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Research Article

Immobilization of *Thermomyces lanuginosus* lipase through isocyanide-based multi component reaction on multi-walled carbon nanotube: application for kinetic resolution of rac-ibuprofen

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ABSTRACT

Thermomyces lanuginosus lipase (TLL) was immobilized on epoxy functionalized hydroxyl multi-walled carbon nanotube (MWCNT-OH) via an isocyanide-based multicomponent reaction. The immobilization process was carried out in deionized water (pH 7.0) at room temperature resulting in loading of 600 mg enzyme/g of support with specific activity 16.9 U/mg. An immobilization yield of 100% was obtained with the expressed activity 60%. The immobilized preparation exhibited an increased thermal stability with 49% residual activity at 75 °C compared with 19% for the free enzyme at the same temperature. Solvent stability in a high ratio of DMSO was improved from 52% in free TLL to 75% in immobilized TLL. The immobilized preparation was used for kinetic resolution of rac-ibuprofen through esterification of ibuprofen in isooctane as solvent. The best result was obtained with ethanol at 45 °C and molar ratio of 2.5:1 ethanol:ibuprofen in 1 ml isooctane with 99% ee_p and E-value 300.

1. Introduction

One of the most important goals in pharmaceutical science is the preparation of chiral pharmaceuticals as single enantiomers. Ibuprofen, which is one of the important members of the non-steroidal anti-inflammatory drugs (NSAIDs), is available mainly in form of a racemate in pharmaceutical industry. However, pharmacological activity mainly belongs to *S*(+)-enantiomer [1]. (*S*)-ibuprofen was reported to be 160 times more reactive in their analgesic effect than (*R*)-ibuprofen [2]. This fact signifies the importance of developing methods to separate the enantiomers of a racemic mixture to provide pure drugs with minimum side effects. For this purpose, various methods have been reported by some researches including chiral chromatography, membrane separation, enantioselective liquid-liquid extraction (ELLE) and enzymatic kinetic resolution. Chiral liquid chromatography is an effective and useful method applied in many industries but it needs a large amount of solvents and high-voltage instruments which make it a relatively costly method [3]. ELLE is an efficient and low-cost method but its main disadvantage includes a low operative selectivity and low versatility [4]. Enzymatic kinetic resolution is an efficient method for the separation of chiral compounds, in which, enzymes that are the most perfect natural catalysts, are responsible for the selective and in some cases, specific substrate-catalyst interactions at an increased rate [5, 6].

Lipases (triacylglycerol acyl hydrolase, EC 3.1.1.3) are a class of enzymes which catalyze the hydrolysis of long chain triglycerides. They belong to the serine hydrolase class and do not require cofactors. Lipases are ideal biocatalysts for the resolution of racemic esters and alcohols, because they accept a wide range of non-natural substrates, they are stable and active in organic solvents, do not require cofactors, and are readily available from several microorganisms [7, 8]. The mechanism of action of lipases in both hydrophilic and hydrophobic environments is explained by their interfacial activation. Lipases have two different conformations including the closed form, where the active site is isolated from the reaction medium by mobile polypeptide chain referred to as the "lid", and the open form, where the lid moves upon contact with a hydrophobic surface and the active site is fully exposed to the reaction

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medium [9–11].

In organic solvents, which solubility of hydrophobic substrates increases, lipases maintain their enantioselectivity [12, 13]. Possible approaches to the enzymatic resolution of chiral ibuprofens include the enantioselective hydrolysis [14–16], transesterification [17] as well as direct esterification in non-aqueous medium using microbial lipase [18, 19]. One of key advantages of lipase-catalyzed kinetic resolutions in organic media is that they can be performed at high substrate concentration where a complete specificity should be expected, via hydrolysis, esterification, amination, transesterification, etc. These exceptional features of enzymes have made enzymatic kinetic resolution of racemic mixtures practical for industrial applications. However, in order to apply this method effectively, some parameters are needed to be considered including the enzyme's sensitivity to temperature and its reusability and activity under various conditions. In this regard, immobilization of enzymes is a promising method for increasing the enzymes resistance to temperature as well as its reusability. Immobilization may greatly alter enzyme specificity and/or selectivity [20]. In some reactions such as the resolution of racemic mixtures, as a result of the changes in K_M or K_{cat} towards one or both enantiomers, the enantioselectivity of the enzyme and the obtained enantiomeric excess may be affected significantly [9, 21].

