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The Utilization of Two-Phase Catalytic System in Enantioselective Biotransformation of Racemic Atenolol

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Abstract: There are several methods that allow enantiomerically pure compounds to be obtained. In the study presented herein, the enantioselective biotransformations of (*R*,*S*)-atenolol were performed with the use of various catalytic systems containing ionic liquids and toluene as a reaction medium, vinyl acetate as an acetylating agent as well as lipases from Candida rugosa. The conducted studies profs that, the use of the two-phase reaction system enables the reuse of the biocatalyst in another cycle and allows to achieve satisfactory kinetic resolution parameters.

Keywords: *Candida rugosa* lipase; ionic liquid; racemic atenolol; enantioselective biotransformation

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1. Introduction

Cardiovascular disorders are the leading cause of death in the world. More than 75% of heart disease and stroke-related fatalities take place in low- and middle-income nations. Elevated blood pressure, often known as hypertension, is a serious medical condition that dramatically raises the risk of cardiovascular disorders. Specific systolic and diastolic blood pressure values or the documented usage of antihypertensive drugs can be used to diagnose hypertension. Only 14% of the estimated 1.4 billion people with high blood pressure have it under control. There are, nevertheless, affordable therapy choices [1–3].

Due to the fact that β-blockers, including atenolol, in their chemical structure have an asymmetric carbon atom, which is their chiral center, they exist in the form of two enantiomers, i.e., (*R*)-enantiomer and (*S*)-enantiomer [4,5]. (*S*)-enantiomers of β-blockers are usually responsible for the therapeutic action, since the (*R*)-enantiomers of β-blockers have significantly lower affinity to the β-adrenergic receptors and could cause additional adverse events. Nevertheless, β-blockers are still mainly administered as racemates, instead of pure eutomers and thus could be responsible for unnecessary side effects.

Currently, there are three main ways to obtain optically pure compounds (including therapeutic agents). It is an organic synthesis using a "chiral pool" of optically pure substrates; racemate separation; as well as asymmetric synthesis with the use of pro-chiral substrates. Enzymatic transformations are appreciated by many good features such as high selectivity, milder reaction conditions, and biocompatibility, which become an alternative powerful tool in organic synthesis. Therefore, the kinetic resolution (racemate resolution) with the use of enzymes, which relies on carrying out a stereoselective biotransformation, is one of the most frequently used methods due to the fact that, compared to the use of a "chiral pool", it is significantly less expensive and does not require the use of toxic and environmentally hazardous chemical compounds [6–17].

In the process of stereoselective biotransformation of racemic forms of active pharmaceutical ingredients, it is still common to use organic solvents that act as a reaction medium. Nevertheless, most of these compounds are toxic and dangerous to the environment, and in many cases, they can cause organic contamination of the final synthesis product, e.g., β-blocker derivatives. On the other hand, the use of ionic liquids as the reaction medium

brings many advantages. Many ionic liquids have been developed to solve specific synthetic problems and are therefore also referred to as so-called "design solvents". Their unique properties make them useful in many technological processes [18]. Ionic liquids are considered also as "green solvents" that exhibit several unique characteristics, such as high ionic conductivity, high solvation power, thermal stability, low volatility, and recyclability [19–21]. These "green" solvents are environmentally friendly and the transformations with the use of ionic liquids are often faster. Additionally, the ionic liquids can be recovered from the bioreactor and reused in subsequent catalytic cycles, which reduces the overall cost of the biotransformation [22–27]. Therefore, nowadays, more and more processes in the pharmaceutical industry, including stereoselective biotransformations, are carried out with the use of ionic liquids [28–31].

2. Results and Discussion

2.1. Enantioselective Biotransformation of Racemic Atenolol

Candida rugosa OF and MY lipases, which are commercially available, were used to study the enantioselective biotransformation of (*R*,*S*)-atenolol in a variety of two-phase reaction conditions (Figure 1). Atenolol has very little solubility in organic solvents. Therefore, the research that was undertaken was concentrated on exploring different reaction systems to omit the racemic compound's solubility issue. The types of ionic liquid and enzyme isoforms used in the developed and tested catalytic systems varied from one another. It directly led to the acquisition of numerous products of appropriate quality for a given catalytic system. However, some of the evaluated reaction systems demonstrated adequate kinetic resolution performance criteria (Table 1). It was seen during the studies that, in every case, the value of conversion was rising with the reaction time.

