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Enantioselective acetylation of (*R*,*S*)-atenolol: The use of *Candida rugosa* lipases immobilized onto magnetic chitosan nanoparticles in enzyme-catalyzed biotransformation

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ABSTRACT

This paper describes the enzyme immobilization protocol as well as the enzymatic method for the direct resolution of (*R*,*S*)-atenolol. The used magnetic enzyme carriers possess on their surface new-synthetized chitosan derivatives with free amine groups distanced by ethyl or butyl chain. Additionally the catalytic activity of two types of commercially available lipases from *Candida rugosa* immobilized onto two different magnetic nanoparticles were compared. The highest values of enantioselectivity (E = 66.9), enantiomeric excess of product (ee_p = 94.1%) and conversion (c = 41.84%) were obtained by using lipase from *Candida rugosa* OF immobilized onto Fe_3O_4-CS-EtNH_2. The study confirmed that even after 5 reaction cycles the immobilized lipase maintain its high catalytic activity.

1. Introduction

Nowadays, the use of biotechnology is an alternative approach, which offers more environmentally and economically attractive ways to obtain bioactive compounds [1–3]. Lipases (E.C. 3.1.1.3) are ubiquitous enzymes, widely used in several industrial applications because of their ability to catalyse enantioselective biotransformation [2,4]. In recent years, these enzymes have been useful in organic synthesis and kinetic resolution of racemic compounds, which are well described in numerous papers [1,5–11]. However, lipases as proteins are extremely sensitive to reaction media as well as temperature, which may influence their enantioselectivity and catalytic activity. Additionally, the utilization of a native enzyme from a reaction mixture is another serious issue, particularly in industrial processes, because of the difficulty in its separation, recycling and reuse, which is directly related to high total cost of biotransformation [12,13]. Thus, the application of lipases in the industry is limited. Nevertheless, the process of immobilization of enzyme on the surface of nanoparticles could provide many additional advantages over the use of native lipases, because it does not only allow to reuse the bounded enzyme in another catalytic system but also increases the catalytic activity and operational stability of biocatalysts [14,15], and therefore many immobilization techniques have been investigated.

Magnetic nanoparticles are promising materials for many applications: biomedical, catalytic, analytical and industrial [14,16–18]. The immobilization of enzymes onto a support gives the opportunity for its reusability [19–21]. The application of magnetic nanoparticles as enzyme carriers allows to easily separate the bounded biocatalyst from reaction mixture by attracting with an external magnetic field. It gives the possibility to reuse the enzyme in another catalytic system, since the removal of enzymes from the reaction mixture is easy, it allows for simple product purification [12,13,22,23]. Additionally, magnetic supports based on Fe_3O_4 are usually nontoxic [16]. Due to the simplicity of their synthesis and modification of commonly large surfaces, magnetite (Fe_3O_4) nanoparticles are very popular and have gained a great attention in materials science.

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Atenolol chemically known as 2-(4-(2-hydroxy-3-(propan-2-ylamino)propoxy)phenyl)acetamide is one of the most important β-adrenolytic drug widely used in treatment of hypertension and cardiovascular disorders [24]. Due to the fact, β-blockers possess asymmetric carbon atom in their structure, they are presented in two enantiomeric forms [25,26]. It was reported by many studies, only the *S*-enantiomers of these drugs possess the desired therapeutic effect, whereas the administration of the racemate may cause dangerous side effects such as bronchoconstriction or diabetes [6,27,28]. Nevertheless, β-blockers are still commercially available drugs mainly used in medicine as racemates.

The main aim of the study presented herein was to perform the kinetic resolution of (*R*,*S*)-atenolol with the use of two lipases from *Candida rugosa* (OF and MY) immobilized onto two types of not commercially available magnetic nanoparticles, which were *de novo* synthesised. Additionally, the reusability of immobilized enzyme was investigated, and the high catalytic activity of enzyme after five reaction cycles was confirmed. What is more, the new analytical method with the use of chiral stationary phases and UPLC system coupled with mass spectrometry was suggested in order to determine the quality and quantity of the atenolol enantiomers and its derivatives.

2. Materials and methods

2.1. Chemicals

(*R*,*S*)-atenolol, (*R*)-atenolol, toluene, isopropenyl acetate, 2-propanol, acetonitrile, Iron(II) chloride tetrahydrate, iron(III) chloride hexahydrate, chitosan (low molecular weight), glutaraldehyde, epichlorohydrine, sodium periodate, ethylenediamine, acetic acid, sodium hydroxide, EDC (*N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride), sulfo-NHS (*N*-hydroxy-sulfosuccinimide sodium salt), glycine, acetic acid, ethanol, diethylamine, 1,4-diaminobutane, acetyl chloride were purchased from Sigma-Aldrich Co. (Stainhaim, Germany). Sodium sulphate anhydrous, sodium sulphate decahydrate, molecular sieves 4 Å, ethanol were purchased from POCH S.A. (Gliwice, Poland). Lipases OF and MY from *Candida rugosa* were a gift from Meito Sangyo Co., Ltd. (Japan). Water used in the study was prepared using a Milli-Q Water Purification System (Millipore, Bedford, MA, USA). All incubations were performed at adjusted temperature and number of rotation.