A new field in enzyme immobilization based on three component reaction was introduced by Mohammadi et al in 2016 [22]. They reported a fast and efficient lipase immobilization on epoxy-functionalized silica based on three functional groups (epoxide, isocyanide and carboxylic acid) that was proposed for the first time in 2003 by Kern [23]. Shahedi et al. in 2021 [2] reported immobilization of *Candida antarctica* lipase (CALB) and *Rhizomucor miehei* lipase (RML) on epoxy functionalized silica with the same approach. Also, Salami *et al.* in 2018 immobilized laccase (a multicopper oxidase) by this method [24].

Different supports are used for immobilizing enzymes such as iron oxide-silica core-shells [25], arabic gum and carbon nanotubes [26–28]. In recent years, carbon-based materials such as graphene, carbon nanotubes (CNTs), and activated carbons have been widely used as supports for immobilization of enzymes [29, 30]. These materials provide high surface areas which allow high immobilization yield compared with other organic and inorganic supports. They also have high stability against organic solvents and high temperatures. In addition, their surfaces can be modified through oxidation process in acidic media, for introducing the hydroxyl, epoxide, carbonyl, and carboxyl groups. Mohammadi and co-workers reported immobilization of RML on the carboxylated graphene sheets (GrCOOH) and multi-wall CNTs (MWCNTs-COOH) by Ugi four component reaction. Initial activity of both immobilized enzymes was higher than native enzyme [22].

Epoxy-functionalized supports are relatively ideal systems to develop simple protocols for enzyme immobilization. However, epoxy groups are hardly reactive for enzyme immobilization under mild experimental conditions such as neutral pH and low ionic strength [31–33]. The immobilization on epoxy-functionalized supports takes place in a two-step procedure: i) the physical adsorption of the enzyme on the support [34–36]; in this step, high ionic strength is often required to drive the hydrophobic adsorption of the proteins on the supports, ii) previously adsorbed enzyme is attached covalently to the epoxy groups on the surface of the support surface by N-C or O-C bonds formation [37].

In this work, the covalent attachment of TLL on epoxy-functionalized support, takes place via isocyanide-based multi component reaction in one step without the need for physical pre-adsorption of the enzyme. Using multicomponent method in deionized water, the enzyme immobilization proceeds more rapidly at lower ionic strength, while in conventional methods, the immobilization requires higher ionic strength and is carried out in longer periods of time.

The lipase from *Thermomyces laguginosus*, a quite stable enzyme with high activity is used in most of the reaction media. The enzyme has many different industrial applications, from biodiesel production and modification of fats and oils to fine chemical processes such as enantio- or regioselective synthesis or hydrolysis of prochiral esters. Stabilization of TLL by intense multipoint covalent attachment between the enzyme and the support, increases the rigidity of the enzyme's structure and results in an increased stability of enzyme against any inactivating reagent [38, 39].

It is noteworthy that esterification is a well-known thermodynamically controlled reaction and the enzymatic synthesis of esters in aqueous solutions is unfavorable due to the competing hydrolysis reaction. Enzyme-catalyzed synthesis in dry organic solvents, addition of one of the substrates in excess or the in-situ removal of water as side product, are among the practical solutions for maximizing ester synthesis and minimizing the reverse hydrolysis reaction. As reported by Sousa et al., the use of hydrophobic supports an ultrasonication may reduce the adverse effect of accumulation of water inside the biocatalyst particles [40].

According to the results by Kern and our previous reports [2, 22, 23, 41], TLL was immobilized on epoxy functionalized hydroxyl multi-walled carbon nanotube by the proposed mechanism (Fig. 1).

The first step includes the conventional epoxy functionalization of the carbon nanotube. In the next step, the nucleophilic attack of isocyanide results in opening of the epoxide ring and a carboxylic acid group from the enzyme reacts simultaneously with isocyanide to form an activated carboxylic acid ester **1** which finally forms the immobilized derivative **2**.

In this study, the enzymatic kinetic resolution of ibuprofen using TLL immobilized on MWCNT-OH was investigated. (*S*)-Ibuprofen was separated through selective enzymatic resolution with a significant enantiomeric excess of 99%.

2. Experimental

2.1. Materials

Hydroxylated multiwalled carbon nanotube (MWCNT-OH: OD < 8nm, length: 30 μm, specific surface area > 500 m^2g^{-1}, purity > 95 wt%) was purchased from Neutrino company and used as support for enzyme immobilization. Triethylamine, cyclohexyl isocyanide and solvents including toluene, chloroform, THF, DMSO, isooctane, methanol, ethanol, propanol and butanol were provided by Merck. Lipase enzyme from *Thermomyces lanuginosus* (TLL), p-nitrophenyl butyrate (p-NPB) and the epoxy linker (3-Glycidyloxypropyl) trimethoxysilane (GPTMS) were purchased from Sigma and other chemicals used were all analytical grade.