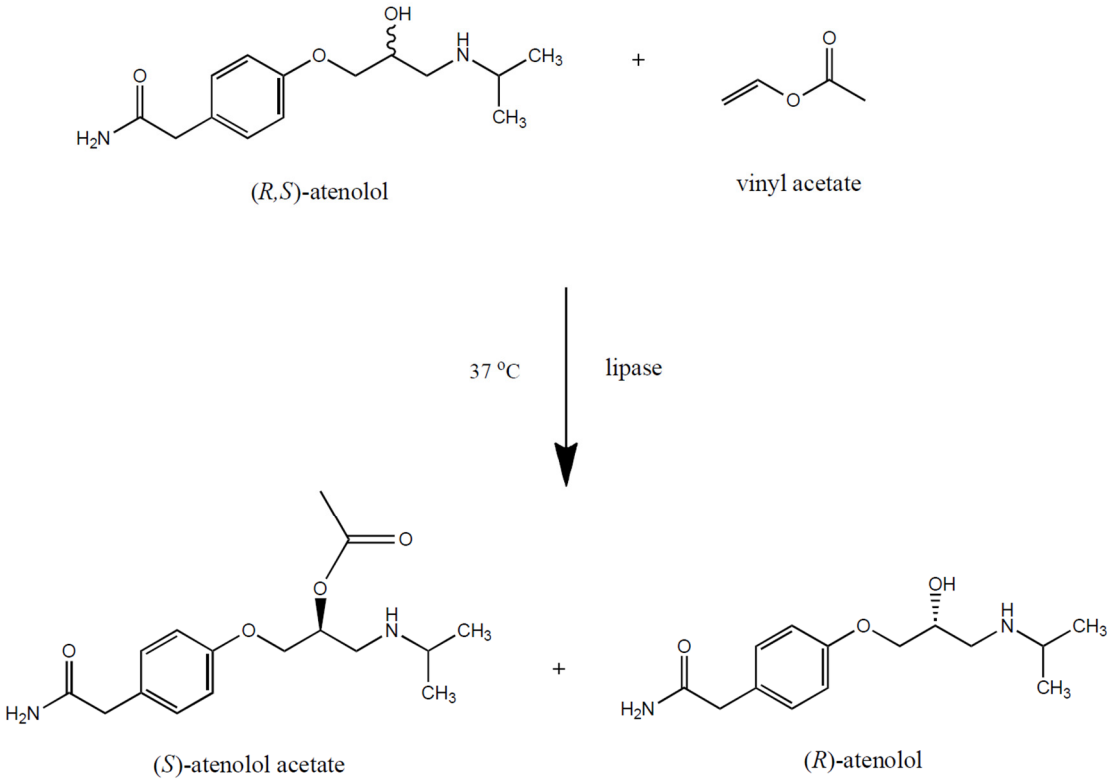


Figure 1. Enantioselective biotransformation of (*R*,*S*)-atenolol with the use of *Candida rugosa* lipase as biocatalyst. The reaction mixture consisted of (*R*,*S*)-atenolol (3.0 mg, 0.01 mM), vinyl acetate (2.0 µL, 0.02 mM), lipases from *Candida rugosa* OF or MY (10.0 mg), and toluene (10 mL) with or without the addition of ionic liquid (500 µL) and was incubated for 120 h along with mechanical shaking (250 RPM) at 37 °C.

Table 1. List of obtained results of performed enantioselective biotransformation of (R,S)-atenolol after 120 h of incubation: enantiomeric excesses of substrates (ee_s), products (ee_p), conversion (c), and enantioselectivity (E).

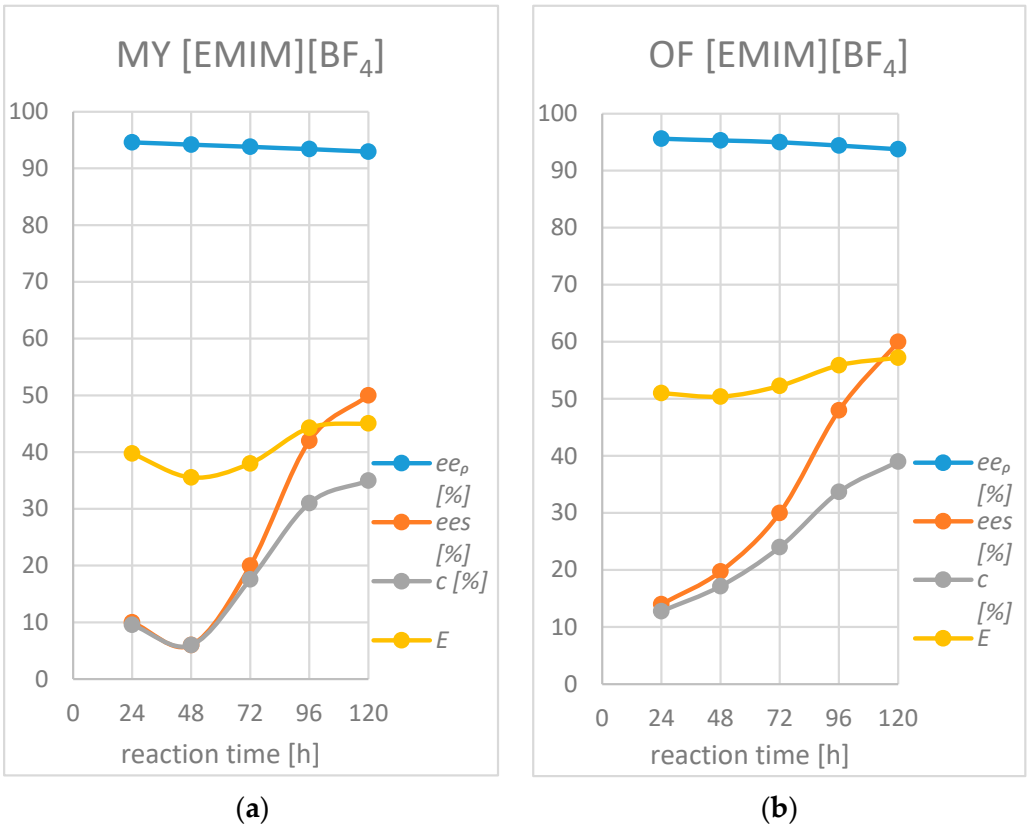
Reaction Medium	Lipase	ee_s	ee_p	c	E
Toluene	*Candida rugosa* OF	61.80%	93.60%	39.77%	56.99
	Candida rugosa MY	52.00%	93.00%	35.86%	46.34
Toluene [EMIM] [BF_4]	*Candida rugosa* OF	59.98%	93.76%	39.01%	57.22
	Candida rugosa MY	50.00%	92.96%	34.97%	45.07
Toluene [EMIM] [OTf]	*Candida rugosa* OF	58.78%	79.60%	42.48%	15.88
	Candida rugosa MY	52.24%	77.60%	40.23%	13.27
Toluene [EMIM] [$EtSO_4$]	*Candida rugosa* OF	1.60%	1.40%	53.30%	1.04
	Candida rugosa MY	1.86%	0.36%	83.89%	1.02