2.2. Instrumentation

The Refrigerated CentriVap Concentrator was purchased from Labconco, the Inkubator 1000 and Unimax 1010 were purchased from Heidolph, the FT-IR Spectrometer model Spectrum Two was purchased from Perkin Elmer, the Shimadzu UPLC–MS/MS system (Japan) 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 LCMS-8030. Lux Cellulose-2 (LC-2) column with cellulose tris(3-chloro-4-methylphenylcarbamate) stationary phase and Guard Cartridge System model KJO-4282 were purchased from Phenomenex Co.

2.3. Chromatographic conditions

The most appropriate chromatographic conditions for the resolution of racemic atenolol and its acetylated forms were optimized with Lux Cellulose-2 (4.6 mm × 250 mm × 3 μm) HPLC column, which was chosen for the chromatographic separation in polar/organic phase mode (Fig. 1). The mobile phase consisted of acetonitrile/2-propanol/diethylamine in ratio 98/2/0.1 (v/v/v). The chromatographic process was operated at 30 °C. The detection was made using triple quadrupole mass spectrometer in multiple reaction monitoring mode (MRM). The transitions of MRM 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. In order to determine optical purity and enantioselectivity of enantioselective acetylation, the equations were used basing on peak areas from chromatogram achieved in chromatographic separation of (*R*,*S*)-atenolol and its acetylated forms. The percentage enantiomeric excesses of the substrate (ee_s) and product (ee_p), conversion (c) as well as enantioselectivity (E) were calculated, as below [11]:

$$ee_s = \frac{|R-S|}{R+S} \times 100\%$$

$$ee_p = \frac{|R-S|}{R+S} \times 100\%$$

$$c = \frac{ee_s}{ee_s + ee_p} \times 100\%$$

$$E = \frac{\ln\left[(1-c)\left(1+ee_p\right)\right]}{\ln\left[(1-c)\left(1-ee_p\right)\right]}$$

where R was values of peak areas for (*R*)-atenolol and its ester, whereas S was values of peak areas for (*S*)-atenolol and its ester.

2.4. Synthesis of magnetic chitosan nanoparticles: Fe_3O_4-CS-$EtNH_2$ and Fe_3O_4-CS-$BuNH_2$

Chitosan (0.2 g) was added into 20 mL of 1% acetic acid solution and mechanically stirred at room temperature for 20 min. After that, iron(II) chloride tetrahydrate (0.74 g, 3.75 mmol) and 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 30% solution of NaOH (7 mL). The black mixture was formed, separated by filtration and washed by deionized water for five times. Next, 10 mL of bicarbonate buffer pH – 10 and 10 mL of 5% glutaraldehyde solution were added and the composed mixture was mechanically stirred at room temperature for 1 h. In order to differentiate the synthesis between the Fe_3O_4-CS-$EtNH_2$ and Fe_3O_4-CS-$BuNH_2$ nanoparticles, the 20 mL of aqueous solution of ethylenediamine (2.4 g, 40 mmol) or 1,4-diaminobutane (3.53 g, 40 mmol), respectively, was added and the mixtures were stirred at room temperature for 2 h. The obtained magnetic materials 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 (Fig. 2).

2.5. Lipase immobilization onto chitosan magnetic nanoparticles with the use of EDC and sulfo-NHS

The covalent coupling of *Candida rugosa* lipase onto the surface of chitosan magnetic nanoparticles was performed by the formation of an amide bond between the carboxyl group of lipase and the primary amine group of the nanoparticle. The preparation procedure was performed according to the previously reported methodology [16,19], with few modifications (Fig. 3). In brief, the 36.5 mg of lipase OF and MY were suspended separately in 1.0 mL of 50 mM phosphate buffer (pH 6.4). After that, 2 mg of EDC in 50 μL of phosphate buffer was added to each tube with lipase suspension. The solutions were incubated at 21 °C and shaken for 1 h. After that time, 2.4 mg of sulfo-NHS was dissolved in 50 μL of phosphate buffer (50 mM, pH = 6.4) and added to each of the