2.2. Epoxy functionalization of MWCNT-OH

The hydroxylated multiwalled carbon nanotube (50 mg) was dispersed in toluene (20 ml) and ultrasonicated for 20 minutes. Then, triethylamine (50 μl) was added to the dispersed solution. The resulting solution was refluxed under inert N_2 atmosphere for 15 min using a magnetic stirrer. After that, GPTMS (500 μl) was added to the mixture. After 3 hours, the solution was washed using water and ethanol and centrifuged for 5 times. The black solid was then heated in oven at 80 oC for 4 hours to completely remove the volatile compounds from the support.

2.3. Immobilization of TLL on epoxy-functionalized MWCNT-OH

For the immobilization of TLL on epoxy-functionalized MWCNT-OH, 5 mg of the support was added to 1 ml deionized water (pH 7.0) and ultrasonicated for 20 minutes to optimize the dispersion of the support in the solution. 1, 2 and 3 mg of TLL solution (16 mg ml^{-1}) was then added to the three individual solutions on a magnetic stirrer (400 rpm). After that, 14, 16 and 18 μl of cyclohexyl isocyanide was added respectively, at room temperature (25 oC). Finally, the prepared

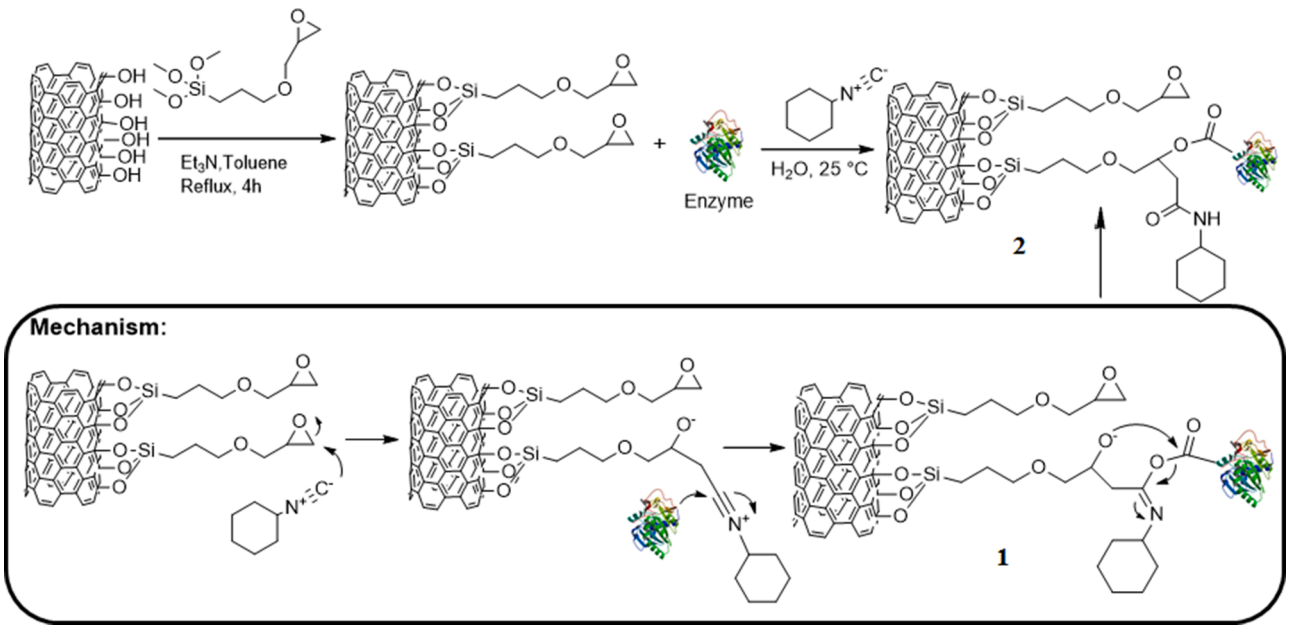

Fig. 1. Preparation of epoxy-functionalized MWCNT-OH and immobilization of TLL by multicomponent reaction

biocatalysts were rinsed by ethanol and deionized water and separated by centrifugation.