ee_s—enantiomeric excesses of substrates; ee_p—enantiomeric excesses of products; c—conversion; E—enantioselectivity.

The lipase from *Candida rugosa* OF had the greatest outcomes among all examined catalytic systems, nevertheless. After 120 h of incubation, the (*S*)-atenolol acetate was obtained, with the highest value of enantioselectivity which equaled E = 57.22, whereas the enantiomeric excesses of the product equaled ee_p = 93.76%. Although the application of lipase from *Candida rugosa* MY allowed to obtain acceptable results, in particular catalytic systems, the enantiomeric purity of achieved products was lower compared to the results obtained with the use of lipase from *Candida rugosa* OF. Finally, the use of *Candida rugosa* MY lipase allowed to obtain the (*S*)-atenolol acetate with the enantioselectivity equaled E = 45.07, whereas the enantiomeric excess of product was ee_p = 92.96% after 120 h of incubating the reaction mixture.

2.2. Effect of Rreaction Time

One of the most crucial aspects of the kinetic resolution of racemic chemicals was found to be the incubation duration of the reaction mixture among all evaluated influencing factors on enzyme-catalyzed biotransformations. Other investigations have shown that the enantioselectivity and enantiomeric excess of both products and substrates rapidly decline when the reaction medium is incubated for an excessively long time. The lack of substrate makes the reaction no longer regarded as enantioselective as a result of the conversion value having the potential to be higher than 50%. Commercially available lipases from *Candida rugosa* OF and MY, vinyl acetate (2 µL) as an acetylating agent, (*R*,*S*)-atenolol (3.0 mg), ionic liquid (500 µL), and toluene (10 mL) were utilized as the reaction medium in the experiment. The biotransformations were carried out for 120 h at 30 °C. According to Figure 2, the reaction duration increased along with the conversion, enantiomeric excess of the substrate, and enantiomeric ratio. Over the same time span, the product's enantiomeric excess slowly diminished. The value of conversion was the highest after 120 h of reaction (Figure 2), and it varies depending on the type of catalytic system (Table 1).



(**a**) (**b**)

Figure 2. Effect of reaction time on the enzymatic parameters of performed kinetic resolution of (*R*,*S*)-atenolol in two-phase catalytic system consisting of [EMIM] [BF_4] and lipase from *Candida rugosa* OF (**a**) or *Candida rugosa* MY (**b**) including values of both enantiomeric excesses of substrates (ee_s) and products (ee_p) as well as conversion (*c*) and enantioselectivity (*E*).

2.3. Effect of Biocatalysts

The enzyme-catalyzed biotransformation of racemic atenolol with the use of vinyl acetate as an acetylating agent was carried out using *Candida rugosa* (OF, MY) lipases in native forms, and their catalytic and enantioselective capabilities were examined. The use of lipases from *Candida rugosa* OF produced the highest levels of enantioselectivity among all evaluated catalytic systems, as indicated in Table 1. It should be highlighted that reactions utilizing lipase OF had greater enantiomeric ratios and enantiomeric excess of product than reactions using lipase MY. Since both enzymes could only be deemed to be enantioselective in one of the investigated reaction mediums, the observed results for both lipases were comparable in terms of their sensitivity to the reaction medium. It should be also noted that the differences between compared isoforms of *Candida rugosa* lipases were mainly related in observed conversion, which affected the enantioselective, rather than enantiomeric excess of products, which were similar.

2.4. Effect of Reaction Medium

The investigated systems were effective in reaction media both with and without ionic liquids. As it was observed, *Candida rugosa* lipase exhibited various catalytical properties depending on type of reaction medium. Due to this, one of the most crucial aspects of improving reaction conditions to increase enantioselectivity is selecting the best reaction medium. Taking into account the addition of ionic liquids, it should be noted that only [EMIM] [BF_4] was appropriate for the enantioselective acetylation of racemic atenolol among the three investigated ionic liquids, [EMIM] [OTf] and [EMIM] [$EtSO_4$], as shown in Table 1. Racemic atenolol was sufficiently soluble in [EMIM] [OTf] and [EMIM] [$EtSO_4$], however, these ionic liquids are ineffective as reaction mediums for the