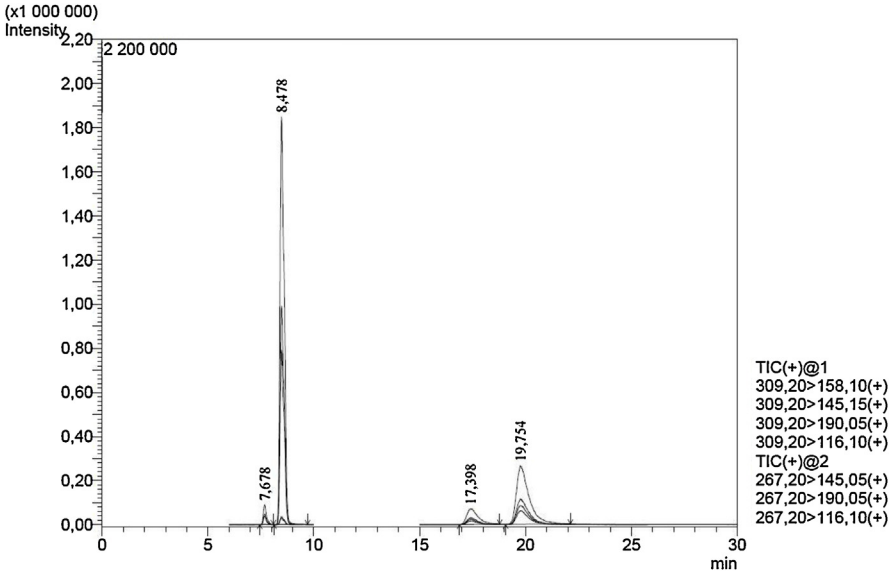

Fig. 1. Chromatogram of racemic atenolol and its esters after 240 h of kinetic resolution of (*R*,*S*)-atenolol with the use of *Candida rugosa* lipase OF immobilized on Fe_3O_4-CS-$EtNH_2$: (*R*)-enantiomer of atenolol acetate (t_R = 7.678), (*S*)-enantiomer of atenolol acetate (t_R = 8478), (*S*)-atenolol (t_R = 17.398), (*R*)-atenolol (t_R = 19.754). 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.

tube containing lipase and EDC. The solutions were also incubated at 21 °C for 1 h and shaken. Further, 50 mg of chitosan magnetic nanoparticles (Fe_3O_4-CS-$EtNH_2$ and Fe_3O_4-CS-$BuNH_2$) were placed into separate 2 mL centrifuge tubes and sonicated for 10 min with 50 mM phosphate buffer (pH 6.4). Then, all prepared solutions with lipase-EDC-sulfo-NHS complex were transferred into separate centrifuge tubes along with the previously rinsed chitosan magnetic nanoparticles. Next, the resulting mixtures were incubated at 21 °C for 2 h and shaken at 600 rpm in a thermomixer. Finally, lipase-immobilized chitosan nanoparticles were rinsed three times with 0.5 mL of 50 mM phosphate buffer (pH 6.4) and were dried overnight at 30 °C. The resulted lipase-immobilized magnetic supports were used in the enantioselective acetylation. The amount of immobilized lipase adsorbed onto the magnetic nanoparticles was determined by measuring the initial concentration of lipase and its final concentration in supernatant after immobilization using the Bradford protein assay method. A calibration curve was constructed with lipase OF and MY suspended in 50 mM phosphate buffer of known concentration (1–9 mg/mL) and then was used in the calculation of protein in the initial solution and supernatant.

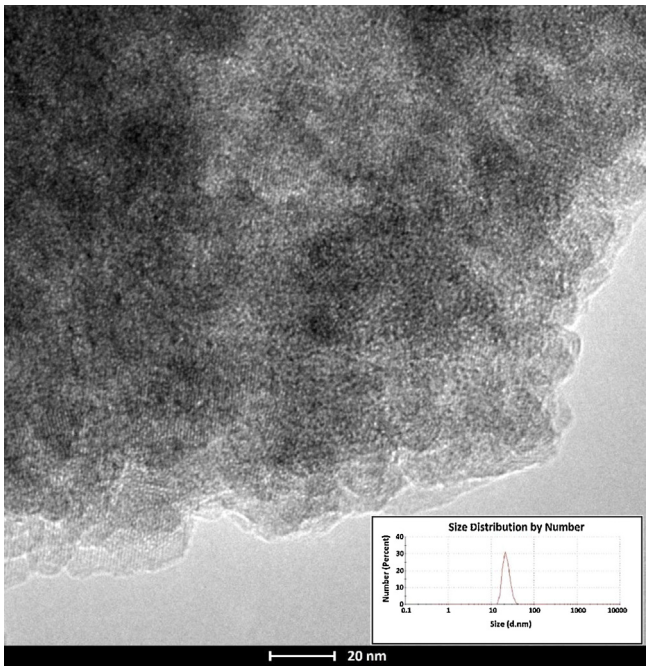

Fig. 2. TEM image of chitosan magnetic nanoparticles – Fe_3O_4-CS-$EtNH_2$.

