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One pot three-component reaction for covalent immobilization of enzymes: Application of immobilized lipases for kinetic resolution of *rac*-ibuprofen

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Abstract

This paper presents a novel strategy for the simple immobilization of biomolecules on epoxy-functionalized supports via a one-pot three component reaction. Lipase B from *Candida antarctica* (CALB) and *Rhizomucor miehei* lipase (RML) as model enzymes were immobilized on epoxy-functionalized silica and mesoporous silica nanoparticles (SBA-15). Investigation on mechanism of this reaction confirmed the participation of three functional groups including carboxylic acid groups from the enzyme surface, epoxy groups from the support and isocyanide from the reaction medium. The results revealed very rapid immobilization of 10 and 40 mg of RML on 1 gr of the supports shortly after 30 minutes of incubation. The loading capacity of the supports was also dramatically improved with the maximum loading of 158 mg of CALB and 77 mg of RML on SBA-15 and silica, respectively. Silica-epoxy-RML showed an enantiomeric excess (*ee*) of 92% and E-value of 29.9 in the enantioselective esterification of (*R*, *S*)-ibuprofen.

KEYWORDS

Covalent immobilization, *Candida antarctica* Lipase B, *Rhizomucor miehei* lipase, Three component reaction, Epoxy-functionalized supports, Enantioselective resolution

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Introduction

Industrial applications of enzymes remain controversial because of their poor stability and difficulty of recovery of soluble enzyme from the reaction medium[1]. Immobilization of enzymes on solid supports greatly enhances their catalytic performances in terms of selectivity, activity, thermal stability, tolerance to high pH values and organic solvents to meet the demands of practical uses[2-5]. Many enzyme immobilization techniques such as adsorption, entrapment and covalent binding have been employed and published so far[6-12]. The most important factors that should be taken into account in the selection of immobilization strategy include: simplicity of the method, good catalytic activity, stability and reusability of the obtained immobilized enzyme.

Among all the strategies used for enzyme immobilization onto the solid supports, covalent immobilization meets the demand of many practical and industrial applications of biocatalysts [13]. This method has advantages of no leaching of enzyme from the support, wide choices of organic linkers and established methods of functionalization and modification of the supports [14]. However reduction or even loss of catalytic activity resulting from conformational changes of the enzyme may take place during covalent attachment [15].

As a carrier for enzyme immobilization, silica-based materials have been explored extensively because of their high stability to chemical forces, porous structures with high capacity of enzyme loading [16, 17]. However, the only existing groups on the surface of silica are hydroxyl groups which are difficult to form chemical bonds with enzyme molecules directly. Therefore, silica supports are always modified with various functional groups to generate chemical bonds with enzymes. Numerous functional groups, including amines, chlorides, thiols, carboxylic acids and epoxy groups have been attached successfully to the surface of siliceous materials [18, 19]. These groups subsequently provide different interactions between the surfaces of the support and the enzyme molecules. By examining the functional

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groups on the surface of an enzyme molecule, a suitable "counter-functional group" on the surface of the support could provide strong interaction for immobilization.

Epoxy-functionalized supports have been widely used for immobilization of different enzymes[20-22]. These activated supports are very stable at neutral pH values even in wet conditions and can be stored for a long time. One example of these systems is commercially available Eupergit C which is amongst the most extensively used matrixes for enzyme immobilization either at laboratory or industrial scales [23]. Epoxy groups can react with nucleophilic groups present on the protein surface; in particular with terminal amino groups [24]. However these groups are not highly reactive in mild conditions and hardly immobilize large amounts of proteins. In fact, it has been proposed a two-step binding mechanism for immobilization of enzymes on epoxy-functionalized supports[25]. It is assumed that, the enzyme is physically adsorbed on the carrier in the first step. This brings nucleophile groups (amino, thiol, or hydroxyl groups) on the surface of the enzyme in close proximity to the oxirane groups of the support. In the second step they react with the oxirane groups to form very stable C-N, C-S and C-O bonds. This two-step mechanism for the covalent immobilization of proteins on epoxy-functionalized supports usually needs long immobilization time. For example in our previous investigation, immobilization of RML on epoxy-functionalized silica using traditional nucleophilic reaction was carried out after 24h of incubation [26]. Mateo and co-workers reported immobilization of Penicillin G acylase on Sepabeads-EP (an epoxy support) after 20 h [25]. In another study, the maximum loading of 14 wt % of CALB on epoxy-functionalized carriers was observed by 5 h incubation while the main part (34%) was the physically adsorbed enzyme [22]. Despite interesting works exploring enzyme immobilization on epoxy-functionalized carriers, it is still a great demand in finding new methods which reduce the immobilization time and also preserve the enzyme activity. Recently we have reported the use of the Ugi four-component reaction for the covalent

