http://dx.doi.org/10.21577/0103-5053.20180063

J. Braz. Chem. Soc., Vol. 29, No. 9, 1876-1884, 2018
Printed in Brazil - ©2018 Sociedade Brasileira de Química

Article

Immobilization of *Burkholderia cepacia* on Pristine or Functionalized Multi-Walled Carbon Nanotubes and Application on Enzymatic Resolution of (*RS*)-1-Phenylethanol

Michele R. G. Dias,[a] Alysson de Pauloveloso,[a] Lilian F. M. do Amaral,[a] Rhaísa T. Betim,[a] Maria G. Nascimento[b] and Cristiane Pilissão[,a]*

[a]*Departamento de Química e Biologia, Universidade Tecnológica Federal do Paraná, 81280-340 Curitiba-PR, Brazil*

[b]*Departamento de Química, Universidade Federal de Santa Catarina, 88040-900 Florianópolis-SC, Brazil*

The immobilization of *Burkholderia cepacia* lipase (BCL) on pristine or functionalized multi-walled carbon nanotubes (MWCNTs) was studied in the resolution of (*RS*)-1-phenylethanol. For the functionalization, three treatments were used, these being in H_2SO_4/HNO_3 (acid, MWCNTs-A), KOH (basic, MWCNTs-B) or in H_2O_2 (oxidizing agent, MWCNTs-O). It was found that the pristine or functionalized BCL/MWCNTs resulted in a 3-fold improvement in the conversion and in a 5-fold improvement in ee_s (enantiomeric excess of the substrate), when compared with free BCL under the same reaction conditions. This pristine or functionalized BCL/MWCNTs system could be reused for up to 8 times without significant decrease in the catalytic efficiency. Under the optimum conditions (pH of immobilization 6.0, 45 ºC and 25 mg/5 mg pristine or functionalized BCL/MWCNTs), the best results were obtained using BCL immobilized on MWCNTs-A. The simple process of physical adsorption of BCL onto MWCNTs-A has improved the catalytic efficiency when compared with free BCL and an increase in the stability was confirmed by thermogravimetric analysis (TGA).

Keywords: multi-walled carbon nanotubes, functionalization, *Burkholderia cepacia* lipase, immobilization, enzymatic resolution

Introduction

Enzyme immobilization can improve the performance of an enzyme, increasing its stability and resistance to a wide range of pH and temperature. Therefore, it preserves the enzyme activity through several catalytic cycles. However, the process to select an adequate support is intricate and depends on the type of enzyme, reaction media, reaction conditions, and safety policy of the field of application.[1-5] Moreover, in order to make this process economically feasible, it is important to take into account some factors such as the type of support and method of immobilization, since they influence activity and reuse of the biocatalyst.[4-9]

Recently, nanomaterials such as carbon nanotubes, nanoparticles, and nanofibers have been increasingly explored as support for enzyme immobilization due to their exclusive properties and various applications.[2,4,5,10,11]

*e-mail: pilissao@utfpr.edu.br

Carbon nanotubes (CNTs), amongst various types of nanomaterials, can have their properties altered by further functionalization on their surface. This functionalization can be either on their walls, tips, or by encapsulating them and it may bring substantially different properties compared with the non-functionalized nanotubes. Their chemically modified structures can be used to facilitate the interaction of nanotubes with organic molecules, such as enzymes. Several studies were conducted to change multi-walled carbon nanotubes (MWCNTs).[2] MWCNTs can be oxidized using several oxidative agents such as acids (nitric and sulfuric acid, HNO_3/H_2SO_4), hydrogen peroxide (H_2O_2) and base (sodium hydroxide, NaOH).[12] The oxidation of MWCNTs occurs particularly at defective sites such as ring defects, edges, dangling bonds and kink sites. In the oxidation process, several functional groups such as carboxyl (–COOH) and hydroxyl (–OH) are formed on the surface of nanotubes. Some oxidants preferably form acidic groups (–COOH) while others preferably form "basic" groups (–OH) as a result of their different properties.

These functional groups can be designed according to the application.[13-15]

MWCNTs have quickly risen as a support for enzyme immobilization because of their high surface area, minimum diffusion limitations, maximum amount of enzyme loading, high mechanical stability, mobility and high mass transference.[16,17]

Immobilization of enzyme on MWCNTs can be achieved by covalent and non-covalent approaches; the latter is preferred since it is carried out without chemical additives, preserving the native conformation of the enzyme.[2,10,18,19] Non-covalent binding of the enzyme on the surface of MWCNTs can occur by different mechanisms: (*i*) adsorption by van der Waals interactions and formation of π–π stacks between aromatic residues of enzymes and the MWCNTs surface; (*ii*) hydrophobic interactions between hydrophobic side chains of amino acids and the MWCNTs surface; (*iii*) amphiphilic binding generating a surfactant-like interaction; (*iv*) electrostatic interactions in which both the isoelectric point of the enzyme and the point of zero charge of MWCNTs play a role; and (*v*) hydrogen bonding between amine terminal groups of enzymes and oxygen-containing groups in oxidized MWCNTs.[19-24]

Lipases are the class of enzymes that shows the highest degree of hydrophobicity with 28-30% of hydrophobic amino acid residues. Furthermore, those hydrophobic residues are usually close to the active site which make them prone to activation after being adsorbed on hydrophobic supports like MWCNTs.[2,16]

Because of their ability to catalyze esterification and transesterification reactions in organic solvents, lipases (Enzyme Commission (EC) No. 3.1.1.3) have been showing several applications in food and pharmaceutical industries.[25]

Thus, in this work, the effect of MWCNTs functionalization and their application as support for the immobilization of *Burkholderia cepacia* lipase (BCL) were studied in the resolution of (*RS*)-1-phenylethanol (**1**) with vinyl acetate. Several experimental conditions such as pH of immobilization, the temperature of reaction, mass support/lipase ratio and reaction time were evaluated. Reuse of immobilized BCL was also analyzed (Scheme 1).