enantioselective biotransformation of racemic atenolol, resulting in decreased enantiomeric excess of product and enantioselectivity. Therefore, it appears that using [EMIM] [BF_4] and toluene as the reaction medium is ideal, and using it led to greater enantiomeric excess of product, with values higher than 93% (Figure 3). Furthermore, the employment of this catalytic system allowed for the achievement of a high value of enantioselectivity (Figure 4). The reactions carried out only in this reaction medium could be identified as being enantioselective since the E-values were in all investigated systems with [EMIM] [BF_4] higher than 40. It should be emphasized that the reaction without the addition of ionic liquids also allowed to obtain comparable kinetic resolution parameters. Nevertheless, the composition of the two-phase catalytic system obtained by direct addition of ionic liquids gave the possibility to easily separate the substrates and products from the reaction medium, by withdrawing ionic liquids containing atenolol and resulted derivatives and by replacing it with fresh ones containing only substrates allowed to reuse the biocatalysts remaining in reaction system.

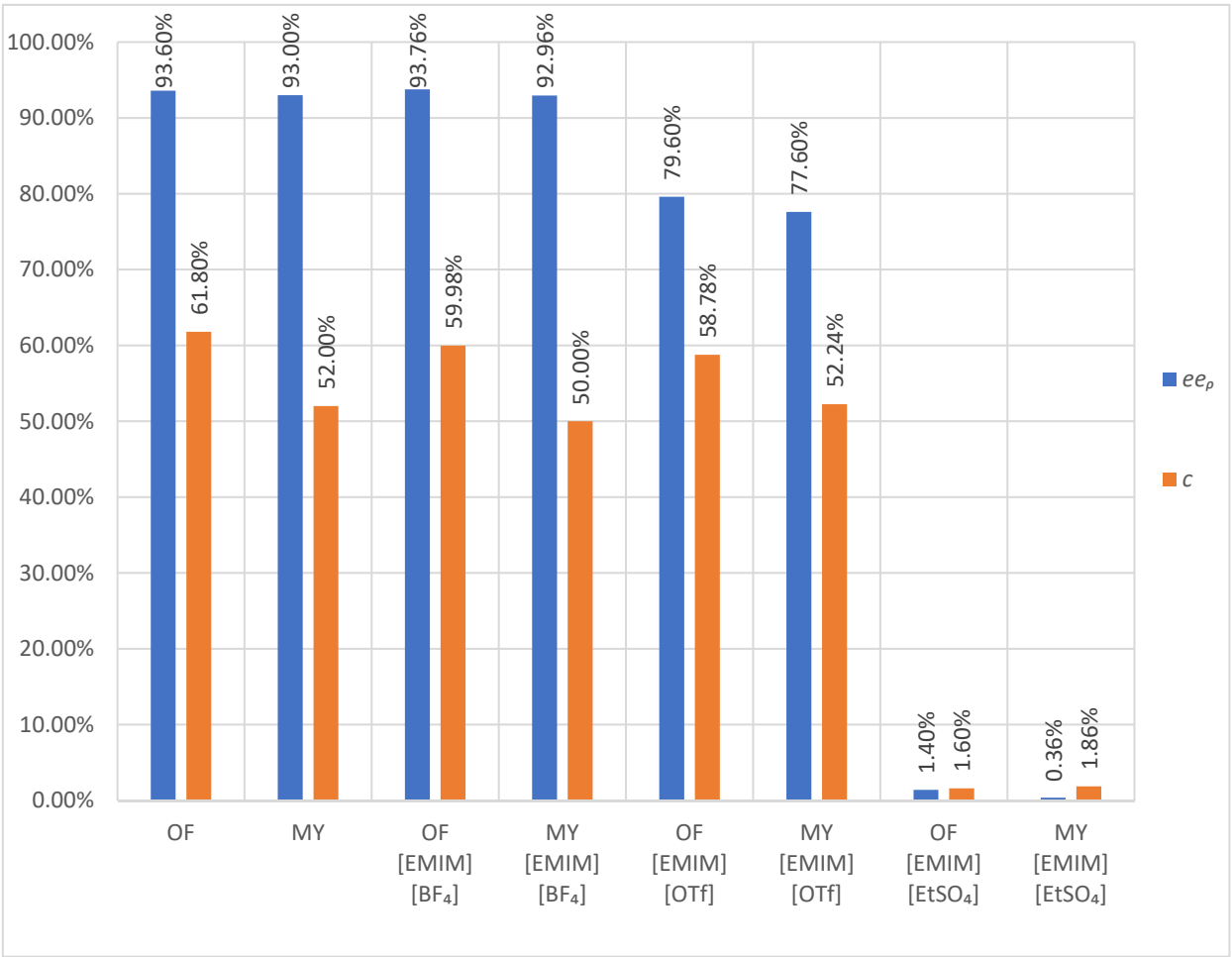


Figure 3. Overview of obtained results of performed enantioselective biotransformation of (*R*,*S*)-atenolol after 120 h of incubation including enantiomeric excesses of products (ee_p) and conversion (c).

