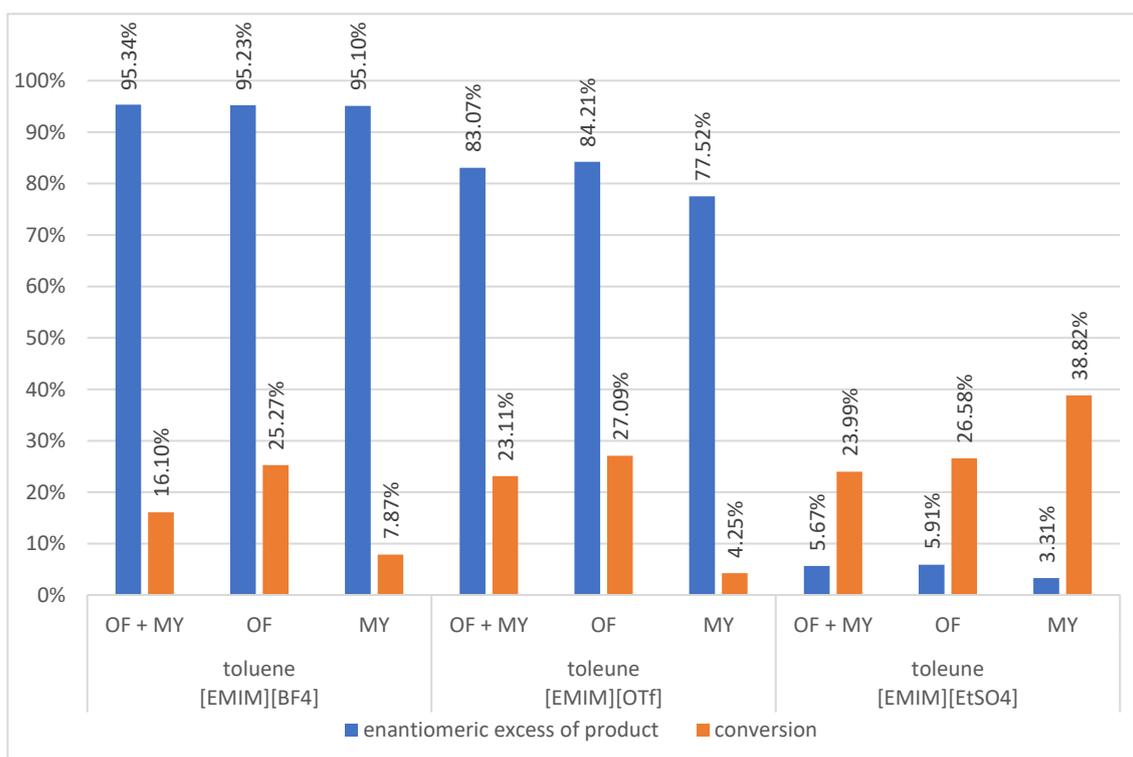


Figure 3. Enzymatic parameters of performed kinetic resolution including: enantiomeric excesses of products (ee_p) and conversion (c) of different reaction medium screened for the enantioselective acetylation of (*R,S*)-atenolol after 240 h of incubation.