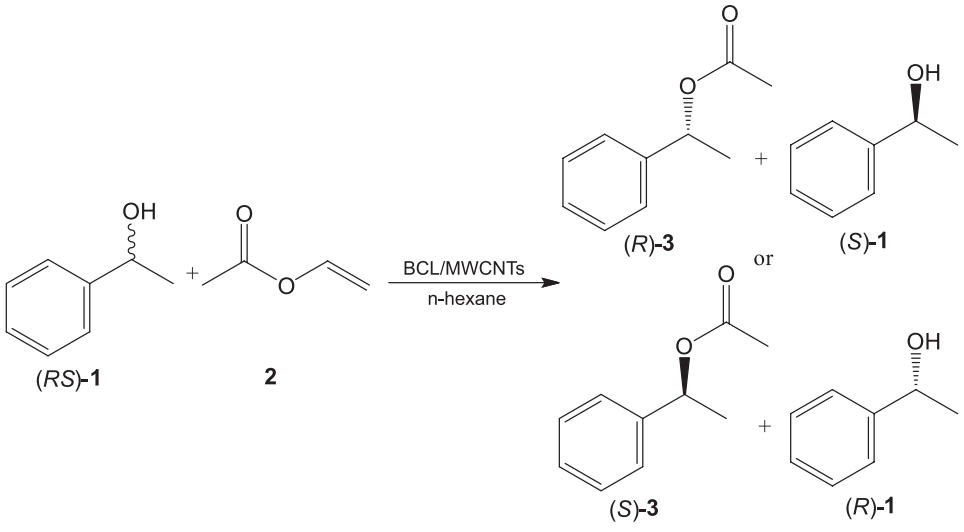
Experimental

Chemicals

Multi-walled carbon nanotubes purchased from Sigma-Aldrich (purity ≥ 98%, 10 ± 0.1 nm × 4.5 ± 0.5 nm × 3-6 μm (o.d. × i.d. × length)) were used without any further treatment (pristine MWCNTs). Sodium borohydride, boric acid, acetic anhydride (97%) and vinyl acetate (99%) were purchased from Vetec. Lipase from *Burkholderia cepacia* (BCL, 30000 U g^{-1}), previously known as *Pseudomonas cepacia*, was donated by Amano Pharmaceutical Co. Other reagents used for the enzymatic resolution were of analytical grade and were obtained commercially.

Synthesis and characterization of standards

(*RS*)-1-Phenylethanol was obtained by the reduction of acetophenone using sodium borohydride and boric acid according to the methodology described by Cho *et al.*[26] (*S*)-1-Phenylethanol was obtained from acetophenone using *Daucus carota* roots, according to the methodology described by Omori *et al.*[27] (*RS*)-1-Phenylethyl acetate was synthesized using the following methodology: acetic anhydride (25 mmol), (*RS*)-1-phenylethanol (5 mmol)



Scheme 1. Kinetic resolution of (*RS*)-1-phenylethanol using BCL immobilized on pristine or functionalized MWCNTs (BCL/MWCNTs).

and sulfuric acid (1%) were dissolved in 30 mL of dichloromethane. The solution was refluxed for 6 h. The reaction mixture was cooled to room temperature and washed with 5% sodium bicarbonate solution (40 mL), and then with water (40 mL). The organic layer was dried using sodium sulfate. The compounds obtained here were used as standard compounds for the gas chromatography analysis.

The esters were also characterized with a Varian-640-IR Fourier transform infrared (FTIR) spectrometer using attenuated total reflectance (ATR) for liquid samples (4000-650 cm^{-1}). The ^1H NMR spectra were recorded at 200 MHz with a Bruker DPX 200 spectrometer using $CDCl_3$ as the solvent and tetramethylsilane (TMS) as the internal standard at Central Analítica, Departamento de Química, Universidade Federal do Paraná (UFPR, Curitiba-PR, Brazil). The pH of immobilization and pH used in the functionalized MWCNTs (MWCNTs-F) were measured using a Marte MB10 pHmeter.

Functionalization and purification of MWCNTs

For the functionalization of the pristine MWCNTs, three treatments were used: acid, basic and oxidizing agent.

Acid treatment

Pristine MWCNTs (20 mg) were mixed with 10 mL nitric acid (3.0 mol L^{-1}) and 10 mL sulfuric acid (3.0 mol L^{-1}), and refluxed for 6 h. Then, the MWCNTs functionalized by acid treatment (MWCNTs-A) were separated from the solution by centrifugation (3000 rpm) and washed with a solution of water/ethanol (1:1 v/v) until pH 7.0. The sample was dried overnight at 50 ºC.

Basic treatment

Pristine MWCNTs (100 mg) were mixed with 100 mL ethanol and 5 g potassium hydroxide, and refluxed for 8 h. Subsequently, the MWCNTs functionalized by basic treatment (MWCNTs-B) were separated from the solution by centrifugation (3000 rpm) and washed with a solution of water/ethanol (1:1 v/v) until pH 7.0. The sample was dried overnight at 50 ºC.

Oxidizing treatment

Pristine MWCNTs (20 mg) were mixed with 80 mL 30% hydrogen peroxide solution and refluxed for 2 h. Then, the MWCNTs functionalized by oxidizing treatment (MWCNTs-O) were separated from the solution by centrifugation (3000 rpm) and washed with a solution of water/ethanol (1:1 v/v) until pH 7.0. The sample was dried overnight at 50 ºC.