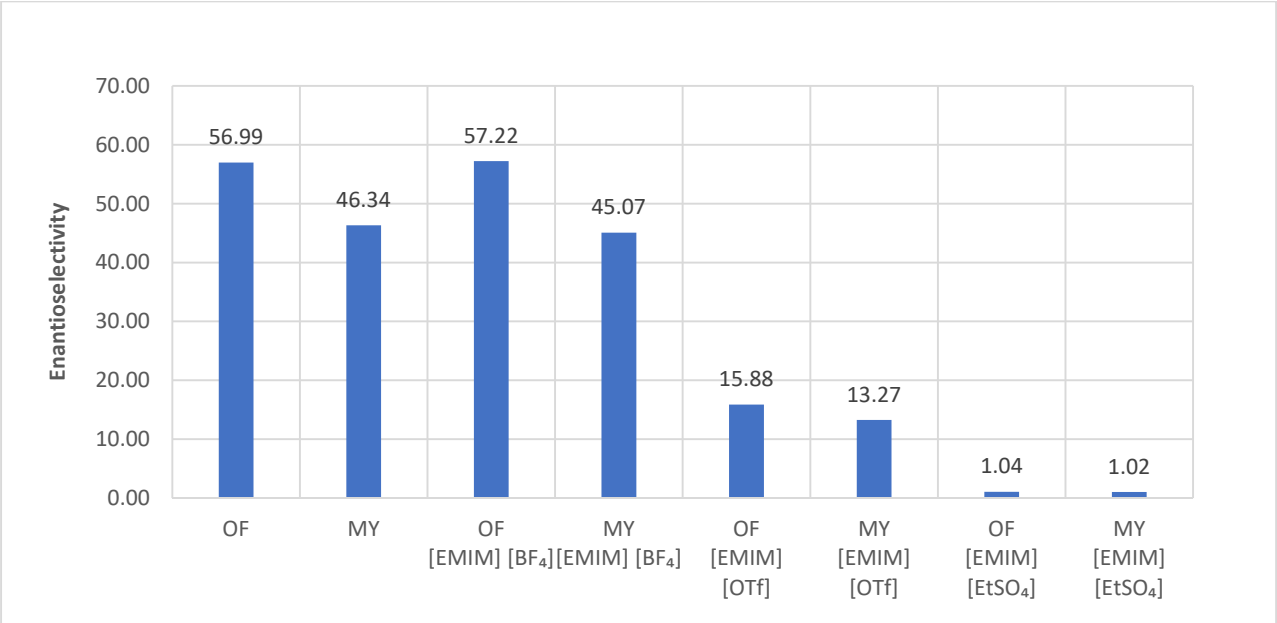


Figure 4. Overview of obtained results of performed enantioselective biotransformation of (*R*,*S*)-atenolol after 120 h of incubation including enantioselectivity (*E*).

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

One of the most significant benefits of using ionic liquids in two-phase enzyme-catalyzed biotransformation is the ability to reuse the enzymes in another catalytic system by simply substituting the ionic liquids with specific substrates and products of reaction. During the study, the effect of native lipases' capacity for reuse on the kinetic resolution of racemic atenolol was investigated. After the indicated substrate substitution method, the *Candida rugosa* lipases OF and MY were employed again for this purpose.

Following the catalytic process, the remaining ionic liquids containing the enantiomers of atenolol and atenolol acetate were transferred to the separated tube. The same lipase that was suspended and remained in toluene was then introduced to the new quantity of ionic liquids containing the racemic atenolol as a reaction substrate. The correct acetylating agent was applied in order to initiate the enantioselective reaction (vinyl acetate). Five reaction cycles were carried out for the purpose of the experiments that were presented, which amount to 600 h of catalytic and operational activity of the enzymes that were used. Enantiomeric excesses of all evaluated reaction mixture products after the fifth reaction cycle were greater than 90% of the initial value (Figure 5).

In a reaction medium made up of toluene and [EMIM] [BF_4], lipase from *Candida rugosa* OF produced the highest value of enantiomeric excess. Nevertheless, it was found that after five reaction cycles, there was no discernible difference in the catalytic activity of any of the used enzymes. The acquired results therefore showed that using ionic liquids not only has direct benefits linked to getting catalytic parameters that are above acceptable levels, but also enables the separation of substrates and products from the catalytic system and the reuse of the enzyme in another cycle.