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immobilization of RML on aldehyde-functionalized supports [18]. High density of enzyme loading and rapid immobilization were the benefits of using this protocol for enzyme immobilization. Here, we propose the use of a three-component reaction not only for the covalent immobilization of CALB and RML, but also as a very interesting and simple method for rapid attachment of other biomolecules on epoxy-functionalized supports at extremely mild conditions. The idea of using this method comes from the only reported research of Kern and co-worker. They have reported a three component reaction in which the epoxide or aziridine group is used instead of aldehyde in the classical three component Passerini reaction [27]. However, the reaction has been carried out in THF as solvent and in the presence of different catalysts under relatively harsh condition (reflux, 3h).

The immobilized derivatives of CALB and RML were used as biocatalyst for the kinetic resolution of (*R,S*)-ibuprofen [2-(4-isobutylphenyl) propionic acid]. Lipases are suitable enzymes for organic syntheses because of accepting a wide range of non-natural substrates without requiring cofactors, stability and activity in organic solvents and availability from several (micro) organisms [28]. They have the natural catalytic function of hydrolyzing fatty acid ester bonds in water and are the most commonly used enzymes in chemical reactions due to their unique capacity of catalyzing different reactions such as esterification, transesterification and hydrolysis [29]. The idea of using lipases for kinetic resolution of racemates is driven by the growing demand for enantiomerically pure compounds by manufacturers of pharmaceuticals and agrochemicals. 2-Arylpropionic acids (profens) with a stereogenic center are known as one of the most commercially successful and important classes of analgesic anti-inflammatory drugs used in the treatment of headache, rheumatoid arthritis, cephalgia and muscular strain [30]. The (*S*)-enantiomer of ibuprofen has the desired therapeutic effect (160 times more active than its (*R*)-enantiomer) in the in vitro inhibition of

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prostaglandin synthesis, while the (*R*)-ibuprofen is inactive and can cause side effects affecting to the gastrointestinal tract, normal lipids metabolism and membrane function[31, 32].

Experimental

Materials

Ibuprofen was extracted from the readily marketed tablets according to the literature procedure [33]. The lipase from *Rhizomucor miehei* (RML) *and* lipase B from Candida antarctica (CALB) Novozymes (Denmark). *p*-Nitrophenyl butyrate (*p*-NPB), cyclohexyl isocyanide, tetraethyl orthosilicate (TEOS), 1-ethyl-3-(dimethylaminopropyl) carbodiimide (EDC), polyuronic acid and 3-glycidoxypropyltrimethoxylsilane (GPTMS) were from Sigma (Steinhiem, Germany). Silica gel (70 –230 mesh), dioxane, 1-propanol and 2-propanol were purchased from Merck. Other used reagents and solvents were of analytical grade. Fourier transform infrared spectra (FT-IR) were recorded on a Bomen FT-IR-MB-series instrument with a KBr pellet technique. Thermogravimetry (TGA) and differential thermal analysis (DTA) were carried out from 10 °C to 800 °C at a heating rate of 20 °C/min in air atmosphere using a STA 503M system from Bähr GmbH, Germany.

Methods

Preparation of the silica nanoparticles

For preparation of pure siliceous SBA-15, the reported procedure in literature was used [15]. For this purpose, 4 g of pluronic P123 triblock copolymer (EO20–PO70–EO20, BASF) were added to 144 mL of an aqueous solution of HCl (2 M) at 40 °C. Successively, TEOS was added dropwise (mass ratio TEOS/P123= 2:1). After 2 h of stirring, the mixture was transferred to Teflon-lined sealed container and kept at 100 °C for 48 h, and then the white

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solid was filtered. In order to remove the template, the white solid was washed with distilled water and calcined at 550°C.

