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Enantioseparation of (*RS*)-atenolol with the use of lipases immobilized onto new-synthesized magnetic nanoparticles

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ABSTRACT

The enzymatic method was used for the direct resolution of racemic atenolol. The catalytic activities of commercially available lipases from *Candida rugosa* (MY and OF) immobilized onto new-synthesized chitosan magnetic nanoparticles [Fe_3O_4-CS-Et$(NH_2)_2$, Fe_3O_4-CS-Et$(NH_2)_3$] in the kinetic resolution of racemic atenolol were compared. The best results were obtained by using *Candida rugosa* lipase OF immobilized onto Fe_3O_4-CS-Et$(NH_2)_3$. Additionally, the enzyme reusability was investigated. It was established that even after 5 reaction cycles, both lipases from *Candida rugosa* maintained their high catalytic activities and operational stabilities. This approach is extremely important from an economical point of view, because it allows for a direct cost reduction of the biotransformation.

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1. Introduction

β-Blockers are drugs, which play an important role in the treatment of many human diseases. Atenolol is an amino-alcohol used for the treatment of hypertension, arrhythmia problem and angina pectoris.[1,2] Since β-blockers possess an asymmetric carbon atom in their structure, they have two enantiomeric forms. Although, it has been proven by many studies that only the (*S*)-enantiomers of the mentioned drugs have the therapeutic effects, β-blockers are still administrated to patients as racemates. Replacing racemic drugs with enantiomerically pure compounds might develop lesser side effects. For this reason, there are many strategies or chemical syntheses that can be used to obtain enantiomerically pure products, including the (*R*)- and (*S*)-enantiomers of the β-blockers.[3–8]

The use of enzymes in organic biotransformations has become an interesting area for scientists. Hydrolases are some of the most frequently used enzymes, because of their broad substrate spectrum and considerable stability. Lipases classified as hydrolyses are ubiquitous enzymes, which catalyse enantioselective biotransformations, e.g. esterifications, transesterification and hydrolysis to give enantiomerically pure compounds.[9–12] Additionally, lipases do not require any cofactors, and maintain their operational stability and catalytic activity both in aqueous and/or organic media. These enzymes are commercially available in free or immobilized forms. However, the use of native enzymes is usually associated with separation issues from reaction mixtures. Additionally, the free enzyme is more sensitive to reaction media as well as temperature. Thus, the application of native enzymes in industry is limited. Nevertheless, in order to make enzyme handling in biotechnological process more convenient, various methods have been employed, including immobilization. One of the greatest advantage of this technique is the possibility of reducing the total cost of the biotransformation with easy removal and transfer of the immobilized enzyme from one reaction mixture to another.[13,14] Furthermore the bonded enzymes are usually characterised by an increased catalytic activity. The immobilization techniques of different enzymes onto various supports have been reported in numerous studies.[15–17]

Magnetic nanoparticles are widely used for the physical and chemical immobilization of various biomolecules, including lipases. Magnetic nanoparticles contain a superparamagnetic core. Therefore, their application as enzyme carriers allows for easy separation of bonded biocatalysts from reaction mixtures, by attracting then with an external magnetic field. This approach allows for the enzyme to be used in another catalytic system and thus lower the total cost of biotechnological process. Nevertheless, pure magnetite (Fe_2O_3) has a poor colloidal stability and thus requires stabilization and surface modification. Chitosan is a non-toxic natural polymer, which is capable for shell covering of magnetic nanoparticles.[18,19] Additionally, the chitosan magnetic nanoparticles could be modified, allowing for covalent bonding of various enzymes, which seems to be more effective, due to the stabilization of enzyme conformation. Furthermore, the simplicity of their synthesis and the modification of the commonly large surfaces, have meant that chitosan magnetic nanoparticles have gained a great attention in material chemistry as promising materials for many applications.[20,21]

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Herein the kinetic resolution of (*RS*)-atenolol with the use of two lipases from *Candida rugosa* (OF and MY) was investigated. The applied biocatalysts were immobilized onto two types of not commercially available magnetic nanoparticles, which were synthesized *de novo*. Furthermore, the reusability of the immobilized enzyme was tested, and the high catalytic activity of the enzyme after five reaction cycles was confirmed.