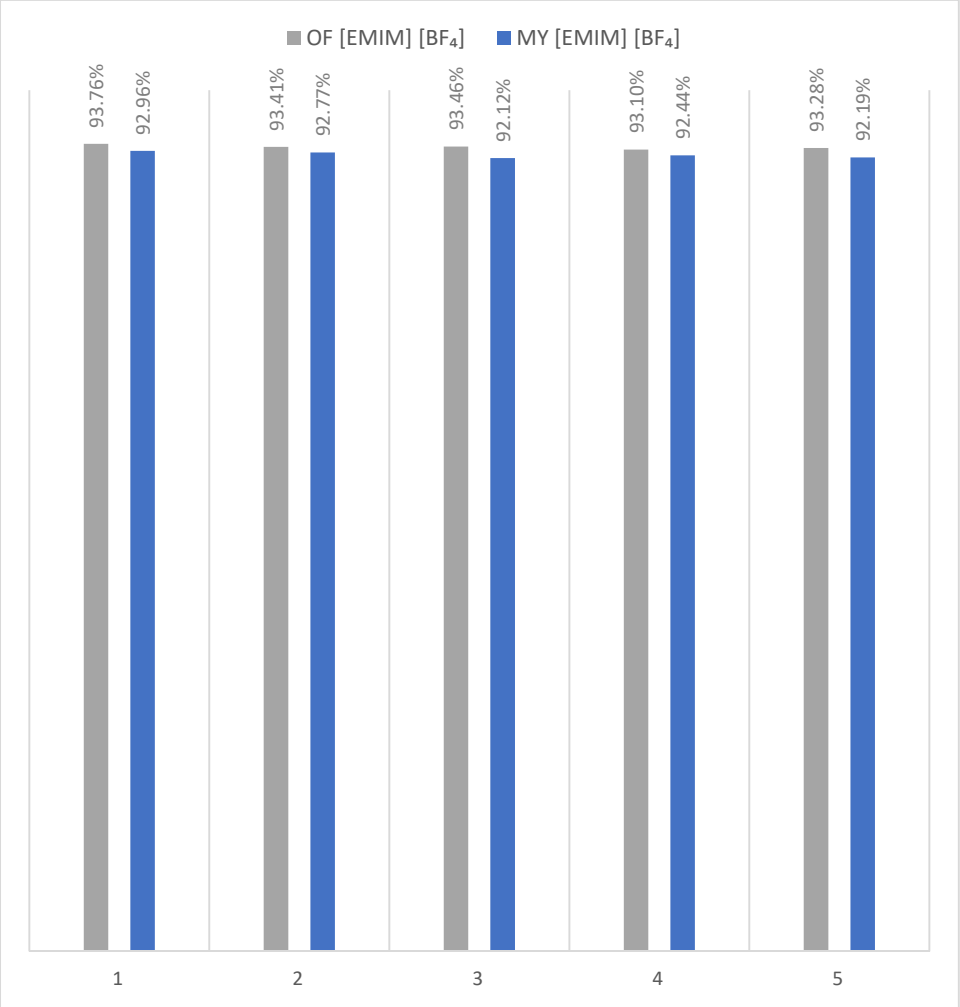


Figure 5. Comparison of the values of enantiomeric excesses of products in five reaction cycles with the reused lipases from *Candida rugosa* in enantioselective biotranformation of (*R*,*S*)-atenolol.

3. Materials and Methods

3.1. Chemicals

Acetonitrile, acetyl chloride, diethylamine, [EMIM] [BF_4], [EMIM] [$EtSO_4$], [EMIM] [OTf], vinyl 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. (Tachikawa, Japan). The activity of lipase from *Candida rugosa* OF is 360,000 U/g powder, whereas the activity of lipase from *Candida rugosa* MY is 30,000 U/g powder. The thermal stability of both lipases is equal or below 37 °C and optimum *pH* is 6–7.

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

3.2. Instrumentation

The Refrigerated CentriVap Concentrator, which was bought from Labconco, was used to purify the HPLC samples.

Shimadzu UPLC-MS/MS system (Japan) used for the HPLC study was equipped with an autosampler (SIL-40AC), two solvent supply pumps with gradient systems (LC-40AD), a degasser (DGU-30A5), a column oven (CTO-40AC), a UV detector (SPD-M20A), and a triple quadrupole mass spectrometer detector (model: LCMS-8045). The Guard Cartridge System model KJO-4282 and Lux Cellulose-2 (LC-2) column with cellulose tris(3-chloro-

4-methylphenylcarbamate) stationary phase, both obtained from Phenomenex Co., were used to perform the chiral resolutions.

All incubations were carried out in specialized incubating apparatuses, models: Inkubator1000 and Unimax 1010, which were bought from Heidolph, at a controlled temperature and rotation (250 RPM) (Schwabach, Germany). Every piece of glass that was used was oven-dried overnight before being cooled in a nitrogen stream.

3.3. Chromatographic Conditions

In previously published papers [32–34], the chiral chromatographic resolution optimization method of (*R*,*S*)-atenolol and its acetylated derivatives was discussed. Finally, baseline chiral separation of the enantiomers of both atenolol and atenolol acetate was accomplished using a chiral column made of Lux Cellulose-2 which was thermostatic at 30 °C. Acetonitrile, isopropanol, and diethylamine were combined in the ideal mobile phase at a volumetric ratio of 98/2/0.1.

The mobile phase flow rate was tuned at 0.8 mL/min in order to get a good resolution. Utilizing a triple quadrupole mass spectrometer in multiple reaction monitoring mode, the detection was made (MRM). Atenolol had MRM transitions of 267.20 > 116.10, 267.20 > 190.05, and 267.20 > 145.05, whereas atenolol acetate had transitions of 309.20 > 116.10, 309.20 > 145.15, 309.20 > 158.10, and 309.20 > 190.05, which is shown in Figure 6 and was in line with other published articles [35,36] as well as the METLIN database, which directly proves that the identified peaks were from atenolol and atenolol acetate. The retention time of (*R*)-atenolol acetate was t_R = 7.717 min, (*S*)-atenolol acetate was t_R = 8.516 min, (*R*)-atenolol was t_R = 19.921 min, and (*S*)-atenolol was t_R = 17.539 min (Figure 7).