Functionalization of the supports

The dry silica gel particles (1 g) or mesoporous silica materials (0.5 g) were dispersed in 50 mL of dry toluene; then 1 mL of GPTMS and 200 µL Et_3N were added. The resulting mixture was refluxed under nitrogen atmosphere and vigorous stirred for 4h. The modified supports were collected by filtration and washed thoroughly with THF. Finally the modified particles were dried at 120 °C for 8 h. The successful functionalization of the supports was confirmed by IR spectroscopy and TGA-DTA analysis.

Determination of epoxy groups on the support

In order to determine the epoxy groups on each support, 100 mg of the support was added to 1.5 mL of 1.3 M sodium thiosulphate solution. This solution was titrated by addition of 0.1 M hydrochloric acid until neutralization. Based on the needed amount of hydrochloric acid for maintaining the neutrality of the mixture, the amount of epoxy groups on the support was calculated [34]. The same reaction was performed using unmodified particles as blank.

Enzyme immobilization on the epoxy- functionalized supports

To a solution of distilled water (2 mL, pH 7) and certain amount of RML or CALB (10 - 100 mg per g of silica-epoxy and 40-200 mg per 1 g of SBA-epoxy), 100 mg of the supports was added. In order to start the reaction, 10 µL of cyclohexyl isocyanide was added to the protein solution under gently stirring at 25 °C. For monitoring the reaction, samples from the supernatant were withdrawn periodically, and then analyzed by enzyme activity assay and the Bradford's method [35] to determine the protein concentration. Finally the immobilized RML or CALB derivatives were filtered and washed by distilled water and stored at 4°C.

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Enzymatic activity assay

The activity assay of the soluble lipases and its immobilized preparations were performed spectrophotometrically based on the increment in absorbance at 348 nm ($\in$= 5150 $M^{-1}cm^{-1}$). Releasing of *p*-nitrophenol resulted by the hydrolysis of *p*-NPB in 25 mM sodium phosphate buffer at pH 7 and 25°C made an increment in the absorbance. Briefly, 0.05-0.2 mL of the lipase suspension or solution (blank or supernatant without further dilution) was added to 1.25 mL of substrate solution (0.4 mM) under magnetic stirring [6]. Enzymatic activity is given as 1µmol of *p*-nitrophenol released per minute per mg of the enzyme (IU) under the conditions described above.

Determination of the amount of protein bound to the carriers

Based on the Bradford's method, the amount of protein in supernatant and blank was determined. The ratio of the amount of protein bound on to the support to the initial amount was calculated and reported as the immobilization's yields which were expressed as percentage.

Thermal inactivation of different RML and CALB immobilized preparations

In order to investigate the thermal stability of the immobilized enzymes, the incubation of the free lipases and their immobilized preparations was carried out in 25 mM sodium phosphate (pH 7) at different temperatures. The suspension of each sample was withdrawn periodically and their activities were measured using the *p*-NPB assay.

Co-solvent stability of the free and immobilized preparations of the lipases

To a solution of sodium phosphate buffer (25 mM, pH7, total volume of 1 mL) containing 10% and 20% of dioxane, 1-propanol and 2-propanol, the free enzymes and their immobilized preparations were added and incubated at 25 °C for 24 h. The activity of each sample was measured using the *p*-NPB assay.

Determination of the optimum pH activity

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In order to investigate the optimum pH activity, the soluble lipases and immobilized preparations were added in a total volume of 1 mL solution containing 25 mM sodium phosphate buffer with different pH from 5 to 9 at 25 °C. The activity of each sample was measured using the *p*-NPB assay.

Leaching experiment

In a solution containing 1 M of NaCl and 0.5% of sucrose laurate, 500 milligram of silica-RML (10 mg/g) and SBA-RML (60 mg/g) were added and incubated with vigorous magnetic stirring for 24 h. By using the Bradford's method, the concentration of the enzymes in the supernatant was measured.

Blocking acid residues of RML

To a solution of RML (1 mg/mL, total volume of 10 ml) in distilled water, 500 µl of an aqueous solution of ethanol amine (1 M) was added. Afterwards, 10 mM of 1- ethyl-3-(dimethylaminopropyl) carbodiimide was added to initialize the reaction and the pH was adjusted to 4.7. After 3 h, the reaction mixture was dialyzed in deionized water for a period of 24h.