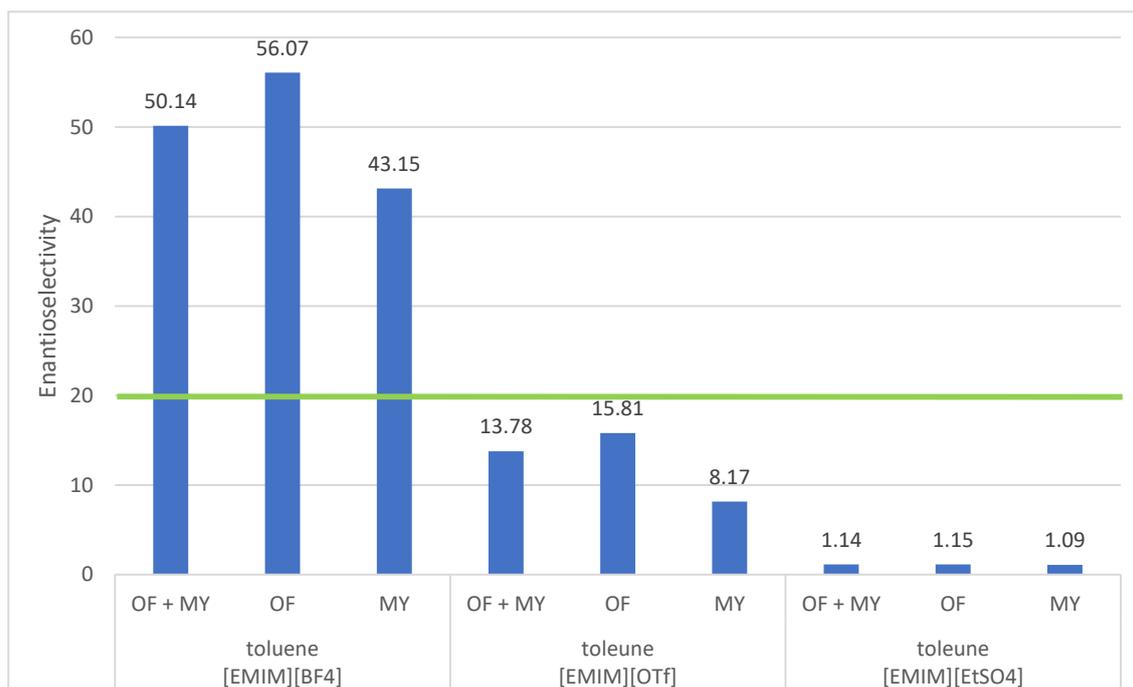


Figure 4. Enzymatic parameters of performed kinetic resolution including enantioselectivity (E) of different reaction medium screened for the enantioselective transesterification of (R,S)-atenolol after 240 h of incubation.

2.5. Effect of Lipase Reusability in Enzyme-Catalyzed Biotransformation of (R,S)-Atenolol

One of the most important advantages of using ionic liquids in two-phase enzyme-catalyzed biotransformation is a possibility to apply a “trojan horse” strategy, which relies on reusing the enzyme in another catalytic system, by simply replacing the ionic liquids with specific substrates and products of reaction. The impact of native lipases reusability on kinetic resolution of racemic atenolol was investigated. For this purpose, the lipases from *Candida rugosa* OF and MY were reused after the specified substrate replacement procedure.

After the catalytic reaction, the residue of ionic liquids containing the enantiomers of both atenolol and atenolol acetate were withdrawn to the separated tube. Afterwards, the new portion of ionic liquids with the racemic atenolol as a reaction substrate was added to the same lipase suspended in toluene. To start the enantioselective reaction the proper acetylating agent was added (isopropenyl acetate). For the purpose of the presented studies, five reaction cycles were performed, which corresponded to 1200 h of catalytic and operational activity of the used enzymes. After the fifth reaction cycles, the enantiomeric excesses of products of all tested reaction mixtures were higher than 90% compared to the initial value (Figure 5). The highest value of enantiomeric excess was obtained with the use of lipase from *Candida rugosa* OF in reaction medium composed by toluene and [EMIM][BF₄]. Nevertheless, it was observed, that the differences of catalytic activity of all utilized enzymes after five reaction cycles were not significant. Thus, the obtained results demonstrated that, the use of ionic liquids not only carries the direct advantages related in obtaining more than acceptable catalytic parameters, but also allows to separate the substrates and products from the catalytic system and reuse the enzyme in another cycle.