2. Materials and methods

2.1. Chemicals

1,4-Diaminobutane, 2-propanol, acetic acid, acetonitrile, chitosan (low molecular weight), diethylamine, EDC [*N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride], epichlorohydrin, ethanol, ethylenediamine, glutaraldehyde, glycine, iron(II) chloride tetrahydrate, iron(III) chloride hexahydrate, isopropenyl acetate, (*R*)-atenolol, (*RS*)-atenolol, sodium hydroxide, sodium periodate, sulfo-NHS (*N*-hydroxy-sulfosuccinimide sodium salt), toluene were purchased from Sigma–Aldrich Co. (Stainhaim, Germany).

Lipases from *Candida rugosa* (MY and OF) were a gift from Meito Sangyo Co., Ltd (Japan). The water used herein was prepared with the use of a Milli-Q Water Purification System (Millipore, Bedford, MA, USA).

2.2. Instrumentation

The FT-IR Spectrometer model Spectrum Two was purchased from Perkin Elmer, the Inkubator 1000 and Unimax 1010 were purchased from Heidolph; the Refrigerated CentriVap Concentrator was purchased from Labconco; the Shimadzu UPLC-MS/MS system equipped with solvent delivery two pumps LC-30AD combined with gradient systems, degasser model DGU-20A5, an autosampler model SIL-30AC, a column oven model CTO-20AC, UV detector model SPD-M20A and triple quadrupole mass spectrometer detector model LCMS-8030. Chiral column Lux Cellulose-2 (LC-2) cellulose tris(3-chloro-4-methylphenylcarbamate) and Guard Cartridge System model KJO-4282 were purchased from Phenomenex Co.

2.3. Chromatographic conditions

The chromatographic process was carried out at 30 °C. (*RS*)-Atenolol and its acetylated derivatives were baseline separated using a chiral column: Lux Cellulose-2 (4.6 mm × 250 mm × 3 μm). The composition of the mobile phase consisted of acetonitrile/2-propanol/diethylamine in a volumetric ratio of 98/2/0.1. The detection was made with the use of triple quadrupole mass spectrometer operating with a multiple reaction monitoring mode. The transitions of the multiple reaction monitoring mode for atenolol were 267.20 > 256.05; 267.20 > 190.05; 267.20 > 116.10, whereas for atenolol acetate were 309.2 > 158.10; 309.20 > 145.15; 309.20 > 116.10. The enantiomeric purity and enantioselectivity of enantioselective acetylation were determined by using the equations basing on peak areas from chromatogram obtained from chromatographic separation of (*RS*)-atenolol and its acetylated forms.

2.4. Synthesis of magnetic chitosan nanoparticles coated with a modified chitosan with two and three long-distanced free amino groups: Fe_3O_4-CS-Et-$(NH_2)_2$ and Fe_3O_4-CS-Et-$(NH_2)_3$

2.4.1. Magnetic nanoparticles coated with modified chitosan with two long-distanced free amino groups (Fe_3O_4 CS-Et-$(NH_2)_2$)

Chitosan (0.2 g) was mixed and mechanically stirred with a 1% acetic acid solution (20 mL) at room temperature for 20 min. Next, both iron (II) chloride tetrahydrate (0.74 g, 3.75 mmol) and iron(III) chloride hexahydrate (2.02 g, 7.5 mmol) were added to the previously obtained mixture and the resulting solution was chemically precipitated at room temperature by adding dropwise 7 mL of 30% solution of NaOH. A solution of epichlorohydrin (0.2 mL, 2.5 mmol) was then added and the resulting mixture was stirred at 50° for 2 h. After cooling to the room temperature, sodium periodate solution (0.16 g in 2.5 mL of water) was added and the mixture was stirred for 30 min. Next, 20 mL of aqueous solution of ethylenediamine (2.4 g, 40 mmol) were added and the mixture was stirred at room temperature for 2 h. The resulting magnetic was material were recovered from the suspension by applying a magnet, washed five times with deionized water and dried under vacuum at 50 °C for 24 h.