2.6. Chemical acetylation of (R,S)-atenolol

The (*R*,*S*)-atenolol was acetylated according to the reported methodology with few modifications. Briefly, (*R*,*S*)-atenolol (0.02 g; 0.075 mmol) was refluxed with dichloromethane (20 mL) and acetyl chloride (8 μL; 0.076 mmol) was very slowly added. After that, the reaction mixture was incubated at 30 °C for 2 h, and further successively washed with equal volumes of saturated aqueous sodium bicarbonate and brine. The organic layer was collected and evaporated to dryness under vacuum to afford atenolol acetate. Finally, resulted derivate of atenolol was used as standard in order to establish optimal chromatographic method which allowed to determine quantitatively racemic atenolol and its acetylated form.

2.7. Enzymatic acetylation of (R,S)-atenolol

Enzymatic acetylation of (*R*,*S*)-atenolol was performed in 25 mL flasks. The reaction was carried out in 10 mL of reaction medium. The reaction mixture was composed of toluene, racemic atenolol (3.0 mg, 0.01 mM) and isopropenyl acetate (2 μL, 0.018 mM) as acetyl donor (Fig. 4). The enzymatic acetylation was started by the direct addition of immobilized lipase. The reaction mixture was shaken at 250 rpm at 35 °C. The process of enantioselective acetylation was monitored with the use of chiral stationary phases. The samples were withdrawn at established time points (0, 24, 48, 72, 96, 120, 144, 168, 192, 214, 240 h) and after that were evaporated and redissolved in acetonitrile and after filtration injected on the UPLC–MS/MS system.

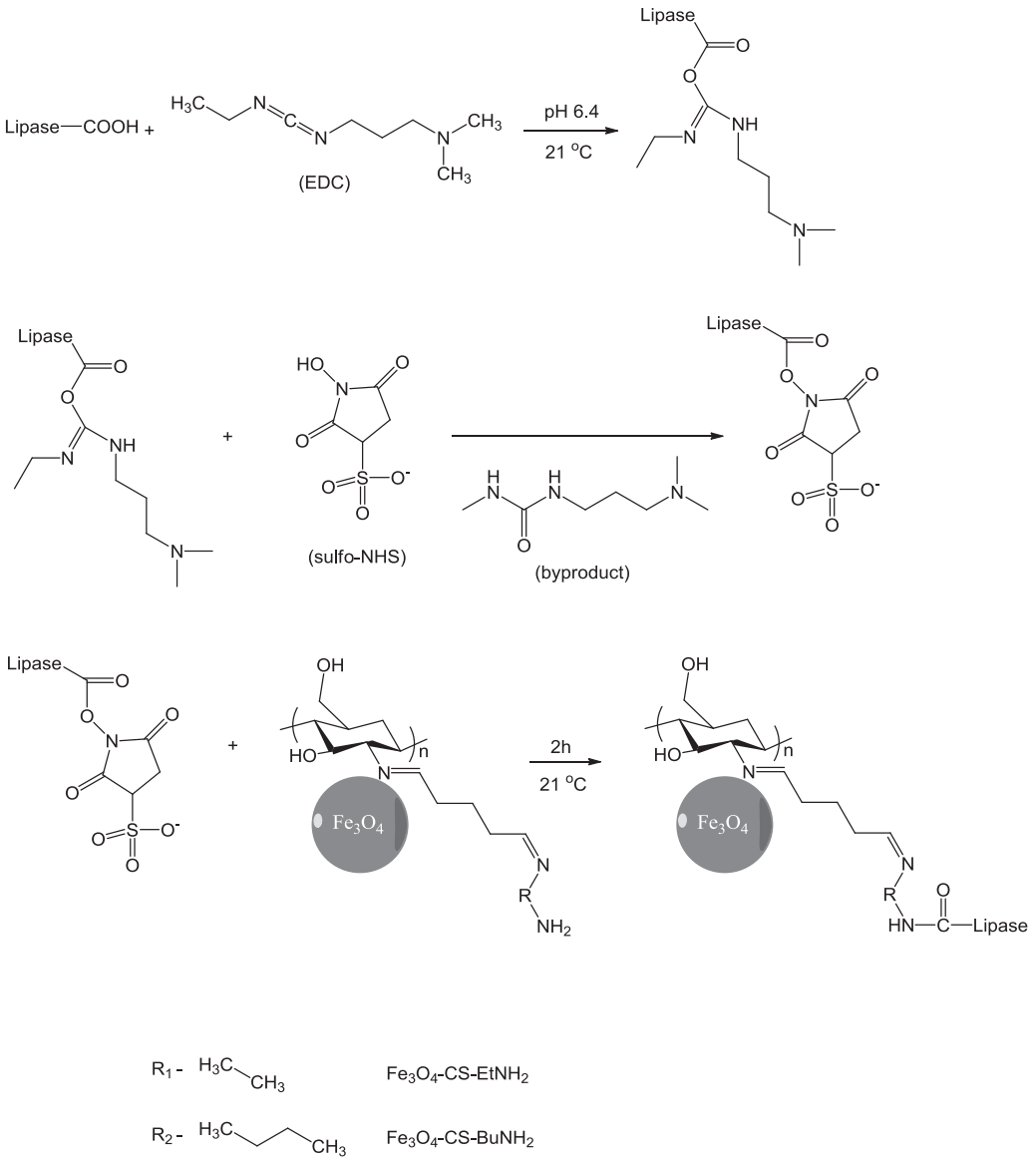

Fig. 3. Immobilization of lipases from *Candida rugosa* using EDC and sulfo-NHS onto the surface of chitosan magnetic nanoparticles.

