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Enzymatic Kinetic Resolution of (*RS*)-1-(Phenyl)ethanols by *Burkholderia cepacia* Lipase Immobilized on Magnetic Nanoparticles

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A lipase proveniente da *Burkholderia cepacia* imobilizada em nanopartículas superparamagnéticas usando diferentes metodologias de imobilização (adsorção e quimiosorção) foi eficientemente aplicada como biocatalisador reciclável na resolução cinética de (*RS*)-1-(fenil) etanols através de reações de transesterificação. Os (*R*)-ésteres e os (*S*)-alcoóis foram obtidos com excelente excesso enantiomérico (> 99%), o que corresponde a um perfeito processo de resolução cinética enzimática (conversão 50%, *E* > 200). As reações de transesterificação catalisadas pela lipase de *B. cepacia* imobilizada pela metodologia com glutaraldeído apresentaram os melhores resultados em termos de conversão após 8 ciclos de reação.

Lipase from *Burkholderia cepacia* immobilized on superparamagnetic nanoparticles using adsorption and chemisorption methodologies was efficiently applied as recyclable biocatalyst in the enzymatic kinetic resolution of (*RS*)-1-(phenyl)ethanols *via* transesterification reactions. (*R*)-Esters and the remaining (*S*)-alcohols were obtained with excellent enantiomeric excess (> 99%), which corresponds to a perfect process of enzymatic kinetic resolution (conversion 50%, *E* > 200). The transesterification reactions catalysed with *B. cepacia* lipase immobilized by the glutaraldehyde method showed the best results in terms of reusability, preserving the enzyme activity (conversion 50%, *E* > 200) for at least 8 successive cycles.

Keywords: superparamagnetic nanoparticles, lipase, transesterification catalysis, enantiomeric resolution

Introduction

Immobilized lipases have been successfully employed in hydrolysis[1] and *trans*-esterification catalysis, exhibiting high enantioselectivity.[2] Industrial use of such expensive biocatalysts suffers yet from a critical point, which is the lack of efficient enzyme recovery processes.[3] This is actually a general problem in catalysis. For this reason great efforts have been made, pursuing the best immobilization strategies for the enzymes on suitable solid supports.[3] As a very promising alternative, superparamagnetic nanoparticles based on magnetite (γ-Fe_3O_4) can provide outstanding support materials for the enzymes, exhibiting striking characteristics, such as large surface area, mobility and high mass transference. More than this, they can be easily recovered by simple application of an external magnetic field.[4] In addition to

their excellent environmental compatibility, the use of such superparamagnetic supports represents an effective green chemistry approach, allowing to extend, through the successive recovery cycles, the useful lifetime of the biocatalyst. Among the several synthetic routes to prepare iron-oxide superparamagnetic nanoparticles,[4] the most employed one for enzyme immobilization is the co-precipitation methodology which makes use of aqueous Fe^{2+}/Fe^{3+} salt solutions. In addition, we can also mention the use of other magnetic materials[5-25] to immobilize enzymes, such as magnetic metallic nanoparticles, magnetic microspheres prepared from copolymers with magnetic particles, hybrid materials (Fe_3O_4–silica–NiO) and magnetite-containing mesoporous silica spheres. A literature survey revealed that several enzymes including lactase,[5] lipase,[6] esterase,[7] β-galactosidase,[8] oxidase,[9] dehydrogenase,[10] α-chymotripsin,[11] chloroperoxidase,[12] Penicillin G acylase,[13] *L*-asparaginase,[14] tyrosinase,[15] horseradish peroxidase,[16] chitosanase,[17] papain,[18] diastase,[19]

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levansucrase,[20] amylase,[21] streptokinase,[22] dispase,[22] dehalogenase,[23] laccase[24] and epoxide hydrolase[25] have been immobilized onto magnetic particles for different purposes.

In this work, we report on the successful application of the immobilized form of *Burkholderia cepacia* lipase (BCL) on superparamagnetic nanoparticles, exploring three different immobilization methodologies and their influence in the enzymatic kinetic resolution of secondary alcohols.