2.4.2. Magnetic nanoparticles coated with modified chitosan with three long-distanced free amino groups (Fe_3O_4 CS-Et $(NH_2)_3$)

Chitosan (0.2 g) was added into 1% acetic acid solution (20 mL) and mechanically stirred at room temperature for 20 min. Next, iron(II) chloride tetrahydrate (0.74 g, 3.75 mmol) as well as iron (III) chloride hexahydrate (2.02 g, 7.5 mmol) were added (1:2 molar ratio) and the resulting solution was chemically precipitated at room temperature by adding dropwise a 30% solution of NaOH (7 mL). To the black mixture that formed was added epichlorohydrin (0.2 mL, 2.5 mmol) and the mixture was stirred at 50° for 2 h. After cooling to room temperature, a solution of sodium periodate (0.16 g in 2.5 mL of water) was added and the mixture was stirred for 30 min. The black precipitate was separated by filtration and washed with deionized water five times. Next, 10 mL of bicarbonate buffer pH – 10 and 10 mL of 5% glutaraldehyde solution were added and the mixture was mechanically stirred at room temperature for 1 h, after which 20 mL of an aqueous solution of ethylenediamine (2.4 g, 40 mmol) were added and the mixture was stirred at room temperature for 2 h. The resulting magnetic material was recovered from the suspension by applying a magnet, washed five times with deionized water and dried under vacuum at 50 °C for 24 h (Fig. 1).

2.5. The immobilization assay of lipases from *Candida rugosa* onto the synthesized magnetic nanoparticles

The enantioselective biocatalysts were covalently bonded to the surface of chitosan magnetic nanoparticles. The resulting amine bond was formatted between the primary amine group of the magnetic nanoparticles and the carboxyl group of the lipase (Fig. 2). The immobilization procedure was performed according to the previously reported methodology, with a few modifications.[12,18,22] In brief, lipases from *Candida rugosa* OF and MY (36.5 mg) were suspended separately in 1.0 mL of 50 mM phosphate buffer (pH 6.4). Next, 2 mg of EDC were dissolved in 50 μL of phosphate buffer and added to each tube with the suspended lipase. The resulted solutions were incubated at 21 °C and shaken for 1 h. Next, 2.4 mg of sulfo-NHS in 50 μL of phosphate buffer (50 mM, pH = 6.4) were added to the mixture containing the lipase and EDC. The solutions were incubated at 21 °C for 1 h and shaken. Next, 50 mg of magnetic nanoparticles (Fe_3O_4-CS-Et-$(NH_2)_2$ and Fe_3O_4-CS-Et-$(NH_2)_3$) were placed into separate 2 mL centrifuge tubes with 50 mM of phosphate buffer (pH 6.4) and sonicated for 10 min. All prepared solutions with lipase–EDC–sulfo-NHS complexes were then transferred into separate centrifuge tubes, along with the previously rinsed chitosan magnetic nanoparticles. The obtained mixtures were incubated at 21 °C for 2 h and shaken at 600 rpm in the thermomixer. Finally, the lipase-immobilized magnetic nanoparticles were washed three times with 50 mM of phosphate buffer (pH 6.4) and were dried overnight at 30 °C. The