Immobilization of BCL on pristine and functionalized MWCNTs by physical adsorption

Pristine MWCNTs and functionalized MWCNTs-F, MWCNTs-A, MWCNTs-B and MWCNTs-O (5 mg) were suspended in 5 mL potassium phosphate buffer (50 mmol L^{-1}) for pH 6.0-8.0 or potassium phosphate buffer (135 mmol L^{-1}) for pH 5.0 and 5.5, containing BCL (5-50 mg). The mixture was incubated at 25 ºC for 24 h with constant stirring at 150 rpm. Then, the suspension was centrifuged for 10 min (3000 rpm) to remove the supernatant. The derived immobilized lipase (pristine or functionalized BCL/MWCNTs) was dried overnight at 30 ºC and ground into a powder, which was used in the kinetic resolution of **1** with vinyl acetate. The immobilization of BCL was further confirmed by FTIR spectroscopy and thermogravimetric analysis (TGA). The experimental data were obtained in triplicate.

Characterization of pristine and functionalized MWCNTs

Pristine MWCNTs, functionalized MWCNTs and BCL/MWCNTs were characterized in a ratio of 1:1000 mass of sample/KBr by FTIR. The spectra were obtained using a Bomem spectrometer (model Varian-640-IR) in transmission mode between 4000 and 400 cm^{-1} at a resolution of 4 cm^{-1}.

The TGA analyses were performed on SDT Q600 instrument (TA Instruments) from 50 to 900 ºC in a nitrogen atmosphere (flow rate: 20 cm^3 min^{-1}; heating rate: 10 ºC min^{-1}) using samples less than 10 mg.

Determination of protein loading

The protein loading was determined by means of the Bradford method in which the free BCL powder contains 1 wt.% protein.[28]

The amount of lipase bound to pristine MWCNTs or functionalized MWCNTs-F was determined indirectly from the difference between the amount of lipase (concentration of protein in the free BCL) introduced into the reaction mixture and the amount of lipase in the filtrate (final protein concentration) after immobilization measured by the Bradford method,[28] using bovine serum albumin (BSA) as the standard. All experiments were performed in triplicate.

The immobilization yield was determined following equation 1:[3]

$$\text{Yield} = \frac{\left(\begin{array}{c}\text{initial protein}\\ \text{concentration}\end{array}\right) - \left(\begin{array}{c}\text{final protein concentration}\\ \text{after immobilization}\end{array}\right)}{\text{initial protein concentration}} \times 100 \quad (1)$$

A yield of immobilization of 98% was achieved after 24 h of immobilization at room temperature.

The immobilized BCL on pristine MWCNTs/BCL or functionalized MWCNTs-F/BCL powder was used in the resolution of **1** with vinyl acetate to evaluate the catalytic efficiency of this system in this reaction.

General procedure for lipase-catalyzed resolution of (*RS*)-1-phenylethanol

The reactions were carried out in 15 mL pure *n*-hexane containing 1 mmol racemic (*RS*)-1-phenylethanol, 1 mmol vinyl acetate and 5-50 mg free or immobilized BCL. The reactions were performed in a 125 mL stoppered flask at 25-65 ºC in a Dubnoff water bath at 150 rpm for 3-24 h. Aliquots were withdrawn at specified time intervals from the reaction mixture, and then analyzed by chiral chromatography. The enantiopreference of the formed product was compared to the chiral (*S*)-alcohol standard by gas chromatography. The conversion and the enantiomeric excesses of the formed products were determined with a gas chromatograph (GC-14B, Shimadzu) equipped with a chiral column (RT-BetaDEX-sm, 30 m × 0.32 mm × 0.25 μm, Agilent). H_2 was used as the carrier gas with a pressure of 120 kPa and flow rate of 1.40 mL min^{-1}. The temperature of the injector and detector was 230 ºC. The column temperature was an isotherm of 100 ºC for 30 min. The retention times observed for *S*-(–) and *R*-(+)-1-phenylethanol were 19.4 and 20.9 min, respectively, and for the corresponding *S*-(–) and *R*-(+)-acetyl esters were 17.6 and 19.2 min, respectively. The enantiomeric ratio (E) was calculated from the enantiomeric excess of the product (ee_p), enantiomeric excess of the substrate (ee_s), and the conversion degree (c) according to the method described by Chen *et al.*[29] An experiment was performed with pristine MWCNTs in the absence of BCL, and no product was detected.

Results and Discussion

Effect of lipase loading, pH and temperature

It is well known that the immobilization conditions have significant effects on the immobilization efficiency.[30] Conditions such as lipase loading-support, pH of immobilization and temperature were firstly evaluated in the transesterification reaction of (*RS*)-1-phenylethanol with vinyl acetate using the system BCL/MWCNTs. When the optimal condition was defined, the effect of functionalized MWCNTs on the resolution was also studied.

Effect of lipase loading

The influence of the amount of free BCL or BCL immobilized on pristine MWCNTs was evaluated in the resolution of (*RS*)-**1** with vinyl acetate using *n*-hexane at 35 ºC for 24 h, and pH of immobilization 7.0 (potassium phosphate buffer). The amount of pristine BCL/MWCNTs used was in the range of 5-50 mg in 5 mg of pristine MWCNTs and 25 mg of free BCL. The results of conversion degrees and ee_s are presented in Figure 1.

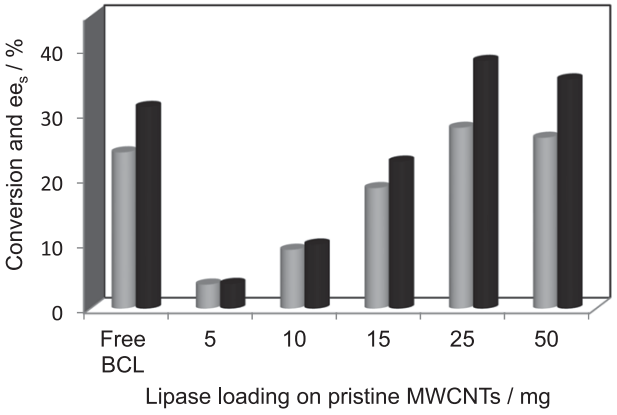


Figure 1. Influence of free BCL or BCL immobilized on pristine MWCNTs on the enzymatic resolution of (*RS*)-1-phenylethanol with vinyl acetate: (■) conversion and (■) ee_s. Reaction conditions: (*RS*)-**1** (1 mmol), vinyl acetate (1 mmol), *n*-hexane (15 mL), 24 h, 35 ºC, pH of immobilization 7.0 (potassium phosphate buffer, 50 mmol), 25 mg of free BCL or 5-50 mg BCL/5 mg pristine MWCNTs.