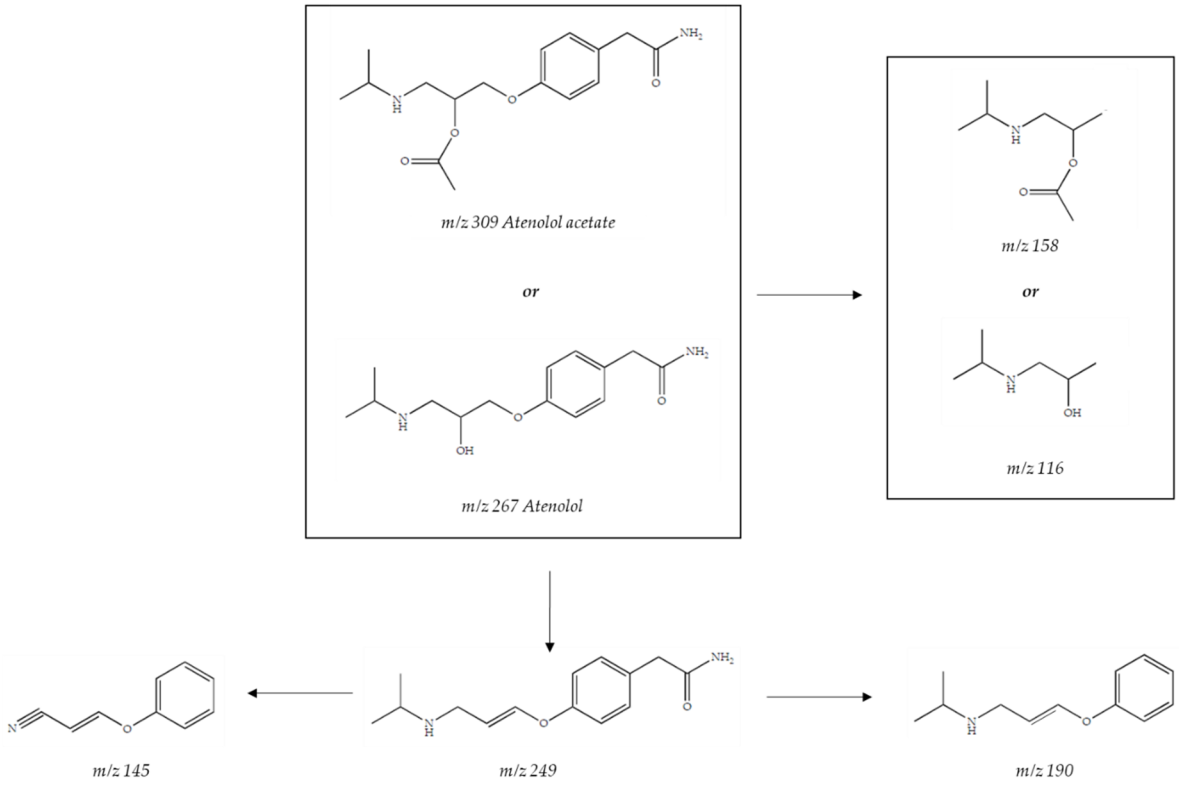


Figure 6. Fragmentation pathways for molecular ions of atenolol.

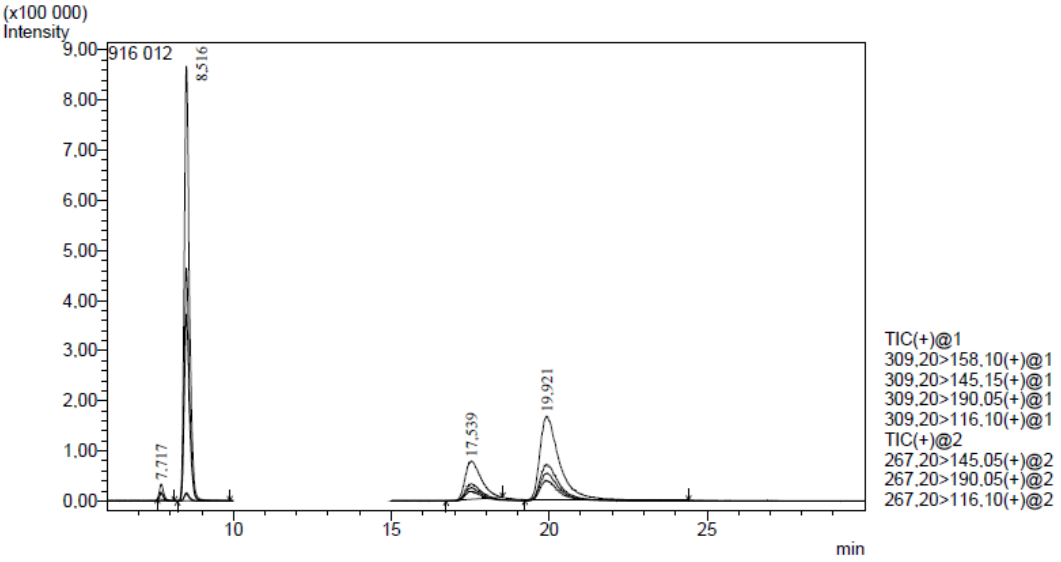


Figure 7. Chromatogram of (*R*,*S*)-atenolol and its esters after 120 h of kinetic resolution of (*R*,*S*)-atenolol with the use of *Candida rugosa* OF in two-phase reaction media containing [EMIM] [BF_4] and toluene: (*R*)-enantiomer of atenolol acetate (t_R = 7.717), (*S*)-enantiomer of atenolol acetate (t_R = 8.516), (*S*)-atenolol (t_R = 17.539), (*R*)-atenolol (t_R = 19.921). Chromatographic conditions: Lux Cellulose-2 (4.6 × 250 mm × 3 µm) column, mobile phase: acetonitrile/2-propanol/diethylamine (98/2/0.1 *v*/*v*/*v*), F = 1 mL/min, t = 30 °C.