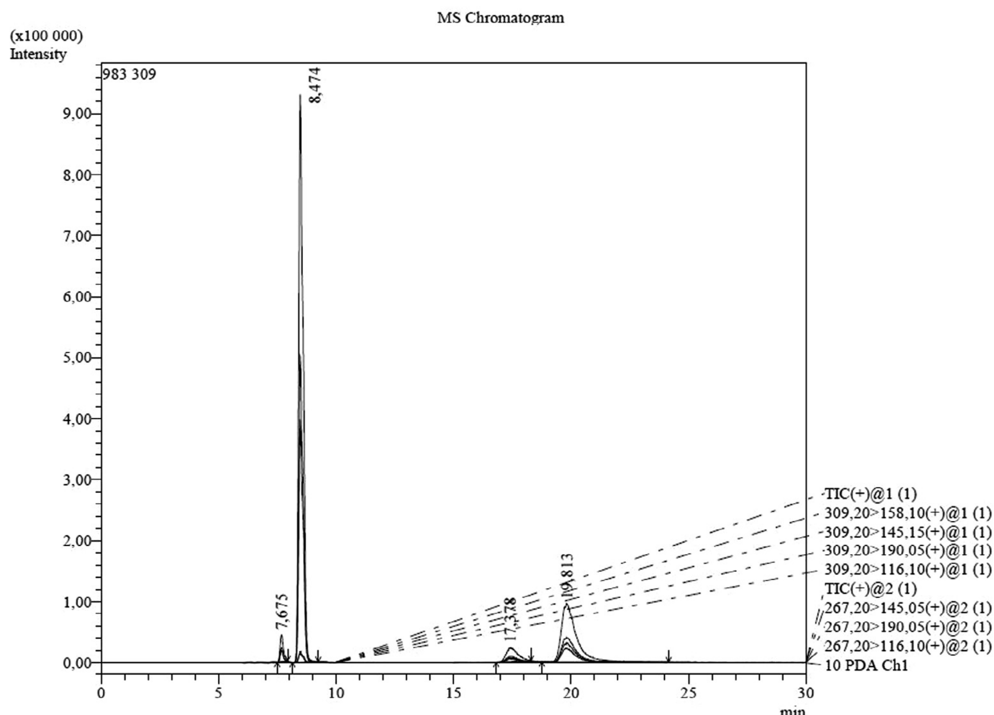

Figure 1. Chromatogram of racemic atenolol and its esters: (*R*)-enantiomer of atenolol acetate (t_R = 7,675), (*S*)-enantiomer of atenolol acetate (t_R = 8,474), (*S*)-atenolol (t_R = 17,378), (*R*)-atenolol (t_R = 19,813). Chromatographic conditions: Lux Cellulose-2 (4.6 × 250 mm × 2 μm) column, mobile phase: acetonitrile/2-propanol/diethylamine (98/2/0.1 v/v/v), *F* = 1 mL/min, *t* = 30 °C.

obtained immobilized lipase on the magnetic supports were used in the enantioselective acetylation of racemic atenolol.

2.6. Kinetic resolution of (*RS*)-atenolol

The kinetic resolution of (*RS*)-atenolol was carried out in 10 mL of reaction medium. The reaction mixture consisted of toluene, racemic atenolol (3.0 mg, 0.01 mM), and isopropenyl acetate (2 μL, 0.018 mM) as the acetyl donor (Fig. 3). The enantioselective biotransformation was started by the direct addition of the lipase immobilized on magnetic supports. The reaction mixture was incubated at 35 °C along with shaking (250 RPM). The enantioselective acetylation was monitored with the use of chiral stationary phases and a UPLC system coupled with triple quadrupole mass spectrometer. The samples were withdrawn at previously established time points and after evaporating and redissolving in pure acetonitrile, were injected on the chiral column of UPLC-MS/MS system.