3. Results and discussion

3.1. Characterization of the native and immobilized lipase from Candida rugosa as well as magnetic chitosan nanoparticles: Fe_3O_4-CS-$EtNH_2$ and Fe_3O_4-CS-$BuNH_2$

In presented study, the magnetic chitosan nanoparticles with amine groups were employed for the immobilization of lipase using coupling reaction between the amino groups of the magnetic support and carboxyl groups of the biocatalysts. The structure of the prepared nanoparticles, native lipases from *Candida rugosa* (OF and MY), and immobilized biocatalysts onto nanoparticles were characterized with the Attenuated Total Reflectance Fourier Transform Infrared (ATR-FTIR) – Spectrum Two™ (Perkin Elmer). Spectra were recorded on over the region from 4000 to 400 cm^{-1} (Fig. 5). The C=N and N—H characteristics vibration peaks at about 1640 and 1540 cm^{-1} were observed for both magnetic chitosan nanoparticles (Fe_3O_4-CS-$EtNH_2$ and Fe_3O_4-CS-$BuNH_2$). The peaks at 1410 cm^{-1} and at 860 cm^{-1} as well as 766 cm^{-1} increased with N—H stretching and N—H wagging vibrations in primary amine groups. These peaks were not observed in nanoparticles coated with pure chitosan [29]. The signal at 571 cm^{-1} was assigned to the Fe—O group of magnetite. On pure lipase spectrum the characteristic band from carboxylic groups at 1649, 1540 cm^{-1} and 1646, 1543 cm^{-1} for *Candida rugosa* lipase OF and MY, respectively, were

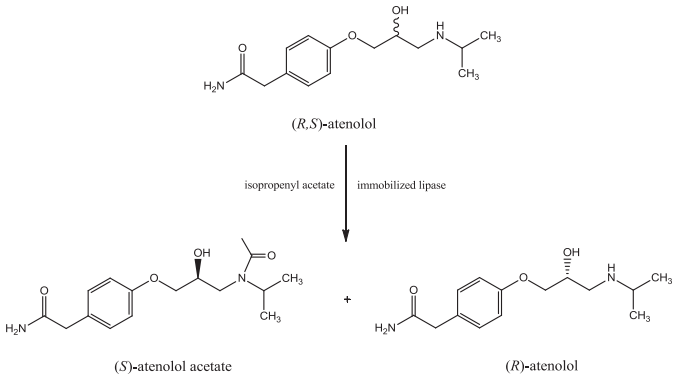

Fig. 4. Enantioselective acetylation of racemic atenolol with the use of immobilized lipase as biocatalyst.