2.4. Assessing the enzymatic activity

The activity of TLL and the prepared biocatalysts was estimated using UV-visible spectroscopy based on the increment in absorbance at 410 nm due to release of *p*-nitrophenol through hydrolysis of *p*-NPB in buffer solutions (sodium phosphate buffer, pH 7, 25 oC). 125 µl of the lipase solution (16 mg ml^{-1}) was added to 1 ml deionized water under vigorous stirring as the blank solution. 10 µl of *p*-NPB (0.8 mmol l^{-1}) was added to the buffer solution. Hydrolysis was followed by measuring the change in absorbance over 2 min (0-120 s, 15 s time intervals). All experiments were performed in triplicate. All data are expressed as the mean ± standard deviation. The immobilization yield and expressed activity were calculated according to Boudrant et al [42].

2.5. Amount of TLL immobilized on epoxy-functionalized MWCNT-OH

Bradford's method is a well-known method to determine the amount of the protein (enzymatic or non-enzymatic) in the solution [43]. The yields of immobilizations were calculated based on the ratio of the protein attached to the support to the initial amount of the protein (equation 1). B_0 is initial and B_1 is residual protein concentration.

$$\text{Theyieldsofimmobilization}(\%) = [B_0 - B_1 / B_0] \times 100 \quad (1)$$

2.6. Leaching experiment

5 mg biocatalyst (MWCNT-TLL) was added to an aqueous solution of NaCl (1 M). This solution was then stirred for 24 hours on a magnetic stirrer. The amount of the protein released into the solution was determined by both the Bradford and the enzymatic activity methods.

2.7. Thermal stability of the free and immobilized TLL

Thermal stability of free TLL and its immobilized preparations in aqueous solution was studied through incubating each sample for 2 hours at temperatures ranging from 40 to 70 oC.

2.8. Solvent stability of the free and immobilized TLL

In order to study the stability of the aqueous enzyme solutions in presence of organic co-solvents (THF, propanol, DMSO and 1,4-dioxane), various amounts of each co-solvent (10, 20 and 50% v/v) were added to the aqueous solution (pH = 7) of the enzyme on a magnetic stirrer (200 rpm) for 24 hours. The free solution contained 2 mg TLL, and the biocatalyst also contained 2 mg TLL immobilized on 5mg support. The final volume of the solutions was 2 ml. The activity of each solution was estimated using activity assay method explained in section 2-4.

2.9. Esterification of racemic ibuprofen using immobilized TLL

Stereoselective esterification of racemic ibuprofen was evaluated by TLL in isooctane using methanol, ethanol, 1-propanol and 1-butanol at two temperatures 25 and 45 oC. In order to establish the optimum conditions for successful esterification, various sets of reactions were carried out using different amounts of solvent, biocatalysts and alcohols. Each reaction operated at temperature 25 and 45 oC with different alcohol: ibuprofen molar ratios. two reaction conditions are shown in Table 1. The first set of reactions ended up with no significant products. Therefore, the other reaction set was run with the second optimized condition at 45 oC. The reactions were carried out in 5 mL screw-capped vials containing anhydrous isooctane (1-2 mL), racemic ibuprofen (10 mM) and alcohol (20-25 mM) as acyl donor, with certain amounts of biocatalysts. The quantitative analysis of the ester products was carried out by gas chromatography (GC).

The certain amounts of immobilized TLL (2.5-10 mg) were added to the reaction vessel and the reaction mixture was stirred (200 rpm) for 24-72 h at 25 and 45°C. Samples of 100 µL of the solution were withdrawn at different times without dilution. The amount of ester (conversion degree) formed during the reaction and the enantiomeric excess

Table 1
The reaction conditions in two sets of the reactions

Entry	Biocatalyst (mg)	Isooctane (ml)	Alcohol: Ibuprofenmolar ratio	T (oC)
1	2.5	2.0	2:1	25
2	10	1.0	2.5:1	45

of the (S)-enantiomer were determined by gas chromatography (GC).

2.10. Gas chromatography analysis

The analysis was performed using a Thermoquest-Finnigan (USA) gas chromatograph equipped with flame ionization detector (FID) and a HP-CHIRAL-20B column (30 m × 0.32 mm × 0.25 μm). The temperature was kept at 260 oC for the injector and 300 oC for the detector. The column temperature was programmed to increase from 100 to 178 oC at a rate of 10 oC per minute in a 15-minute run-time. The flow rate of the carrier gas (H_2) was set to be 0.7 ml/min. All experiments were performed in triplicate.