Results and Discussion

Magnetite (Fe_3O_4) nanoparticles of about 10 nm size, were obtained by the co-precipitation method[26,27] exhibiting a typical superparamagnetic behavior. In order to improve their stability and add reactive amino groups for enzyme immobilization purposes, they were treated with 3-aminopropyltrimethoxysilane (APTS), generating APTS-coated nanoparticles, here referred as APTS-MagNP (Scheme 1).
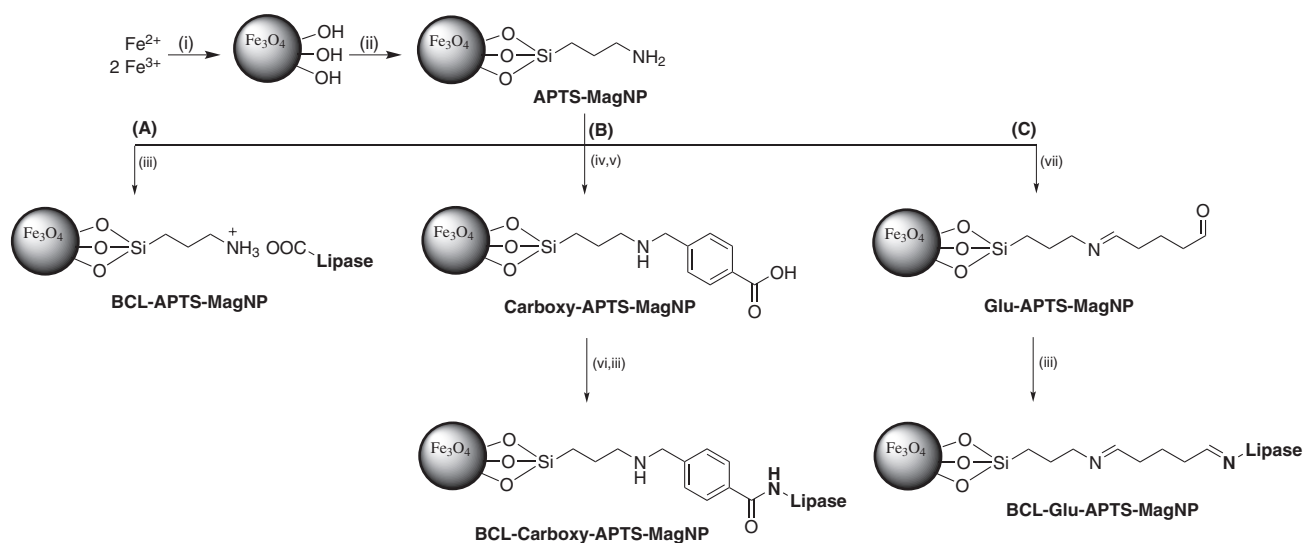
The immobilization of *B. cepacia* lipase on the superparamagnetic nanoparticles was carried out according to three different methods[28] pursuing the best performance in the enzymatic kinetic resolution of secondary alcohols.

The first method consisted in the direct interaction of the enzyme with APTS-MagNP (Scheme 1A). It should be noticed that at pH 7, lipase from *B. cepacia* is negatively charged (isoelectric point = 5.2)[29] while the superparamagnetic nanoparticles exhibit a positive charge due to the protonation of the aliphatic amines (pKa *ca*. 9).[30]
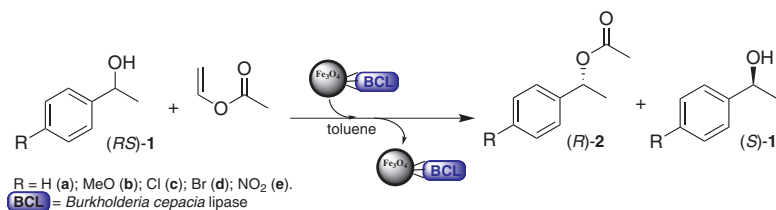
In this way, the APTS shell can act as effective binding site for the lipase protein chains, either *via* hydrogen bonding or electrostatic interactions with the amide and aminoacid residues of the enzyme. Typically, it was employed in the preparations a protein solution (550 µg L^{-1}; 1 mL) for 20 mg of APTS-MagNP. After work-out, the amount of protein immobilized on the superparamagnetic nanoparticles was determined by Bradford method,[31] yielding 0.21 mg protein/20 mg APTS-MagNP.

The second lipase immobilization method consisted in the previous modification of the APTS-functionalized nanoparticles with carboxybenzaldehyde, in order to make the covalent attachment of the lipase (Scheme 1B). In the next step, the amino groups from APTS-MagNP react with the carboxybenzaldehyde, yielding imines, which can be converted into their secondary amines by the reduction with $NaBH_4$. Then, the remaining carboxylic groups from carboxybenzaldehyde moieties can react with EDC (1-ethyl-3-(3-dimethyl aminopropyl) carbodiimide hydrochloride), and finally perform the coupling with the lipase (550 µg L^{-1}). This protocol led to immobilization of 0.23 mg protein on 20 mg Carboxy-APTS-MagNP.