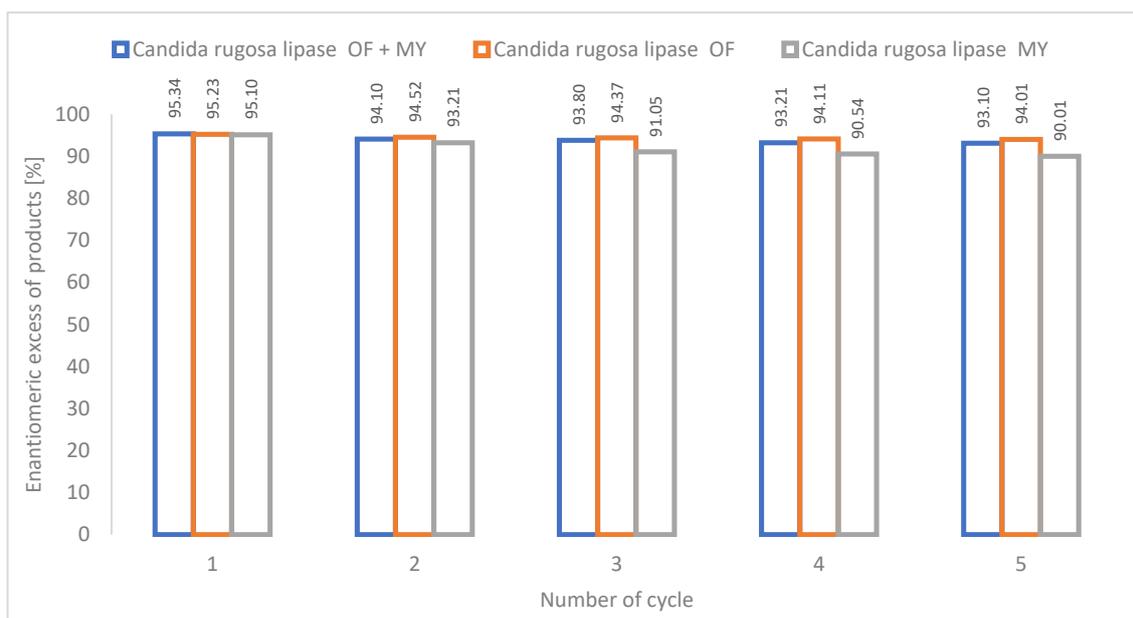


Figure 5. Enantiomeric excess of products in 5 reaction cycles with the reused lipases from *Candida rugosa* in kinetic resolution of (*R,S*)-atenolol.

3. Materials and Methods

3.1. Chemicals

Acetonitrile, acetyl chloride, diethylamine, [EMIM][BF₄], [EMIM][EtSO₄], [EMIM][OTf], isopropenyl acetate, isopropanol, (*R*)-atenolol, (*R,S*)-atenolol, and toluene, were purchased from Merck (Sigma-Aldrich Co., Stainhaim, Germany).

Lipases from *Candida rugosa* MY and OF were a gift from Meito Sangyo Co., Ltd. (Japan).

In the conducted study the water used was obtained using a Milli-Q Water Purification System (Millipore, Bedford, MA, USA).

3.2. Instrumentation

All incubations were performed at controlled temperature and number of rotations (250 RPM) in the dedicated incubating apparatus, model: the Inkubator1000 and Unimax 1010, which were purchased from Heidolph (Schwabach, Germany).

The HPLC samples were purified using the Refrigerated CentriVap Concentrator, which was purchased from Labconco (Kansas City, MO, USA).

The HPLC analysis were performed with the use of the Shimadzu UPLC-MS/MS system (Kyoto, Japan), which was equipped with an autosampler (model: SIL-40AC); two solvent delivery pumps combined with gradient systems (model: LC-40AD); a degasser (model: DGU-30A5); a column oven (model: CTO-40AC); a UV detector (model: SPD-M20A) as well as a triple quadrupole mass spectrometer detector (model: LCMS-8045). The chiral resolutions were conducted by using Lux Cellulose-2 (LC-2) column with a cellulose tris(3-chloro-4-methylphenylcarbamate) stationary phase and a Guard Cartridge System model KJO-4282, which were purchased from Phenomenex Co (Torrance, CA, USA).

All employed glassware was oven dried overnight and then cooled in a stream of nitrogen.