As it can be observed, the conversion degrees increased from 4 to 28% as the amount of BCL increased until 25 mg. The ee_s values were in the range of 4-38% and ee_p > 99% (data not shown), resulting in E > 200, and the enantiopreference was for the (*R*)-**1**, thus forming the *R*-ester. When the reaction was carried out using 50 mg of BCL, no improvement in the conversion degree or ee_s was observed. Therefore, 25 mg of BCL were selected to be used in the following studies.

Effect of pH on BCL immobilization on pristine MWCNTs

Enzymes are highly affected by pH, both during immobilization and along biocatalysis processes.[31] Changing the pH will alter the surface charge of both the enzyme and the MWCNTs, hence affecting the electrostatic interactions between enzyme and support.[1,32]

Thus, the effect of pH on the immobilization of BCL on pristine MWCNTs was evaluated in the resolution of the (*RS*)-**1** in the range of 5.0 to 8.0. The data of conversion degrees ee_s and ee_p are given in Figure 2.

As can be observed in Figure 2, using BCL immobilized on pristine MWCNTs, the best results were achieved at pH 6.0, where the conversion degree was of 48%, ee_s 91%

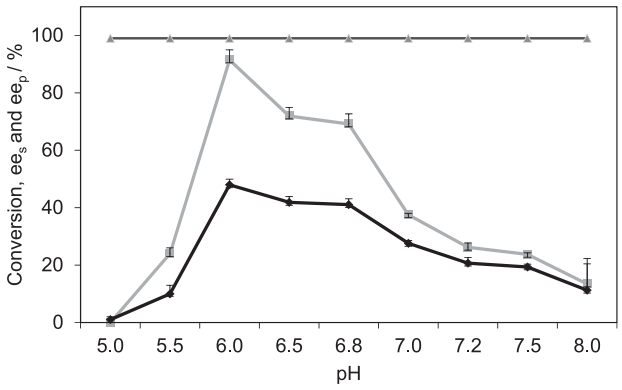


Figure 2. Effect of pH on the immobilization of BCL and its influence on the enzymatic resolution of (*RS*)-1-phenylethanol with vinyl acetate mediated by pristine BCL/MWCNTs. (◆) conversion and (■) ee$_s$. Reaction conditions: (*RS*)-**1** (1 mmol), vinyl acetate (1 mmol), pristine BCL/MWCNTs (25 mg/5 mg), *n*-hexane (15 mL), 35 ºC, 24 h.

and ee$_p$ > 99%, resulting in E > 200. Using pH of 6.5 and 6.8, a small decrease in the conversion degrees and ee$_s$ values was observed, of 42 and 41, and 72 and 69%, respectively. Using pH in the range of 7.0-8.0, a large decrease in both conversion degrees and ee$_s$ values was observed, of 12-27 and 13-37%, respectively. However, no change was observed in the ee$_p$ values (> 99%). When pH 5.0 and 5.5 were used, the conversion degrees were low, of 1 and 10%, ee$_s$ of 0.5 and 24%, but with ee$_p$ > 99%, resulting in E-value > 200.

The results may be related to the interactions of the support and lipase surface, and in this case, can be considered satisfactory for an enzymatic resolution. It is usually described in the literature that the pH of the lipase in free form may be different than immobilized form.[1,22,24,32] As previously reported by Lou *et al.*,[33] the optimum pH of the free BCL is 7.0. However, it is interesting to note that in this study, a change in the optimum pH value from 7.0 to 6.0 was observed after immobilization on MWCNTs.

The same behavior was observed by Pereira *et al.*,[24] using *Candida rugosa* lipase immobilized on chitosan in the synthesis of *n*-butyl butyrate. The pH decreased from 7.0 in the free form to 6.0, after immobilization. Fadiloglu and Soylemez[34] also observed a reduction in the optimal pH from 7.0 to 6.5 after *C. rugosa* lipase immobilization on celite. From these results, pH 6.0 was selected to be used to evaluate the other parameters, such as the influence of temperature and the functionalization of MWCNTs.

Effect of temperature

Another key factor that may affect the rate of a reaction catalyzed by an enzyme is the temperature. The temperature influences the activity, selectivity and stability of the biocatalyst besides the reaction equilibrium.[35]

Thus, to evaluate this parameter in the resolution of the (*RS*)-**1** with vinyl acetate catalyzed by the system BCL/MWCNTs, the temperature was changed from 35 to 65 ºC. The results showed no significant changes in the conversion degrees, being 50% in this temperature range. The selectivity showed small changes, being ee$_s$ > 99% and ee$_p$ from 86 to > 99%, resulting in E-values > 200. These data are interesting and show the high stability of the immobilized BCL on pristine MWCNTs. Based on these data, a temperature of 45 ºC was considered as appropriate to evaluate the influence of MWCNTs functionalization.

MWCNTs functionalization effect on BCL immobilization in the resolution of (*RS*)-1-phenylethanol

The effect of MWCNTs surface chemistry on the efficiency of the immobilization of *Burkholderia cepacia* lipase by simple non-covalent adsorption was explored in the resolution of (*RS*)-1-phenylethanol with vinyl acetate. The results of conversion degrees and ee$_s$ are presented in Table 1.