The third immobilization method was based on glutaraldehyde (Glu) as the coupling agent for making the covalent attachment of the lipase enzyme to the APTS-MagNP (Scheme 1C). Glutaraldehyde reacts with the amino group from APTS-MagNP forming imine bonds, leaving the terminal aldehyde group for reacting with the amino residues of the enzyme. In this case, it was possible to bind 0.26 mg protein to 20 mg of Glu-APTS-MagNP.

Scheme 1. Immobilization of *B. cepacia* lipase on superparamagnetic nanoparticles (A = Adsorption method; B = Carboxybenzaldehyde method; C = Glutaraldehyde method); (i) Fe^{2+}/Fe^{3+} oxides, NaOH (0.5 mol L^{-1}), 0.5 h; (ii) (3-aminopropyl)trimethoxysilane, 12 h; (iii) *B. cepacia* lipase (550 µg lipase/mL), 1 h; (iv) 4-carboxybenzaldehyde, 4 h; (v) NaBH$_4$, 1 h; (vi) 1-ethyl-3-(3-dimethyl aminopropyl) carbodiimide hydrochloride, 20 min.; (vii) glutaraldehyde, 2 h.

Scheme 2. Enzymatic resolution of (*RS*)-1-(phenyl)ethanols *via* enantioselective acetylation catalyzed by *B. cepacia* lipase.

Enzyme activity: enzymatic kinetic resolution of (RS)-1-(phenyl)ethanol derivatives

The lipase activity was determined for the enzymatic kinetic resolution of (*RS*)-1-(phenyl)ethanols (**1**) *via* enantioselective acetylation, yielding the corresponding esters (**2**) (Scheme 2).

In the control experiments, a sample of *B. cepacia* lipase solution, employed in the immobilization studies, was lyophilized and used in the enzymatic resolution of (*RS*)-1-(phenyl)ethanol and (*RS*)-1-(4-nitro-phenyl)ethanol. Typical results are shown in Table 1, and can be taken as reference for comparison purposes with respect to the catalytic activity of the immobilized enzyme (Figure 1).
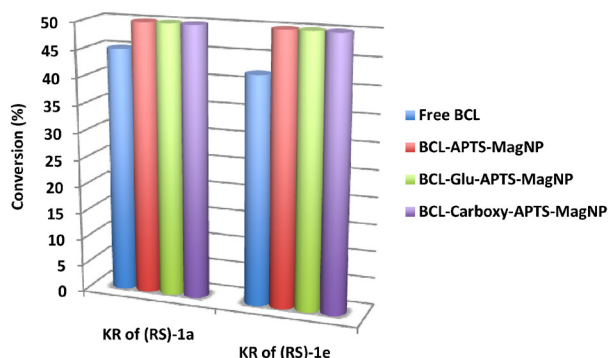

Figure 1. Comparison of the conversion obtained in the KR of (*RS*)-**1a** and (*RS*)-**1e** catalyzed by free (Free BCL) and *B. cepacia* lipase immobilized by adsorption (BCL-APTS-MagNP), glutaraldehyde (BCL-Glu-APTS-MagNP) and carboxybenzaldehyde method (BCL-Carboxy-APTS-MagNP): (*S*)-alcohols and (*R*)-esters (*ee* > 99%; *E* > 200).

As one can see in Figure 1, free *B. cepacia* lipase afforded the ester **2** with high *E*-value, but with lower conversion than in the cases of using immobilized lipase. This is a remarkable aspect, since besides the possibility of recycling the biocatalyst, the immobilization process seems also to improve the enzyme activity, increasing the conversion rates.

The results from the KR of a racemic mixture of substituted (*RS*)-1-(phenyl)ethanols **1a-e** using immobilized *B. cepacia* lipase are shown in Table 1. The substituents attached to the aromatic ring were chosen to

provide a wide range of electron donor and withdrawing characteristics.
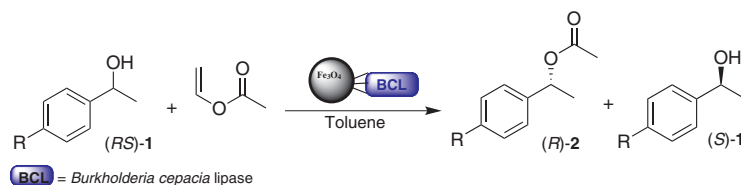
As one can see in Table 1, all reactions yielded the corresponding (*R*)-acetates in excellent conversion rates (50%) with *E*-value > 200. It is important to mention that the stereochemistry of the resolved chiral alcohols and their acetates is in accordance with Kazlauska's rule.[34] Surprisingly, no significant difference between the results of KR using *B. cepacia* lipase immobilized by the three methodologies could be detected.