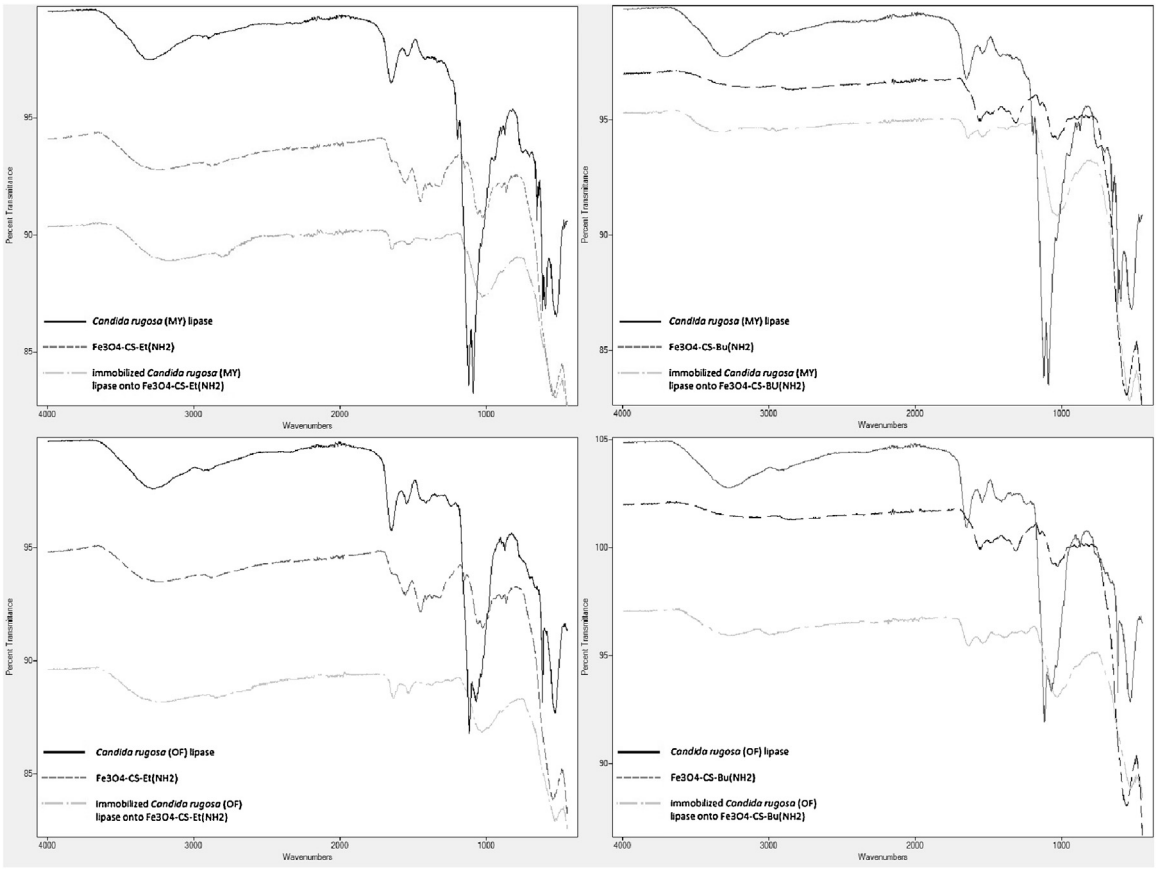

Fig. 5. FTIR spectra of the native lipases from *Candida rugosa* (MY and OF), chitosan magnetic nanoparticles (Fe_3O_4-CS-Et(NH_2) and Fe_3O_4-CS-Bu(NH_2)), and immobilized lipases onto the magnetic nanoparticles.

observed. After lipase immobilization the spectrum showed only two weak peaks at 1632, 1651 cm^{-1} (*Candida rugosa lipase* OF), and 1639, 1563 cm^{-1} (*Candida rugosa* lipase MY) corresponding to the characteristic amide bond formation which proves the covalent lipase bonding, which was previously reported by numerous studies [19,30,31].

3.2. *Application of immobilized* Candida rugosa *lipase onto chitosan nanoparticles*

Two commercially available lipases from *Candida rugosa* OF and MY were coupled to surface of two types of chitosan nanoparticles and further used in enantioselective acetylation of (*R*,*S*)-atenolol. Due to the dissimilar nanomaterials and different type of lipases, both enzymes showed different effects on the kinetic resolution of racemic atenolol. As it is shown in the tables the application of two enzymes preparation showed acceptable parameters of performed reaction. However, the use of lipase from *Candida rugosa* OF (Table 1) allowed to obtain the product with higher enantiomeric excess compared to the result with the use of *Candida rugosa* MY lipase (Table 2), both on Fe_3O_4-CS-EtNH_2 as well as Fe_3O_4-CS-BuNH_2. On the other side, the immobilization of lipases onto Fe_3O_4-CS-EtNH_2 as the enzyme support resulted in better enzyme activity which is described by conversion. The conversion of enantioselective acetylation of racemic atenolol is higher for both lipases immobilized on Fe_3O_4-CS-EtNH_2 compared to Fe_3O_4-CS-BuNH_2. On the other hand, the enantiomeric excesses of products were similar while the same type of enzyme coupled with different chitosan magnetic nanoparticles is considered. The main difference of ee_p value was observed, while the results

Table 1
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* OF.

	Reaction time [h]	E	ee_s%	ee_p%	c%
Candida rugosa lipase OF immobilized onto Fe_3O_4-CS-EtNH_2	24	48.76	18.14	95.22	16
	48	37.01	19.69	93.66	17.37
	72	34.78	23.54	93.02	20.2
	96	27.13	25.2	91	21.69
	120	45.48	30.64	94.24	24.54
	144	43.5	33.99	93.79	26.6
	168	58.51	39.74	95.07	29.48
	192	53.68	42.11	94.52	30.82
	216	65.17	52.41	94.95	35.57
	240	66.9	67.71	94.1	41.84
Candida rugosa lipase OF immobilized onto Fe_3O_4-CS-BuNH_2	24	20.06	10.5	89.52	10.49
	48	22.84	10.87	90.71	10.7
	72	28.82	10.33	92.6	10.03
	96	36.32	10.66	94.07	10.18
	120	32.16	10.91	93.31	10.47
	144	30.46	11.09	92.94	10.66
	168	29.29	11.29	92.65	10.86
	192	27.96	11.53	92.3	11.11
	216	33.55	11.89	93.52	11.28
	240	34.04	13.39	93.52	12.53

obtained with the use of lipase from *Candida rugosa* OF and MY were compared. Nevertheless, after 240 h of the reaction the highest values of enantioselectivity (E = 66.9), conversion (c = 41.84%) and both enantiomeric excesses of substrate (ee_s = 67.71%) and product (ee_p = 94.10%) were observed with the use of *Candida rugosa* OF lipase immobilized onto Fe_3O_4-CS-EtNH_2.