3.3. Chromatographic Conditions

The optimization process of chiral chromatographic resolution of (*R,S*)-atenolol and its acetylated derivatives was described in previously published papers [46–48]. Finally, the use of chiral column: Lux Cellulose-2 thermostated in 30 °C allowed to achieve baseline chiral separation

of the enantiomers of both atenolol and atenolol acetate. The optimal mobile phase consisted of acetonitrile/isopropanol/diethylamine in volumetric ratio 98/2/0.1.

In order to obtain satisfactory resolution, the flow rate of mobile phase was set on 0.8 mL/min. The detection was made with the use of triple quadrupole mass spectrometer operating in multiple reaction monitoring mode (MRM). The transitions of MRM for atenolol were 267.20 > 116.10, 267.20 > 190.05, and 267.20 > 256.05, whereas for atenolol acetate were 309.20 > 116.10, 309.20 > 145.15, and 309.20 > 158.10. The optical purity of both substrates and products as well as enantioselectivity of conducted enzyme-catalyzed biotransformation was calculated by using the equations based on peak areas from chromatogram of (*R,S*)-atenolol and its acetylated forms, which were described and employed in numerous studies [49,50].

3.4. Kinetic Resolution of (*R,S*)-Atenolol

Enantioselective biotransformation of racemic atenolol was carried out in 20 mL glass flask. The reaction mixture consisted of isopropenyl acetate (2 μ L) utilized as an acetyl donor and (*R,S*)-atenolol (3.0 mg, 0.01 mM) dissolved in 0.5 mL of selected ionic liquid placed in 10 mL of toluene, which together created a two-phase reaction medium. As part of the study the following ionic liquids were tested: [EMIM][BF₄], [EMIM][OTf] and [EMIM][EtSO₄]. The enzyme-catalyzed biotransformation of (*R,S*)-atenolol was started by the direct addition of 10 mg of native lipase from *Candida rugosa* OF, MY or equal hybrid mixture of OF and MY to the previously composed bioreactor. The reaction mixture was incubated along with shaking (250 RPM) at 37 °C. The process of enantioselective biotransformation was monitored by using chiral stationary phases and UPLC system coupled with triple quadrupole mass spectrometer operating in MRM mode. The samples of 30 μ L of ionic liquid were withdrawn at previously established time points every 24 h for 240 h. Further, racemic atenolol and its acetylated form were extracted from the ionic liquid by vigorous shaking for 10 minutes with 500 μ L pure acetonitrile, and after centrifugation and filtering the prepared samples were placed to the vials and injected on the chiral column of UPLC-MS/MS system.

4. Conclusions

The performed study confirmed the ability to catalyse enantioselective acetylation of racemic atenolol by lipases from *Candida rugosa* OF and MY. In the presented study, the use of ionic liquids was investigated. As it turned out, the two-phase catalytic systems composed by ionic liquid and toluene, as well as lipase from *Candida rugosa*, and acetylating agent allowed to obtain high enantioselective parameters. However, the utilized ionic liquids exhibited various kinetic properties, which resulted in obtaining different values of enantioselectivities and enantiomeric excesses of substrates and products. It was reported previously that the direct addition of [EMIM][EtSO₄] to reaction mixture inhibits the enzymatic transesterification [37], whereas the results presented herein show that transesterification reaction proceeded smoothly by addition of [EMIM][EtSO₄] resulted in the highest values of conversion. Nevertheless, the *E*-value dropped significantly and, thus, this reaction system could not have been accepted as enantioselective. Additionally, this result may indicate, that the conducted reaction in the catalytic system containing [EMIM][EtSO₄] is non-enzymatic esterification, since this IL could act as a catalyst for esterification. Among all of tested catalytic systems, the best result was obtained by using native *Candida rugosa* lipase OF in a system containing [EMIM][BF₄] (*E* = 56.07, ee_p = 95.23%). Additionally, the use of tested ionic liquids gave the possibility to remove substrates and products from catalytic system and reuse the enzyme, which was investigated. The performed study proved, that even after 5 reaction catalytic cycles, both lipases from *Candida rugosa* OF and MY maintained their high operational stabilities and catalytic activities. The proposed approach could be very important from economical viewpoint, because it allows for direct and extensive total cost reduction of performed enzyme-catalyzed biotransformation.

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