In order to evaluate the recycling potential of the different immobilized forms of the *B. cepacia* lipase on magnetic nanoparticles [(immobilized by adsorption method (BCL-APTS-MagNP), by carboxybenzaldehyde method (BCL-Carboxy-APTS-MagNP) and by glutaraldehyde method (BCL-Glu-APTS-MagNP)], the KR of (*RS*)-**1a** was carried out in several repetitive reaction cycles (24 h). The immobilized *B. cepacia* lipase was collected with a magnet, and used again in a new experiment (Figure 2).

As can be seen in Figure 2, the glutaraldehyde method provided the best lipase reusability, exceeding 8 cycles with the same conversion efficiency (50%) and enantioselectivity (> 99%), while the other two methods collapsed after the fifth cycle. These results can be attributed to the efficient covalent binding of lipase to the magnetic nanoparticles using glutaraldehyde, which is known as a good cross-linking agent between enzyme and solid supports containing amino groups.[3] It should be noted that the long period of each reaction cycle (24 h) employed in the experiments can be contributing to the decrease of enzymatic activity. Since the enzymatic kinetic resolution is carried out in organic solvent, some protein denaturation can also occur, and consequently, an undesired loss of lipase activity is observed.[3]
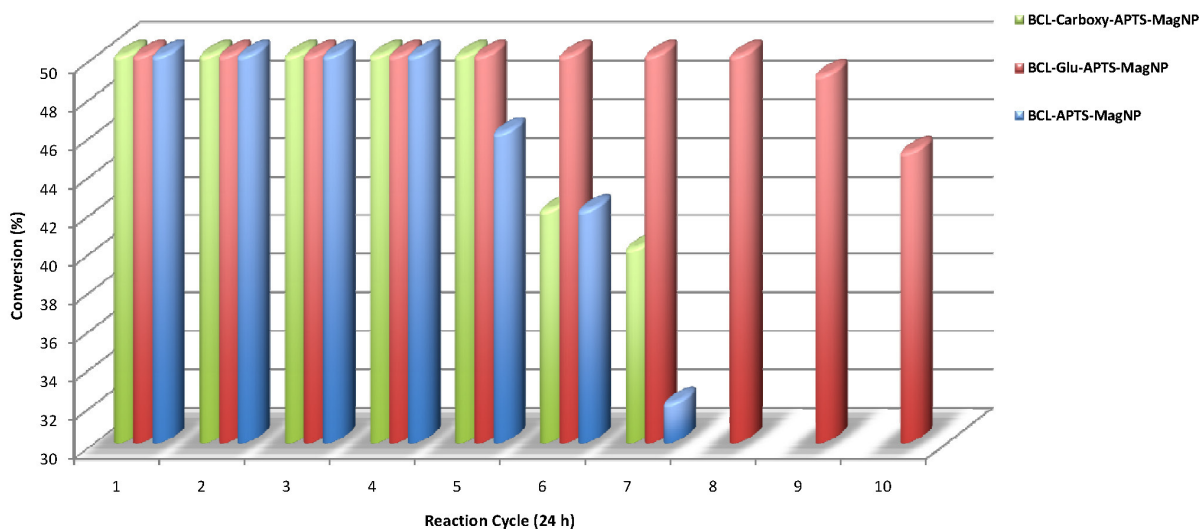
Conclusions

Immobilization of lipase from *B. cepacia* onto superparamagnetic nanoparticles using different methodologies afforded a magnetically recoverable, highly efficient biocatalyst. The lipase activity determined in the KR of different secondary alcohols afforded excellent

Table 1. Enzymatic kinetic resolution of (*RS*)-1-(phenyl)ethanols by *B. cepacia* lipase immobilized by different methodologies[a]