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	ee_s%	ee_p%	c%
Candida rugosa lipase MY immobilized onto Fe_3O_4-CS-$EtNH_2$	24	18.7	8.74	88.98	8.94
	48	13.87	10.22	85.23	10.71
	72	12.91	9.97	84.25	10.59
	96	12.34	10.06	83.58	10.74
	120	20.51	11.82	89.61	11.66
	144	23.44	15.25	90.56	14.41
	168	20.61	17.21	89.14	16.18
	192	19.47	22.92	87.93	20.68
	216	23.23	30.77	89.02	25.69
	240	34.96	45.5	91.42	33.23
Candida rugosa lipase MY immobilized onto Fe_3O_4-CS-$BuNH_2$	24	8.81	2.18	79.22	2.67
	48	13.17	2.92	85.5	3.3
	72	15.41	3.47	87.41	3.82
	96	19.85	3.94	90.04	4.2
	120	16.66	4.11	87.87	4.45
	144	18.24	4.2	89.26	4.49
	168	13.72	4.3	85.86	4.77
	192	15.06	4.94	86.96	5.38
	216	18.07	5.3	88.98	5.62
	240	22.11	5.68	90.87	5.88

3.3. Effect of reaction temperature on kinetic resolution of (R,S)-atenolol

The temperature plays main role in enantioselective biotransformation of chiral compounds with the use of enzymes as catalysts. The influence of temperature (25–45 °C) on the enantioselective acetylation of (*R*,*S*)-atenolol with the use of *Candida rugosa* lipase OF and MY immobilized onto Fe_3O_4-CS-$EtNH_2$ as well as Fe_3O_4-CS-$BuNH_2$ was investigated (Fig. 6). Generally, when the temperature reached 45 °C the values of conversion, enantiomeric excesses of substrate and product as well as enantioselectivity of all performed reactions were the lowest. The enantiomeric excesses of product were the highest for all reactions thermostated at 25 °C, however their values are not significantly higher compared to the reaction performed at 35 °C. Additionally, the enantioselectivity of mentioned reaction was the highest at 35 °C (E = 66.9), whereas at 25 °C and 45 °C the values were E = 34.0 and E = 12.9, respectively. The application of *Candida rugosa* MY lipase immobilized onto Fe_3O_4-CS-$BuNH_2$ in reaction performed at 25 °C resulted in obtaining the conversion value almost two times greater comparing to the reaction incubated at 35 °C as well as 45 °C. Nevertheless, the most significant changes were observed in case of lipase from *Candida rugosa* OF immobilized onto Fe_3O_4-CS-$EtNH_2$. The conversion increased rapidly from 7.63% to 41.84% with the increase of temperature from 25 °C to 35 °C, however, with further temperature rise up to 45 °C the conversion value decreased to 5.74% which was probably related with the partially enzyme destruction, due to the high temperature.

3.4. Influence of reused immobilized lipases on kinetic resolution of (R,S)-atenolol

The ability to reuse the immobilized enzyme in the next catalytic system is extremely important from economical point of view. In order to investigate the efficiency of immobilized lipases OF and MY the same nanoparticles were reused after the specified washing procedure. After the first and further reaction cycles of the enantioselective acetylation of (*R*,*S*)-atenolol the immobilized enzyme was recovered from the reaction mixture with the use of magnet and washed 3 times with toluene. Next, the lipase-coated magnetic nanoparticles were dried overnight in order to remove organic solvent, and further were placed into new reaction system. The study was repeated up to five reaction cycles with the same immobilized lipase. The reaction parameters of the performed reactions were determined. During the five cycles of the kinetic resolution of (*R*,*S*)-atenolol slight changes of the enantioselectivity expressed as enantiomeric excess of product were observed. However, the obtained results demonstrated that the performed immobilization procedure is efficient and allowed to maintain the stability and catalytic activity of used lipases after 5 reaction cycles (Fig. 7).

4. Conclusions

The process of enzyme immobilization allows to reuse the bounded enzyme in another catalytic system, which is extremely important from economical point of view. Additionally, it increases