3. Results and discussion

3.1. Characterization of the magnetic nanoparticles: Fe_3O_4-CS-Et-$(NH_2)_2$ and Fe_3O_4-CS-Et-$(NH_2)_3$, native and immobilized lipase from *Candida rugosa*

The synthesised magnetic chitosan nanoparticles with free amine groups were employed for the immobilization of lipase. The coupling reaction was performed between the amino groups of the magnetic support and the carboxyl groups of the biocatalysts. The structure of the native lipases from *Candida rugosa* (MY and OF), the prepared nanoparticles, and the immobilized biocatalysts onto nanoparticles were characterized with the Attenuated Total Reflectance Fourier Transform Infrared (ATR-FTIR) – Spectrum Two™ (Fig. 4). Spectra were recorded in the range of 4000 to 400 cm^{-1}. The N—H and C═N characteristics vibration peaks at about 1540 cm^{-1} and 1640 were observed for both types of magnetic nanoparticles (Fe_3O_4-CS-Et-$(NH_2)_2$ and Fe_3O_4-CS-Et-$NH_2)_3$. The peaks at 766 cm^{-1}, 860 cm^{-1} and at 1410 cm^{-1} were observed to increase with N—H stretching and N—H wagging vibrations in primary amine groups, however these peaks were not present in the nanoparticles coated with pure chitosan.[19] The signal at 571 cm^{-1} was assigned to the Fe—O group of the magnetite. In the spectrum of the native lipases, the characteristic bands from the carboxylic groups at 1649, 1540 cm^{-1} and 1646, 1543 cm^{-1} for *Candida rugosa* lipase OF and MY, respectively, were observed. After lipase immobilization onto the magnetic nanomaterial, the spectrum showed two peaks at 1639, 1563 cm^{-1} (*Candida rugosa* lipase MY) and 1632, 1651 cm^{-1} (*Candida rugosa lipase* OF), which corresponded to the amide bond formation which directly proves the covalent bonding, which was also reported by numerous studies.[23,20,21]

3.2. Enantioselective acetylation of (*RS*)-atenolol with the use of immobilized lipases from *Candida rugosa* onto magnetic nanoparticles

The enantioselective acetylation of racemic atenolol was performed with the use of lipase from *Candida rugosa* OF and MY covalently bonded to the chitosan magnetic nanoparticles. The prepared magnetic nanomaterials differed from each other in terms of the number of free amine groups on their surface. This resulted in various amount of immobilized lipases. Nevertheless, two commercially available lipases attached to two types of magnetic nanomaterials showed acceptable parameters of performed biotransformation (Tables 1 and 2). In all cases, the conversion value increased with an increase in time. However, among all of the tested reaction systems, the best result was obtained with the use of *Candida rugosa* lipase immobilized onto Fe_3O_4-CS-Et $(NH_2)_3$. After 240 h of incubation, the (*S*)-atenolol acetate was obtained, with the highest enantiomeric excesses of the both

Figure 2. Immobilization of lipases from *Candida rugosa* using EDC and sulfo-NHS onto the surface of chitosan magnetic nanoparticles.

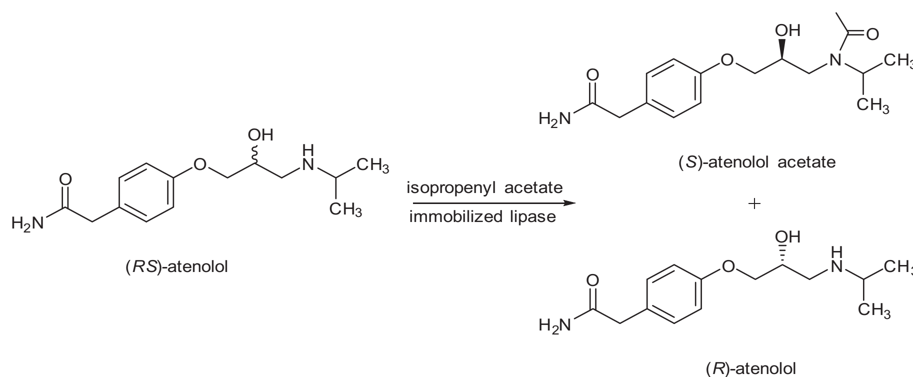

Figure 3. Enantioselective acetylation of racemic atenolol with the use of lipase as biocatalyst.