2.11. Quantitative analysis

The main parameter of the enzyme-catalyzed reactions, enantiomeric excess (ee%), indicates the enantiomeric purity of the product in the final solution. The degree of conversion is calculated using equation 2:

$$c = \frac{ees}{ees + eep} \quad (2)$$

the enantioselectivity (E) is then calculated by equation 3:

$$E = \frac{\ln[1 - c(1 + eep)]}{\ln[1 - c(1 - eep)]} = \frac{\ln[(1 - c)(1 - ees)]}{\ln[(1 - c)(1 + ees)]} \quad (3)$$

3. Results and discussion

3.1. Preparation, functionalization and characterization of the support

The hydroxyl-functionalized multiwalled carbon nanotube (MWCNT-OH), was used as support for enzyme immobilization and further experiments. The support was first functionalized using GPTMS to improve the immobilization efficiency through a multicomponent reaction involving isocyanide in aqueous solution.

In this strategy, nucleophilic attack of the isocyanide group to epoxy group followed by the attack of enzyme to this very reactive intermediate leads to an efficient immobilization with high yield (600 mg/g support) in a short time (12 h) relative to immobilization without isocyanide group which takes place in more than 24 h with low immobilization yield (10 mg/g support) [44].

The crystalline property of the support during functionalization and immobilization steps was assessed using XRD analysis. The similar spectral pattern in all steps reveals the intact structure of the MWCNT-OH during the immobilization process (Fig. 2).

The shape and morphology of the support was also studied during the three stages (Fig. 3). Images indicate that functionalization and