Using equations based on peak areas from chromatograms of (*R*,*S*)-atenolol and its acetylated forms, it was possible to determine the optical purity of both substrates and products as well as the enantioselectivity of the enzyme-catalyzed biotransformation that was carried out.

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

In a 20 mL glass flask, enantioselective biotransformation of racemic atenolol was performed. The reaction mixture contained (*R*,*S*)-atenolol (3.0 mg, 0.01 mM) dissolved in 0.5 mL of chosen ionic liquid put in 10 mL of toluene, which combined, constituted a two-phase reaction medium. Vinyl acetate (2 µL, 0.02 mM) was used as an acetyl donor in the reaction. The ionic liquids [EMIM] [BF_4], [EMIM] [OTf], and [EMIM] [$EtSO_4$] were investigated as part of the investigation. By directly adding 10 mg of native lipase from *Candida rugosa* OF or MY to the previously assembled bioreactor, the enzyme-catalyzed biotransformation of (*R*,*S*)-atenolol was initiated. At 37 °C, the reaction mixture was incubated while being shaken (250 RPM).

The enantioselective biotransformation of (*R*,*S*)-atenolol was monitored using a chiral stationary phase and an UPLC system coupled with a triple quadrupole mass spectrometer in MRM mode. Samples of 30 µL of ionic liquid were collected at predetermined time points every 24 h for 120 h. Next, racemic atenolol and its acetylated forms were extracted from the ionic liquid by vigorous shaking with 500 µL of acetonitrile for 10 min, and after centrifugation and filtration via syringe filters, the prepared samples were placed into the vials and furtherly injected into a UPLC chiral column.

4. Conclusions

The results of the experiment supported the hypothesis that *Candida rugosa* lipases, both OF and MY, can catalyze the enantioselective acetylation of racemic atenolol with the use of vinyl acetate as acetylating agent. It turned out that using two-phase catalytic systems with toluene and ionic liquid, as well as *Candida rugosa* lipase and vinyl acetate, allowed for the production of highly enantioselective parameters. According to the previously published paper related to kinetic resolution of (*R*,*S*)-atenolol, toluene was the most suitable

reaction solvent, whereas the acetylating agent was vinyl acetate or isopropenyl acetate. Nevertheless, all previously published studies emphasized that the solubility of atenolol in toluene is slight [32–34,37,38]. The substrate concentration was at the same level as it is reported herein. The main aim of the performed study was to verify the possibility to perform kinetic resolution of racemic atenolol in a two-phase catalytic system with the use of vinyl acetate. According to available literature, it was decided to test three various ionic liquids, e.g.,: [EMIM] [BF_4], [EMIM] [OTf], and [EMIM] [$EtSO_4$] [39–44]. The used ionic liquids, however, displayed a variety of kinetic characteristics, leading to varying enantioselectivities and enantiomeric excesses of substrates and products. According to a previous report, enzymatic transesterification is inhibited by the direct addition of [EMIM] [$EtSO_4$] to the reaction mixture, but the results presented here demonstrate that a smooth transesterification reaction caused by the addition of [EMIM] [$EtSO_4$] produced the highest values of conversion [29]. However, the E-value dramatically decreased, making it impossible to regard this reaction system as enantioselective. Additionally, given that this IL may function as an esterification catalyst, this result may suggest that the reaction carried out in the catalytic system including [EMIM] [$EtSO_4$] is non-enzymatic.

Although the native *Candia rugosa* lipase OF in a system including [EMIM] [BF_4] produced the best results among all evaluated catalytic systems (E = 57.22, ee_p = 93.76%), the obtained results were comparable to the results obtained without the addition of ionic liquid (E = 56.99, ee_p = 93.60%). Nevertheless, the usage of the tested ionic liquids provided the opportunity to remove substrates and products from the enzyme's catalytic system and reuse it. The conducted investigation shown that both lipases from *Candida rugosa* OF and MY maintained their high operational stabilities and catalytic activity even after five reaction catalytic cycles. It should be emphasized that the two-phase catalytic systems containing ionic liquids could be highly significant from an economic perspective because they permit a direct and significant overall cost reduction of the carried out enzyme-catalyzed biotransformation by the easy separation of substrates and products from the reaction mixture and reuse of the biocatalyst in another reaction. Additionally, it should emphasize that the solubility of atenolol in organic solvents, which are compatible with the biocatalysts such as toluene, is rather low and requires higher volumes of reaction medium, which has a negative impact on the environment. Therefore, the future prospects of the presented study should be referred in seeking new functionality of reaction mixtures, which apart from resulting in obtaining better and more efficient reaction parameters, should also be more "green" and gives the possibility of reusing.

Author Contributions: Conceptualization, J.C. and A.S.; methodology, J.C. and A.S.; investigation, J.C. and A.S.; writing—original draft preparation, J.C.; writing—review and editing, M.P.M.; visualization, J.C.; supervision, M.P.M.; funding acquisition, A.S. All authors have read and agreed to the published version of the manuscript.

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