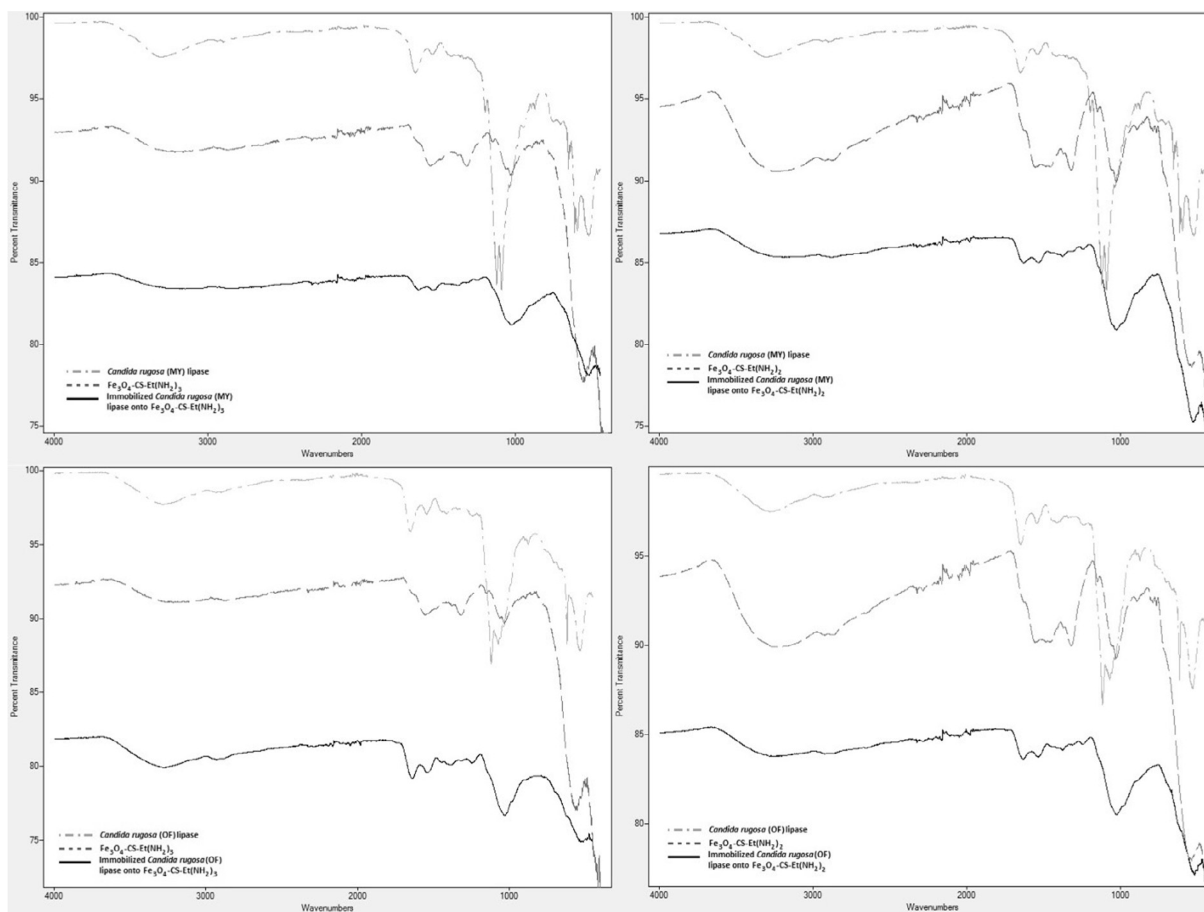

Figure 4. FTIR spectra of the native lipases from *Candida rugosa* (MY and OF), chitosan magnetic nanoparticles (Fe_3O_4-CS-Et$(NH_2)_2$ and Fe_3O_4-CS-Et$(NH_2)_3$), and immobilized lipases onto the magnetic nanoparticles.