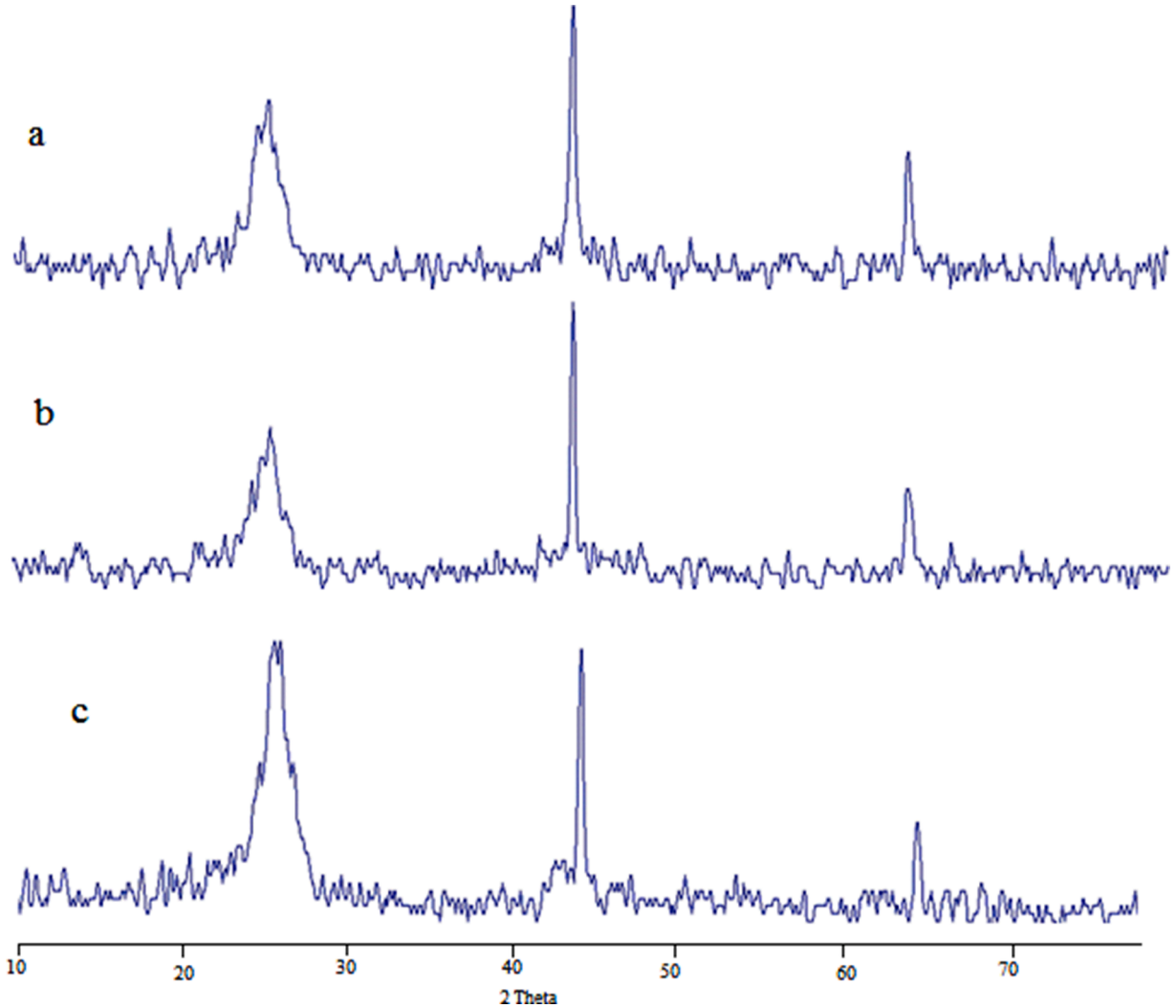

Fig. 2. XRD pattern of (a) MWCNT-OH, (b) epoxy functionalized MWCNT-OH and (c) MWCNT-TLL.

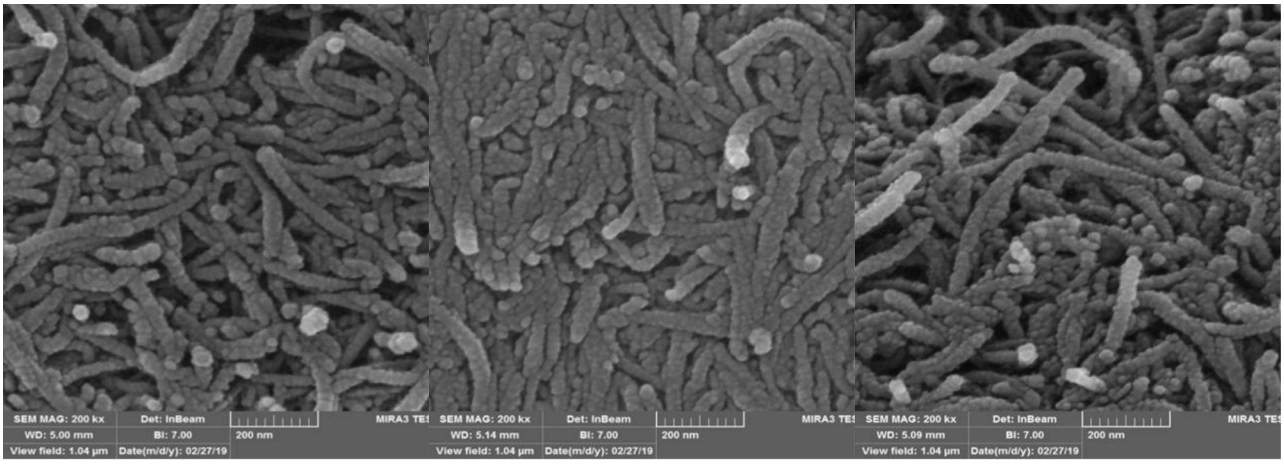

Fig. 3. FE-SEM images of (a) MWCNT-OH, (b) epoxy functionalized MWCNT-OH and (c) MWCNT-TLL.

immobilization of the enzyme on the support alter neither its shape nor its morphology, thus approving the applicability and effectiveness of the immobilization method.

3.2. Immobilization of TLL on the epoxy-functionalized MWCNT-OH through a multicomponent reaction

TLL was immobilized on the epoxy-functionalized CNT in an aqueous solution using various amounts of isocyanide. 200, 400 and 600 mg TLL/g support were successfully obtained according to the offered enzyme in different conditions described in Table 2 after 3-12 hours. The maximum capacity of the support was 600 mg of the enzyme per gram of the support, but after the evaluation of activity of different amounts of enzyme on the support, the highest specific activity was obtained in 400 mg/g. With this method, the immobilization yield in all amounts of the offered enzyme was 100%, and the expressed activity was around 61.7-64.5 %.

The prepared biocatalyst was also studied in desorption tests. According to the results of the activity test after the treatment, no enzyme was leached into the solution, confirming the strong covalent attachment of the enzyme to the support.