Table 1. Effect of functionalization of MWCNTs in the immobilization of BCL and application in the resolution of (*RS*)-1-phenylethanol

entry	Support/BCL	Reaction time / h							
		3		6		12		24	
		c / %	ee$_s$ / %	c / %	ee$_s$ / %	c / %	ee$_s$ / %	c / %	ee$_s$ / %
1	free BCL	8 ± 1	9 ± 1	12 ± 2	13 ± 2	17 ± 2	20 ± 3	24 ± 3	31 ± 5
2	pristine MWCNTs	33 ± 3	50 ± 2	36 ± 3	69 ± 5	42 ± 2	79 ± 1	45 ± 4	82 ± 13
3	MWCNTs-A	41 ± 3	72 ± 2	45 ± 2	87 ± 2	46 ± 1	88 ± 2	47 ± 2	87 ± 5
4	MWCNTs-B	35 ± 3	57 ± 5	38 ± 2	59 ± 1	42 ± 3	66 ± 1	43 ± 2	73 ± 3
5	MWCNTs-O	32 ± 1	46 ± 2	40 ± 3	62 ± 2	43 ± 2	70 ± 2	44 ± 1	76 ± 2

BCL: *Burkholderia cepacia* lipase; c: conversion degree; ee$_s$: enantiomeric excess of the substrate; MWCNTs: multi-walled carbon nanotubes; MWCNTs-A, MWCNTs-B and MWCNTs-O: MWCNTs functionalized by acid, basic and oxidizing treatment, respectively. Reaction conditions: (*RS*)-**1** (1 mmol), vinyl acetate (1 mmol), free BCL (25 mg), MWCNTs (25 mg BCL and 5 mg of pristine and functionalized MWCNTs (MWCNTs-A, MWCNTs-B, and MWCNTs-O)), *n*-hexane (15 mL), 45 ºC, immobilization pH 6.0.

Four types of MWCNTs (pristine MWCNTs, MWCNTs-A, MWCNTs-B and MWCNTs-O) were used to immobilize BCL. When pristine or functionalized MWCNTs were used, the results were better than those obtained using free BCL. With their use as catalysts, the conversion degrees were 32-47%, $ee_p > 99\%$, ee_s of 46-88%, resulting in $E > 200$ in 3-24 h of reaction. Using free BCL, the conversion degrees were 8-24%, $ee_p > 99\%$ and ee_s of 9-31% in the same time. These values represent an increase of more than 3-fold in the conversion and 5-fold in the ee_s. These results showed high activity catalytic and selectivity of the BCL when used in immobilized form in the resolution of (*RS*)-**1**.

The best results were obtained using BCL immobilized on MWCNTs-A, forming the *R*-ester in conversion degrees of 41-47% in 3-24 h of reaction, ee_s 72-88% and $ee_p > 99\%$, resulting in $E > 200$. After 6 h of reaction, no significant increase was observed in both conversion degrees and ee_s values. Similar results were obtained when BCL was immobilized on pristine MWCNTs in 24 h of reaction. The main product was the (*R*)-**3**.

The chemical functionalization of nanotubes has been intensely analyzed to attach to the surface of the tube active chemical groups that can anchor through covalent or non-covalent bonds other groups or molecules. Among the various groups used for functionalization, the carboxylic group stands out. In this work the best results were obtained when BCL was immobilized on MWCNTs-A. In the acid treatment (H_2SO_4/HNO_3) the polar groups (COO–) are introduced into the non-polar surface and at the ends of the support MWCNTs. Thus, the surface of the MWCNTs is fixed to other polar portions (NH_2, OH) present in the BCL protein. Therefore, the role of the MWCNTs is to anchor the BCL protein to the MWCNTs through the oppositely charged carboxyl moiety (electron rich) and hydrogen (electron poor) of the back-bone and side-chain of polar amino acids present on the outer surface of the BCL protein.[17]

The difference in conversion degrees and selectivity values using the pristine or functionalized MWCNTs may be related to the interaction between both phases of the lipase and support. These interactions depend on the surface and properties of the support, and these properties may be of primary importance in enzyme catalyzed reactions, since they are capable of affecting the conformation of the enzymes and, consequently, their reactivity.[2,16]

The hydrophobic surface of pristine MWCNTs provides a suitable microenvironment for lipase, where the interaction among BCL and MWCNTs can be through π–π stacks interactions, as well as by hydrophobic interactions.[19-21] The possibility of surface functionalization of pristine MWCNTs can modify their properties, and thus improve the interaction between enzyme and support.[16,17,36]

The results presented in this work showed this improvement in catalytic activity and stability when the carbon nanotube was functionalized in acid medium, hence, showing a better interaction between BCL and MWCNTs-A.

Reuse of BCL/MWCNTs system

One of the advantages of the immobilization is the improvement in stability and activity of the enzymes. Likewise, the reuse of the system is another advantage of the immobilization. In this study, the BCL/MWCNTs systems were used in the resolution of (*RS*)-1-phenylethanol with vinyl acetate in 6 h. After each reaction, the immobilized lipase was separated, washed with *n*-hexane, desiccated in vacuum and then reused for 8 successive cycles. The results are presented in Table 2.

Table 2. Reuse of free or immobilized BCL on pristine or functionalized MWCNTs using different methodologies in the resolution of (*RS*)-1-phenylethanol