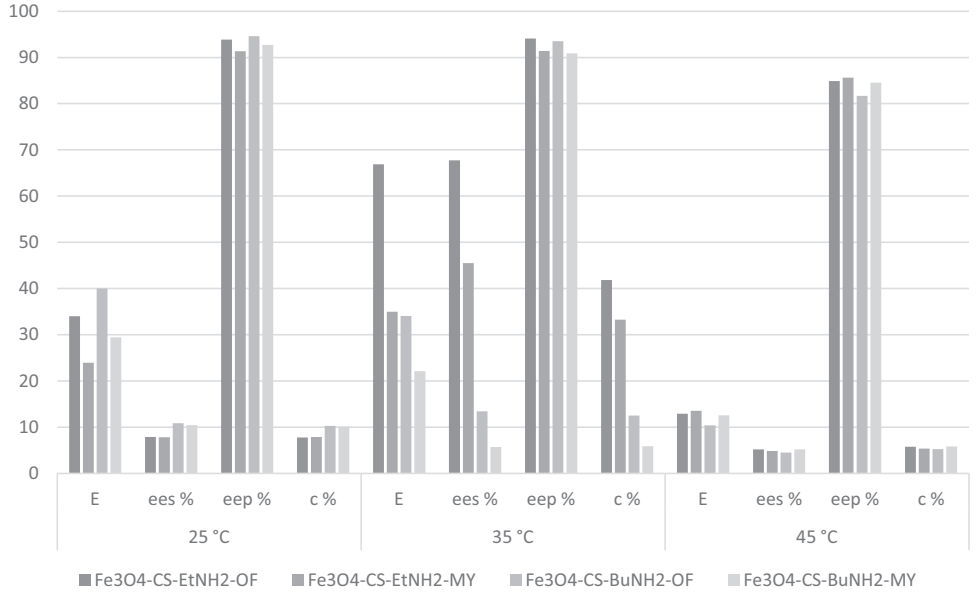

Fig. 6. Enzymatic parameters including enantioselectivity (E), enantiomeric excesses of both substrate (ee_s) and product (ee_p) as well as conversion (c) of enantioselective acetylation of (*R*,*S*)-atenolol performed in different reaction temperatures.

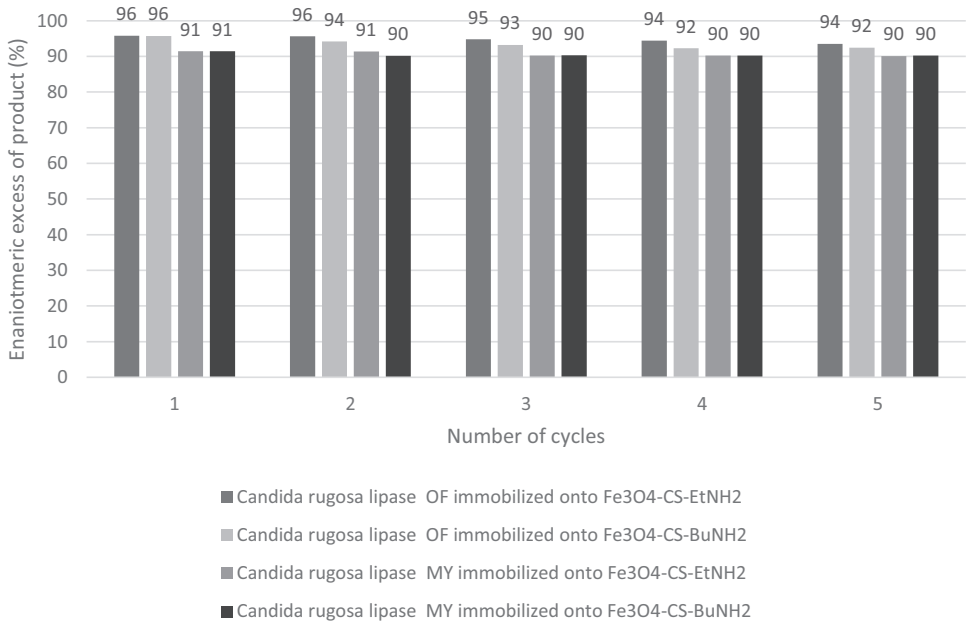

Fig. 7. Influence of reused immobilized lipases from *Candida rugosa* onto magnetic nanoparticles on the kinetic resolution of (*R,S*)-atenolol.

the catalytic activity and operational stability of biocatalysts, and therefore many immobilization techniques have been investigated [32–34]. The performed study proved, that the magnetic nanoparticles, with new-synthetized chitosan derivatives possessing free amine group on their surface distanced by ethyl or butyl group could be used as enzyme carries. The reported results confirmed that two commercially available lipases from *Candida rugosa* OF and MY immobilized onto new-synthesised chitosan magnetic nanoparticles have different effects on the kinetic resolution of (*R,S*)-atenolol. Among all tested catalytic systems the one, which allowed to achieve the highest enantioselectivity (E = 66.9), enantiomeric excess of product (ee_p = 94.1%) and conversion (c = 41.84%) was the system containing lipase from *Candida rugosa* OF immobilized onto Fe_3O_4-CS-$EtNH_2$. Additionally, the presented study demonstrated that, the immobilized lipase could be facilely transferred from one catalytic system to another allowing to reuse the biocatalyst, which is an important advantage from the economic point of view. Thus, the studied magnetic chitosan nanoparticles might be of special importance for the future industrial application of the kinetic resolution of β-adrenolytic drugs.

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