3.3. Thermal resistance of the free and immobilized enzyme

To study the thermal stability of the biocatalyst, the immobilized and free enzyme samples were incubated at various temperatures (45, 50, 55, 60, 65 and 70 oC) under stirring (200 rpm) for 2 hours. The activity test was used for each sample and compared to the activity of the enzyme at room temperature. The results are shown in Fig. 4. According to the results, both free and immobilized TLL preparations were completely active at 45 oC. The immobilized TLL retained its complete activity at 55 oC, but free TLL shows 91% of its original activity at the same temperature. At 75 oC, the activity of free TLL drops to 25.6 % while the immobilized TLL retains 53.2 % of its original activity. As it

Table 2
Immobilization of TLL on epoxy-functionalized MWCNT-OH

Offered enzyme (mg/g)	Time (h)	Immobilized amount(mg/g)	Specific activity (U/mg)	Expressed activity (%)
Free TLL	-	-	27.4 ± 0.5	-
200	3	200	17.1 ± 0.3	62.4
400	5	400	17.7 ± 0.7	64.5
600	12	600	16.9 ± 0.6	61.7

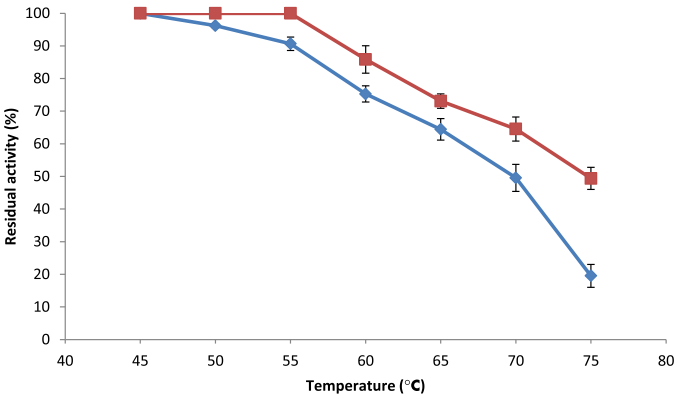

Fig. 4. Thermal stability of the free (◆)and immobilized enzyme)ν(

can be seen, the immobilization has significantly increased the thermal resistance of the enzyme, confirming the potential applicability of the immobilized enzyme at higher temperatures for more reactions over a wide range of temperatures.

3.4. The stability of the free and immobilized TLL in the presence of various organic solvents

Immobilization of enzymes may induce modifications in their activity, specificity or selectivity [20]. In most instances, immobilized enzymes exhibit lower catalytic performance due to alterations made in enzyme's structure (distortion of the enzyme due to the interaction with the support). However in some instances, enzyme's properties may be enhanced by immobilization.

In order to study the changes in the structure and behavior of TLL in various solvents and mixtures, individual solutions containing the enzyme and the biocatalyst were prepared as mixtures of deionized water and various volume fractions (10, 20 and 50% v/v) of propanol, THF, DMSO and dioxane as organic co-solvents. For all solvents, the 50% v/v of organic solvent caused a decrease in the activity of the free and immobilized enzyme with the highest deactivation for THF (Fig. 5). In all other fractions, no significant decrease was observed for the immobilized TLL, whereas free TLL deactivation was observed at 50:50 v/v of propanol:H_2O, 20:80 v/v DMSO:H_2O and 50:50 v/v of dioxane:H_2O. For all solvents except THF (50:50 v/v), a higher stability was observed for the immobilized preparations, indicating the increased stability of the enzyme in the immobilized state and therefore

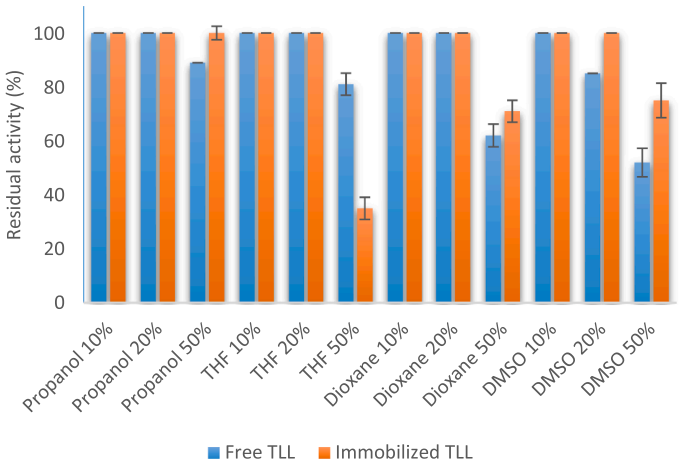

Fig. 5. Assesing the activity of free and immobilized enzyme in organic solvents

confirming the wide range of conditions at which the biocatalyst can be operative.