Cycle	Free BCL		MWCNTs		MWCNTs-A		MWCNTs-B		MWCNTs-O	
	c / %	ee_s / %	c / %	ee_s / %	c / %	ee_s / %	c / %	ee_s / %	c / %	ee_s / %
1	12 ± 2	13 ± 2	36 ± 3	69 ± 5	45 ± 2	87 ± 2	38 ± 2	59 ± 1	40 ± 3	62 ± 2
2	6 ± 1	6 ± 1	45 ± 3	76 ± 1	50 ± 2	99 ± 2	44 ± 2	78 ± 6	48 ± 3	82 ± 5
3	6 ± 2	7 ± 3	35 ± 2	54 ± 4	41 ± 3	68 ± 4	33 ± 3	50 ± 4	37 ± 3	59 ± 4
4	2 ± 2	2 ± 2	36 ± 2	56 ± 4	46 ± 2	85 ± 5	37 ± 4	64 ± 2	38 ± 3	61 ± 5
5	2 ± 2	2 ± 2	41 ± 1	70 ± 1	50 ± 2	98 ± 3	42 ± 2	75 ± 7	42 ± 4	73 ± 4
6	0 ± 0	0 ± 0	40 ± 4	75 ± 4	49 ± 1	94 ± 3	37 ± 0	58 ± 1	40 ± 2	69 ± 5
7	0 ± 0	0 ± 0	36 ± 3	56 ± 5	48 ± 1	92 ± 2	37 ± 0	59 ± 1	34 ± 3	52 ± 6
8	0 ± 0	0 ± 0	33 ± 5	51 ± 7	46 ± 1	83 ± 5	36 ± 1	56 ± 5	29 ± 1	42 ± 1

BCL: *Burkholderia cepacia* lipase; MWCNTs: multi-walled carbon nanotubes; MWCNTs-A, MWCNTs-B and MWCNTs-O: MWCNTs functionalized by acid, basic and oxidizing treatment, respectively; c: conversion degree; ee_s: enantiomeric excess of the substrate. Reaction conditions: (*RS*)-**1** (1 mmol), vinyl acetate (1 mmol), free BCL (25 mg), MWCNTs (25 mg BCL and 5 mg of pristine and functionalized MWCNTs (MWCNTs-A, MWCNTs-B, and MWCNTs-O)), *n*-hexane (15 mL), 45 ºC, 6 h, immobilization pH 6.0.

As can be observed in Table 2, using free BCL, the conversion degrees to (*R*)-**3** were 2-12%, ee_s of 2-13% and ee_p > 99% (data not shown). These results showed a decrease in the catalytic activity and selectivity after the first reaction cycle.

When the pristine MWCNTs or functionalized MWCNTs-F (MWCNTs-A, MWCNT-B, and MWCNTs-O) systems were used, better results were achieved. No significant change in the conversion degrees, ee_s and ee_p, values was observed after 8 reaction cycles. The conversion degrees were 29-50%, ee_s of 42-99% and ee_p > 99% (data not shown in Table 2). The results herein obtained indicated that BCL did not show any deactivation during the reaction or washing procedure. Therefore, these results can be attributed to the immobilization of BCL on pristine or functionalized MWCNTs, which maintained the catalytic activity and stability during the reuse. These results yield, as expected, another advantage of the process: its simplicity and scalability to industrial proportions can be explored economically.

Characterization of functionalized MWCNTs and immobilized BCL/MWCNTs

BCL immobilized on functionalized MWCNTs-A was confirmed and characterized by FTIR and TGA and their corresponding details will be described in the following sub-sections.

FTIR analysis

The successful immobilization of lipase onto MWCNTs-A was confirmed by FTIR spectroscopy. FTIR spectra of MWCNTs-A, free BCL and immobilized BCL/MWCNTs-A are presented in Figure 3.

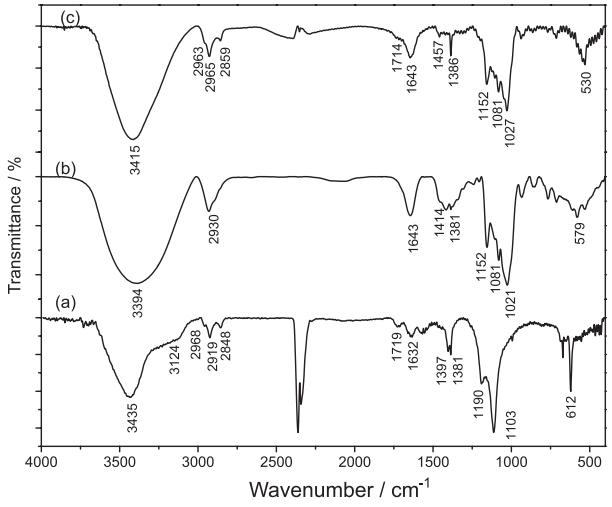


Figure 3. FTIR spectra of (a) raw MWCNTs-A, (b) free BCL and (c) immobilized BCL/MWCNTs-A (KBr = 0.1%).

The MWCNTs-A spectrum (Figure 3a) showed some characteristic bands. The band at 3435 cm^{-1} corresponds to –OH stretching vibration of the surface groups, and another at 1631 cm^{-1} originating from conjugated –C=C– bonds. The band at 1719 cm^{-1} is due to the carbonyl stretch of the carboxylic group, where the conjugation of C=O with C=C results in a lower vibration frequency of carbonyl group.[17] The band at 1103 cm^{-1} is assigned to C–O stretching vibrations, confirming the oxidation of sp^2 hybridized carbon in pristine MWCNTs to sp^3.[10,17] The absorption bands in the region of 2850-2970 cm^{-1} refer to the asymmetric stretching of aliphatic C–H bonds. It is clearly observed that all characteristic bands of the protein are present in the spectrum of the immobilized enzyme, which undoubtedly confirmed that this process was successful.

The free lipase spectrum (Figure 3b) is typical for a protein, with the most prominent band at 1643 cm^{-1} from –C=O stretching and –NH bending vibrations and a band at 1021 cm^{-1}, which was attributed to the C–N bond, thus confirming the presence of the amide. The band at 3394 cm^{-1} was due to amide stretching (N–H), which confirms the presence of this group. Differences in the spectra of free BCL and BCL immobilized on MWCNTs-A (Figure 3c) are significant in the 1386-1457 and 900-1150 cm^{-1} regions. Also, after immobilization, the band due to the vibration of –OH group shifted from 3435 to 3415 cm^{-1}, indicating that the hydroxyl groups formed hydrogen bonds with the lipase.[2]

Thermogravimetric analysis

The thermogravimetric method is often used to monitor any reaction that involves oxidation or dehydration. By measuring the decomposition or weight loss of the sample, TGA reveals the change in thermal stability of the support used in the immobilization process, indicating if it has been modified.[37] It is well known that different structural forms of carbon can exhibit different oxidation behaviors depending each time on the available reactive sites. Herein, the thermal decomposition of functionalized MWCNTs-A, as well as free and immobilized BCL on MWCNTs-A, was investigated. This analysis was performed in the range of 50-900 ºC, and the data is presented in Figure 4.