product and substrate, which were 93.22% and 70.21%, respectively. The conversion value was c = 42.96%, whereas the enantioselectivity was 59.96%. The same enzyme immobilized onto Fe_3O_4-CS-Et$(NH_2)_2$, after 240 h of incubation of the reaction mixture, allowed for us to obtain the product with ee_p = 91.78%. However, in this case, the E value was 38.30, and the conversion was c = 35.22%. The application of *Candida rugosa* lipase MY resulted in the products being obtained with lower enantiomeric excesses for the two types of magnetic nanomaterials compared to the results obtained with the use of *Candida rugosa* lipase OF. The use of lipase from *Candida rugosa* MY immobilized onto Fe_3O_4-CS-Et$(NH_2)_3$ allowed us to obtain the (S)-atenolol acetate with ee_p = 91.61%, with c = 37.32% and E = 39.47, after 240 h of incubating the reaction mixture. The application of lipase from *Candida rugosa* MY and the Fe_3O_4-CS-Et$(NH_2)_2$ resulted in obtaining worse parameters describing the kinetic of resolution racemic atenolol, similar to when lipase OF immobilized onto two types of magnetic nanoparticles was used.

3.3. Reusability of immobilized lipases from *Candida rugosa* in enantioselective acetylation of (*RS*)-atenolol

The main advantage of using immobilized biocatalysts is the possibility of reusing the enzyme in another catalytic system.

Table 1
Enzymatic parameters including enantioselectivity (E), enantiomeric excesses of both substrate (ee_s) and product (ee_p) and conversion (c) of the enantioselective acetylation of (*R*,*S*)-atenolol with the use of lipase from *Candida rugosa* OF

	Reaction time [h]	E	ees %	eep %	*c* %
Lipase from *Candida rugosa* OF immobilized onto Fe_3O_4-CS-Et(NH_2)$_3$	24	34.19	16.99	93.32	15.40
	48	34.87	25.35	92.92	21.43
	72	34.52	31.53	92.42	25.44
	96	37.08	39.48	92.36	29.95
	120	38.20	43.35	92.29	31.96
	144	39.41	48.03	92.15	34.26
	168	41.81	55.27	92.00	37.53
	192	46.52	61.35	92.26	39.94
	216	56.64	67.53	93.09	42.04
	240	59.96	70.21	93.22	42.96
Lipase from *Candida rugosa* OF immobilized onto Fe_3O_4-CS-Et(NH_2)$_2$	24	27.68	15.23	91.95	14.21
	48	27.75	18.01	91.76	16.40
	72	28.23	21.85	91.60	19.26
	96	29.39	25.50	91.64	21.77
	120	33.37	33.50	92.02	26.69
	144	34.79	38.74	91.94	29.65
	168	31.54	40.64	90.98	30.88
	192	31.34	43.50	90.67	32.42
	216	37.26	47.30	91.78	34.01
	240	38.30	49.89	91.78	35.22

Table 2
Enzymatic parameters including enantioselectivity (E), enantiomeric excesses of both substrate (ee_s) and product (ee_p) and conversion (c) of enantioselective acetylation of (*R,S*)-atenolol with the use of lipase from *Candida rugosa* MY

	Reaction time [h]	E	ees %	eep %	*c* %
Lipase from *Candida rugosa* OF immobilized onto Fe_3O_4-CS-Et(NH_2)$_3$	24	19.78	10.20	89.41	10.24
	48	19.99	14.74	89.07	14.20
	72	19.71	16.64	88.73	15.79
	96	20.52	21.46	88.67	19.47
	120	22.79	25.47	89.35	22.18
	144	23.88	33.48	89.03	27.33
	168	25.34	40.89	88.87	31.51
	192	32.05	46.94	90.55	34.14
	216	33.93	52.10	90.56	36.52
	240	39.46	54.56	91.61	37.33
Lipase from *Candida rugosa* MY immobilized onto Fe_3O_4-CS-Et(NH_2)$_2$	24	17.89	13.18	88.04	13.02
	48	17.93	15.79	87.78	15.24
	72	18.66	16.54	88.14	15.80
	96	19.13	18.03	88.26	16.96
	120	18.02	18.25	87.56	17.25
	144	18.07	20.92	87.29	19.33
	168	21.79	27.30	88.70	23.53
	192	22.85	33.10	88.61	27.19
	216	25.54	37.49	89.31	29.57
	240	30.17	47.88	89.89	34.75