3.5. Kinetic resolution of rac-ibuprofen using TLL immobilized on the epoxy functionalized MWCNT-OH

The immobilized preparation was used to study its catalytic ability in the enantioselective esterification of the racemic ibuprofen using alcohol substrates (methanol, ethanol, propanol and butanol). The enantiomeric excess (ee%) and the E-values of representative reactions are shown in Table 3. The results clearly demonstrate that the immobilized TLL favor the interactions with (R)-Ibuprofen.

According to the results, methanol revealed the lowest efficiency and enantioselectivity in TLL-catalyzed ibuprofen esterification. The rest of the alcohols indicated high activity and low selectivity in esterification reaction, implying that both ibuprofen enantiomers were esterified at high amounts. The best results were obtained for ethanol at 45 oC and 2 ml of solvent used with molar ratio of 2.5:1 ethanol:ibuprofen. (R)-ibuprofen was esterified by 99% and (S)-remained almost entirely intact.

3.6. Reuse of the biocatalyst

Immobilization of an enzyme is a requisite for its application as an industrial biocatalyst in most areas such as pharmaceutical chemistry, food modification and energy production [45]. Immobilization enables the simple reuse of the enzyme and simplifies the overall design and performance control of the bioreactors. Experiments investigating the reusability of the biocatalyst indicate that immobilized TLL on epoxy functionalized carbon nanotube could be used repeatedly. As illustrated in Fig. 6, the immobilized TLL can hydrolyze batches of p-NPB while retaining 81.0% of its initial activity after 3 cycles, and 63.7% of its initial activity after 6 cycles.

4. Conclusions

Immobilization of enzymes has been recognized as a promising and efficient method for directing specific chemical reactions in racemic environments. In order to separate ibuprofen enantiomers efficiently, a biocatalyst was prepared using multiwalled carbon nanotube as a support for immobilization of *Thermomyces lanuginosus* lipase (TLL) through an isocyanide-based three-component reaction. The support demonstrated a significant capacity of enzyme loading (600 mg/g). The desorption test also proved the strong and irreversible covalent enzyme immobilization. The immobilized enzyme was indicated to be stable and active over a wide range of temperatures and in various organic solvents. This method of immobilization is applicable to an extensive range of supports and enzymes. The immobilized preparations were used in enantioselective esterification of racemic ibuprofen with ethanol and other alcohols in anhydrous isooctane under various conditions. The best result was obtained by increasing the amount of the biocatalyst and its enzyme specific content at 45 oC, providing a 99% stereoselectivity of (R)-ibuprofen esterified with ethanol in 24 hours. According to the high enantioselectivity of the designed reaction, high stability of epoxy functionalized MWCNT-OH as support and the promoted stability of the prepared biocatalyst under various conditions, shorter reaction time and availability of ethanol as the esterifying reagent, this research work could be considered as a promising method for the purpose of purification of racemic ibuprofen both in the laboratory and in industry.

Table 3
Quantitative analysis of the kinetic resolution of rac-ibuprofen catalyzed by MWCNT-TLL

Alcohol	Reaction time (h)	Esterified (S)-ibuprofen	Esterified (R)-ibuprofen	ee_s (%)	ee_R (%)	E
Methanol	48	24	16	13	20	1.69 ± 0.3
Ethanol	24	0.1	49.9	99	96	307.6 ± 13
Propanol	48	31	46	65	19.4	2.61 ± 0.4
Butanol	72	44	47	31.8	3	1.30 ± 0.2

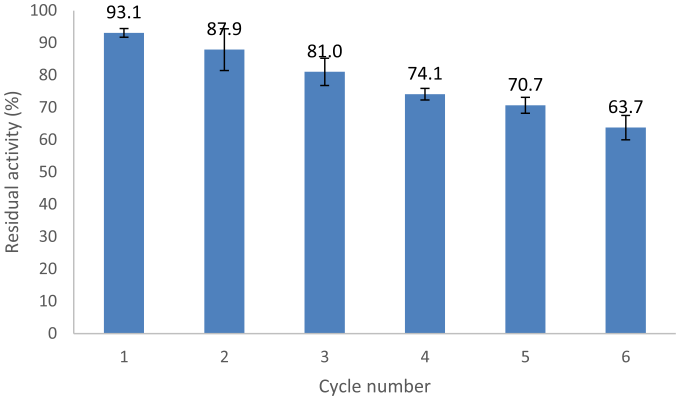

Fig. 6. Reusability of immobilized TLL.

Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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