In this study, TGA was used as a tool to judge the thermal stability of immobilization of BCL on MWCNT-A. It was observed that free BCL was totally decomposed at 50-480 ºC, in which 15 wt.% was below 70 ºC, due to evaporation of the water present in the lipase. When the temperature increased until 150 ºC, the sample showed a large degradation (45 wt.%), which may be associated with elimination of water molecules present in close vicinity

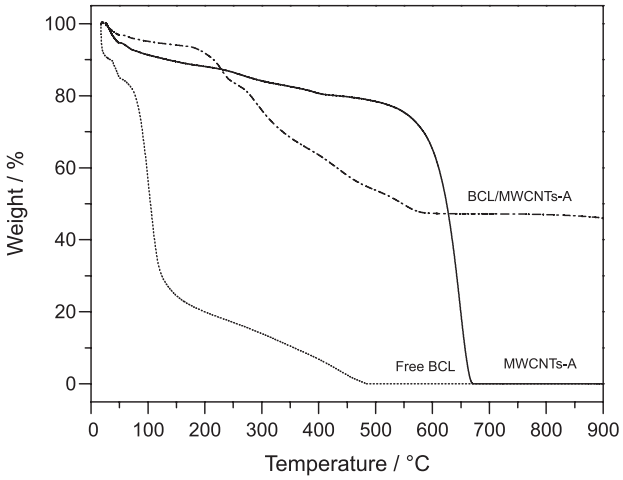


Figure 4. Termogravimetric analysis for (—) MWCNTs-A, (···) free BCL and (----) BCL/MWCNTs-A. Experimental conditions: nitrogen atmosphere, flow rate: 20 cm^3 min^{-1}, heating rate: 10 ºC min^{-1}.

of the enzyme. It had totally decomposed at temperatures between 150-480 ºC. Similar results were observed by Turner and Vulfson.[38]

The MWCNTs-A started to decompose around 50 ºC with a weight loss of approximately 10 wt.%. This mass loss can be related to the evaporation of adsorbed water. The second stage of decomposition, from 50-550 ºC, is attributed to the decarboxylation of the carboxylic groups and by the elimination of hydroxyls present on the MWCNTs wall and are totally burned when the temperature reaches 667 ºC. A different behavior was observed for the BCL/MWCNTs system, in which three stages of decomposition were observed. The first was between 50-200 ºC, the second at 200-250 ºC and the third in the range of 250-580 ºC. In this temperature range, the mass loss was 50 wt.%. Moreover, it can be clearly seen that thermal stability of the immobilized lipase is considerably higher as compared to the free form. As described, free BCL presented a mass loss of 45 wt.% up to 150 ºC, and after immobilization (BCL/MWCNTs-A) the mass loss was 7% up to 200 ºC and 43 wt.% up to 580 ºC. These results can be attributed to the simultaneous BCL and MWCNTs-A decomposition. Additionally, they are in agreement with the data presented in the resolution of (*RS*)-**1**, in which the BCL/MWCNTS-A system can be reused in 8 consecutive cycles, maintaining high catalytic activity (see data in Table 2).

Conclusions

In this study, the pristine or functionalized MWCNTs (MWCNTs-A, MWCNTs-B and MWCNTs-O) were used to immobilize *Burkholderia cepacia* lipase (BCL). These systems were applied in the resolution of (*RS*)-**1** with vinyl acetate. A significant enhancement in the catalytic efficiency and enantioselectivity of the immobilized lipase was observed, under the optimum conditions (pH 6.0 of immobilization, 45 ºC and a BCL/MWCNTs molar ratio of 25 mg/5 mg). The best results were obtained using BCL immobilized on MWCNTs-A, forming the *R*-ester (**3**) in conversion degrees of 45-47%, ee_s of 82-87% and $ee_p > 99\%$ (E > 200), in 6-24 h of reaction. After 6 h, no significant increase in the conversion degrees was observed. The pristine or functionalized lipase/MWCNTs systems could be reused for 8 cycles without significant decrease in the catalytic efficiency. In summary, the MWCNTs present great advantages as supports for BCL immobilization and offer potential advantages for the applications in (*RS*)-**1** resolution.

Supplementary Information

Supplementary data are available free of charge at http://jbcs.sbq.org.br as PDF file.

Acknowledgments

This work was supported by Universidade Tecnológica Federal do Paraná (UTFPR), Pós-Graduação em Química (PPGQ)-Universidade Federal de Santa Catarina (UFSC), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq, process No. 476159/2013-0), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), and Laboratório Multiusuários de Análise Químicas (LAMAQ-UTFPR). We also thank Amano Pharmaceutical Co. (Japan) for the donation of BCL, Prof Hugo A. Gallardo (UFSC) for the termogravimetric analysis, technician Rubia Bottini (UTFPR), and Departamento de Química da Universidade Federal do Paraná for the NMR analysis.