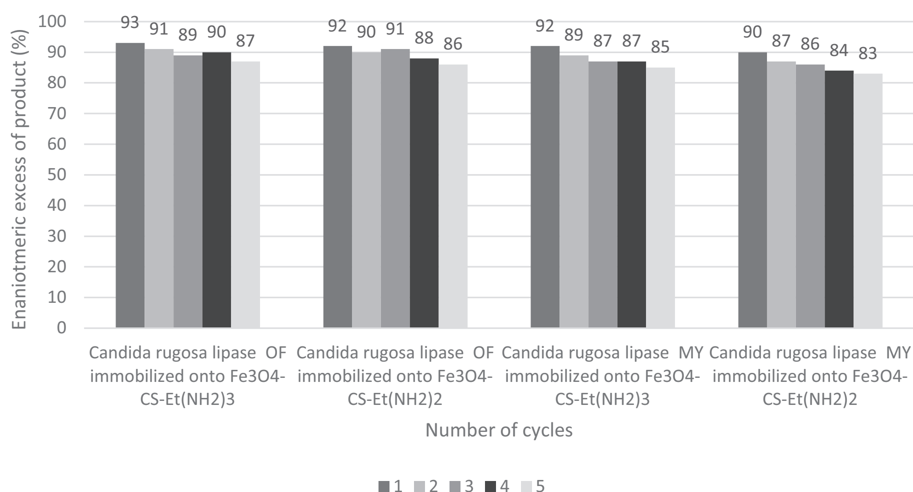

Figure 5. Reusability of the immobilized lipases from *Candida ruogosa* in enantioselective acetylation of (*RS*)-atenolol.

The influence of the reusability of immobilized lipases on the kinetic resolution of racemic atenolol was investigated. For this purpose, the bonded lipases from *Candida rugosa* OF and MY were reused after the specified washing procedure. The complexes of the magnetic nanoparticles and the biocatalysts after the reaction cycle were recovered from the reaction mixture by external magnetic fields and washed with pure toluene. The immobilized lipases were then dried overnight and placed into new reaction mixtures to perform the next catalytic cycle. After 5 reaction cycles, the enantiomeric excesses of products of all tested reaction mixtures were higher than 90% of the initial value (Fig. 5). The highest value of enantiomeric excess was obtained with the use of the lipase from *Candida rugosa* OF immobilized onto Fe_3O_4-CS-Et$(NH_2)_2$. Nevertheless, the differences of catalytic activity of lipases coupled with the two types of magnetic nanoparticles after 5 reaction cycles were not significant. Additionally, it should be stressed that 5 reaction cycles were 1200 h of operational stability of used enzymes. Thus, the obtained results demonstrated that immobilization procedure and the new-synthesised chitosan magnetic nanoparticles allowed us to enhance the stability and catalytic activity of the exploited lipases.

4. Conclusions

Herein the enantioselective acetylation of racemic atenolol has been catalysed by lipases from *Candida rugosa*. Two types of new magnetic nanoparticles were used as carriers for the biocatalysts. However, the immobilized enzymes exhibited various kinetic properties, which resulted in different enantioselectivities. Among all of the tested catalytic systems, the best result was obtained when using lipase from *Candida rugosa* OF immobilized onto Fe_3O_4-CS-Et$(NH_2)_3$ ($E = 59.96$, $c = 42.96\%$, eep = 93.22%). Additionally, the enzyme reusability was investigated. Even after 5 reaction cycles, both lipases from *Candida rugosa* maintained high catalytic activities and operational stabilities. This approach is extremely important from an economical point of view, since it allows for a direct cost reduction of the biotransformation.

Acknowledgments

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