Entry	Substrate	R	Immobilized form of *B. cepacia* lipase	*ee* (%)[c]		Conversion (%)[d]	E[e]
				(*R*)-2	(*S*)-1		
1	**1a**	H	BCL-APTS-MagNP	> 99	> 99	50	> 200
2	**1b**	MeO	BCL-APTS-MagNP	> 99	> 99	50	> 200
3	**1c**	Cl	BCL-APTS-MagNP	> 99	> 99	50	> 200
4	**1d**	Br	BCL-APTS-MagNP	> 99	> 99	50	> 200
5	**1e**	NO$_2$	BCL-APTS-MagNP	> 99	> 99	50	> 200
6	**1a**	H	BCL-Carboxy-APTS-MagNP	> 99	> 99	50	> 200
7	**1b**	MeO	BCL-Carboxy-APTS-MagNP	> 99	> 99	50	> 200
8	**1c**	Cl	BCL-Carboxy-APTS-MagNP	> 99	> 99	50	> 200
9	**1d**	Br	BCL-Carboxy-APTS-MagNP	> 99	> 99	50	> 200
10	**1e**	NO$_2$	BCL-Carboxy-APTS-MagNP	> 99	> 99	50	> 200
11	**1a**	H	BCL-Glu-APTS-MagNP	> 99	> 99	50	> 200
12	**1b**	MeO	BCL-Glu-APTS-MagNP	> 99	> 99	50	> 200
13	**1c**	Cl	BCL-Glu-APTS-MagNP	> 99	> 99	50	> 200
14	**1d**	Br	BCL-Glu-APTS-MagNP	> 99	> 99	50	> 200
15	**1e**	NO$_2$	BCL-Glu-APTS-MagNP	> 99	> 99	50	> 200

[a]BCL immobilized by adsorption method (BCL-APTS-MagNP), by carboxybenzaldehyde method (BCL-Carboxy-APTS-MagNP) and by glutaraldehyde method (BCL-Glu-APTS-MagNP); [b]General conditions: Substrates (0.01 mmol), vinyl acetate (30 μL), toluene (1 mL), lipase (adsorption method = 0.21 mg lipase/20 mg APTS-MagNP; carboxybenzaldehyde method = 0.23 mg lipase/20 mg APTS-MagNP; glutaraldehyde method = 0.26 mg lipase/20 mg APTS-MagNP), 800 rpm, 24 h, 32 °C. [c]*ee* = Enantiomeric excess was determined by chiral GC analysis. [d] Conversion = $ee_S/(ee_S + ee_P)$. [e]$E = \{\ln[ee_P(1 - ee_S)]/(ee_P + ee_S)\}/\{\ln[ee_P(1 + ee_S)]/(ee_P + ee_S)\}$.[32] The absolute configurations of all compounds were determined by comparison of the sign of the measured specific rotation with those in the literature.[33]

Figure 2. Reusability of the *B. cepacia* lipase immobilized by different methodologies (BCL-Carboxy-APTS-MagNP, BCL-Glu-APTS-MagNP and BCL-APTS-MagNP) in the KR of (*RS*)-**1a** to afford the (*R*)-ester **2a** (*ee* > 99%; E > 200).

conversion (50%) and enantiomeric ratio ($E > 200$). The lipase immobilized by glutaraldehyde method showed the best results in terms of reusability, preserving the enzyme activity for at least 8 successive cycles. These results validate a new green approach to be used in the KR of secondary alcohols.

Experimental

General procedure for enzymatic kinetic resolution of substituted (RS)-1-(phenyl)ethanols (1a-e)

To 2 mL microtube (eppendorff®) containing 20 mg of magnetic nanoparticles with immobilized lipase by appropriate methodology (see Table 1), substituted (*RS*)-1-(phenyl)ethanols (**1a-e**) (0.01 mmol) and vinyl acetate (0.3 mmol) were dispersed in toluene and stirred with 800 rpm at 32 °C for 24 h. After the reaction, the samples were analyzed by GC analysis using a chiral capillary column.

Reusability of B. cepacia lipase on the enzymatic kinetic resolution of (RS)-1-(phenyl)ethanol (1a)

To 2 mL microtube (eppendorff®) containing 20 mg of magnetic nanoparticles with immobilized *B. cepacia* lipase by appropriate methodology (see Figure 2), (*RS*)-1-(phenyl)ethanol (**1a**) (0.01 mmol) and vinyl acetate (0.3 mmol) were dispersed in toluene and stirred at 32 °C for 24 h under 800 rpm. After 24 h reaction, the magnetic nanoparticles were controlled by magnetic and supernatant, containing the alcohol **1a** and ester **2a**, was collected for determination of the enantiomeric excess and conversion. Then, the immobilized *B. cepacia* lipase on the magnetic nanoparticles was washed with toluene (3×1 mL) and then used for the next cycle.

Acknowledgments

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