References

1. Mateo, C.; Palomo, J. M.; Fernandez-Lorente, G.; Guisan, J. M.; Fernandez-Lafuente, R.; *Enzyme Microb. Technol.* **2007**, *40*, 1451.
2. Prlainović, N. Ž.; Bezbradica, D. I.; Knežević-Jugović, Z. D.; Stevanović, S. I.; Avramov Ivić, M. L.; Uskoković, P. S.; Mijin, D. Ž.; *J. Ind. Eng. Chem.* **2013**, *19*, 279.
3. Sheldon, R. A.; Pelt, S. V.; *Chem. Soc. Rev*. **2013**, *42*, 6223.
4. Ansari, S. A.; Husain, Q.; *Biotechnol. Adv*. **2012**, *30*, 512.
5. Rebelo, L. P.; Netto, C. G. C. M.; Toma, H. E.; Andrade, L. H.; *J. Braz. Chem. Soc*. **2010**, *21*, 1537.
6. Spahn, C.; Minteer, S. D.; *Recent Pat. Eng*. **2008**, *2*, 195.
7. Khan, A. A.; Alzohairy, M. A.; *Res. J. Biol. Sci.* **2010**, *5*, 565.
8. Tran, D. N.; Balkus, J. J.; *ACS Catal.* **2011**, *1*, 956.

9. Datta, S.; Christena, L. R.; Rajaram, Y. R. S.; *3 Biotech* **2013**, *3*, 1.
10. Pavlidis, I. V.; Tsoufis, T.; Entotiadis, A.; Gournis, D.; Stamatis, H.; *Adv. Eng. Mater*. **2010**, *12*, 179.
11. Hong, S. G.; Kim, H. S.; Kim, J.; *Langmuir* **2014**, *30*, 911.
12. Datsyuk, V.; Kalyva, M.; Papagelis, K.; Parthenios, J.; Tasis, D.; Siokou, A.; Kallitsis, I.; Galiotis, C.; *Carbon* **2008**, *46*, 833.
13. Peng, Y.; Liu, H.; *Ind. Eng. Chem. Res*. **2006**, *45*, 6483.
14. Marzuki, N. H. C.; Mahat, N. A.; Huyop, F.; Buang, N. A.; Wahab, R. A.; *Appl. Biochem. Biotechnol.* **2015**, *177*, 967.
15. Chen, J.; Chen, Q.; Ma, Q.; *J. Colloid Interface Sci*. **2012**, *370*, 32.
16. Boncel, S.; Zniszczol, A.; Szymanska, K.; Mrowiec-Bialon, J.; Jarzebski, A.; Walczak, K. Z.; *Enzyme Microb. Technol*. **2013**, *53*, 263.
17. Mohamad, N. R.; Buang, N. A.; Mahat, N. A.; Lok, Y. Y.; Huyop, F.; Hassan, Y.; Aboul-Enein, H. Y.; Wahab, R. A.; *Enzyme Microb. Technol.* **2015**, *72*, 49.
18. Zhao, D.; Xun, E.; Wang, J.; Wang, R.; We, X.; Wang, L.; Wang, Z.; *Biotechnol. Bioprocess Eng.* **2011**, *16*, 638.
19. Bolivar, J. M.; Mateo, C.; Godoy, C.; Pessela, B. C. C.; Rodrigues, D. S.; Giordano, R. L. C.; Fernandes-Lafuente, R.; Guisan, J. M.; *Process Biochem*. **2009**, *44*, 756.
20. Gomez, J. M.; Romero, M. D.; Fernandez, T. M.; *Catal. Lett.* **2005**, *101*, 275.
21. Tavares, A. P. M.; Silva, C. G.; Drazic, G.; Silva, A. M. T.; Loureiro, J. M.; Faria, J. L.; *J. Colloid Interface Sci.* **2015**, *454*, 52.
22. Chiou, S. H.; Wu, W. T.; *Biomaterials* **2004**, *25*, 197.
23. Perez, V. H.; Silva, G. S.; Gomes, F. M.; de Castro, H. F.; *Biochem. Eng. J*. **2007**, *34*, 13.
24. Pereira, E. B.; Zanin, G. M.; Castro, H. F.; *Braz. J. Chem. Eng*. **2003**, *20*, 343.
25. Kapoor, M.; Gupta, M. N.; *Process Biochem*. **2012**, *47*, 555.
26. Cho, B. T.; Kang, S. K.; Kim, M. S.; Ryu, S. R.; Na, D. K.; *Tetrahedron* **2006**, *62*, 8164.
27. Omori, A. T.; Portas, V. B.; Oliveira, C. S.; *Quim. Nova* **2012**, *35*, 435.
28. Bradford, M. M.; *Anal. Biochem.* **1976**, *72*, 248.
29. Chen, C. S.; Fujimoto, Y.; Girdaukas, G.; Sih, C. J.; *J. Am. Chem. Soc*. **1982**, *104*, 7294.
30. Carlsson, N.; Gustafsson, H.; Thor, C.; Olsoon, L.; Holmberg, K.; Akerman, B.; *Adv. Colloid Interface Sci.* **2014**, *205*, 339.
31. Gustafsson, H.; Johansson, E. M.; Barrabino, A.; Odén, M.; Holmberg, K.; *Colloids Surf., B* **2012**, *100*, 22.
32. Gomes, F. M.; de Paula, A. V.; Silva, G. S. S.; Castro, H. F.; *Quim. Nova* **2006**, *29*, 710.
33. Lou, W. Y.; Zong, M. H.; Zhang, Y. Y.; Wu, H.; *Enzyme Microb. Technol.* **2004**, *35*, 190.
34. Fadiloglu, S.; Soylemez, Z.; *J. Agric. Food Chem*. **1998**, *8*, 3411.
35. Wang, Y. D.; Chen, Z.; Chen, P.; Jin, L.; Cheng, Y.; Zhoub, J.; Cao, S.; *J. Mol. Catal. B: Enzym*. **2007**, *48*, 51.
36. Calvaresi, M.; Zerbetto, F.; *Acc. Chem. Res*. **2013**, *46*, 2454.
37. Lehman, J. H.; Terrones, M.; Mansfield, E.; Hurst, K. E.; Meunier, V.; *Carbon* **2011**, *49*, 2581.
38. Turner, N. A.; Vulfson, E. N.; *Enzyme Microb. Technol.* **2000**, *27*, 108.

Submitted: October 20, 2017
Published online: March 23, 2018