Blocking amino residues of RML

7 µL of acetaldehyde was added to 1 ml (1 mg/mL) of the RML solution at pH 10 for 24h at 4ºC under vigorous stirring [6]. In order to reduce Schiff bases, 1% of $NaBH_4$ was added under continuous stirring at room temperature for 1.5h. Subsequently, the reaction mixture was dialyzed in deionized water for a period of 24 h.

General procedure for enzymatic esterification

The immobilized enzymes were tested in the esterification of (*R*,*S*)-ibuprofen using 1-propanol as acyl donner. The reaction was performed in 10 mL screw-capped vials containing (*R*, *S*)-ibuprofen (10 mM), 1-propanol (20 mM), anhydrous isooctane (2 mL) and molecular sieves (4 A, 10% w/v). The certain amounts of immobilized preparations were added to the

reaction vessel and the reaction mixture was shaken (200 rpm) for 72 h at 0°C, 25°C and 30°C. Samples of 100 µL of the solution were withdrawn at different times without any dilution. The amount of ester (conversion degree) formed during the reaction and the enantiomeric excess of the *(S)*-enantiomer were determined by gas chromatography (GC).

The recycling experiment was performed with 200 rpm shaking. Samples were centrifuged and washed with isooctane after each cycle, and the same amounts of racemic ibuprofen and 1-propanol were then added.

Chromatographic analysis

Gas chromatography 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). Injector temperature was 260 °C and the detector was 300 °C; oven temperature was maintained at 178 °C. Carrier gas was helium with a flow rate of 0.7 ml/min. An external standard method was employed to quantify the amount of ester and the remaining acid.

Enantioselectivity-value (E-value) calculation

The value of enantioselectivity (E-value) was calculated from the enantiomeric excess of the substrate (ee_s) and the conversion degree (c) according to the equation described by Chen *et al.* [36].

$$c = \frac{ee_s}{ee_s + ee_p} \qquad (1)$$

$$E = \frac{\ln[(1-c)(1-ee_s)]}{\ln[(1-c)(1+ee_s)]} \qquad (2)$$

Results and Discussion

Preparation, functionalization and quantification of the supports

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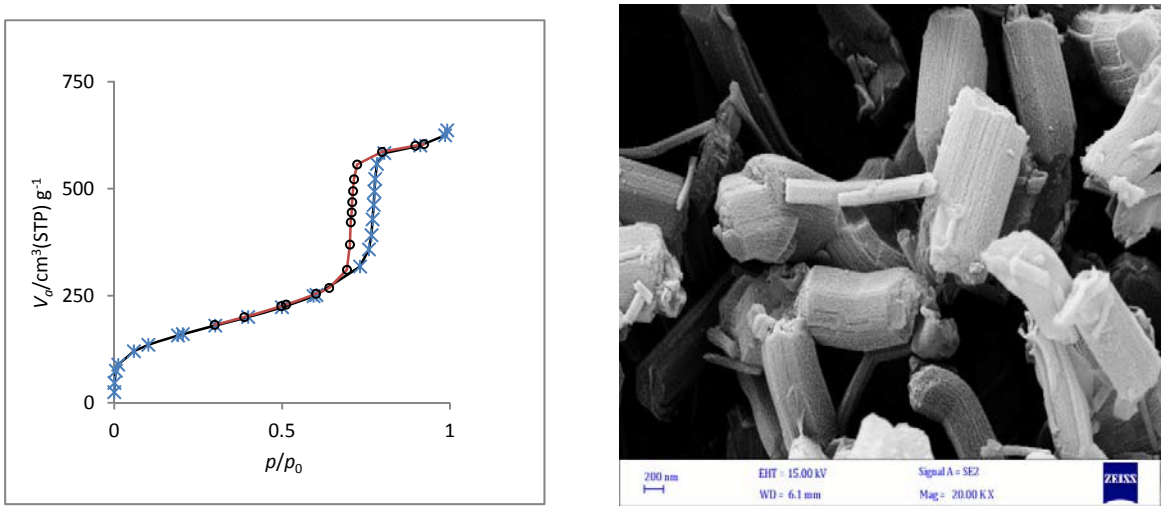
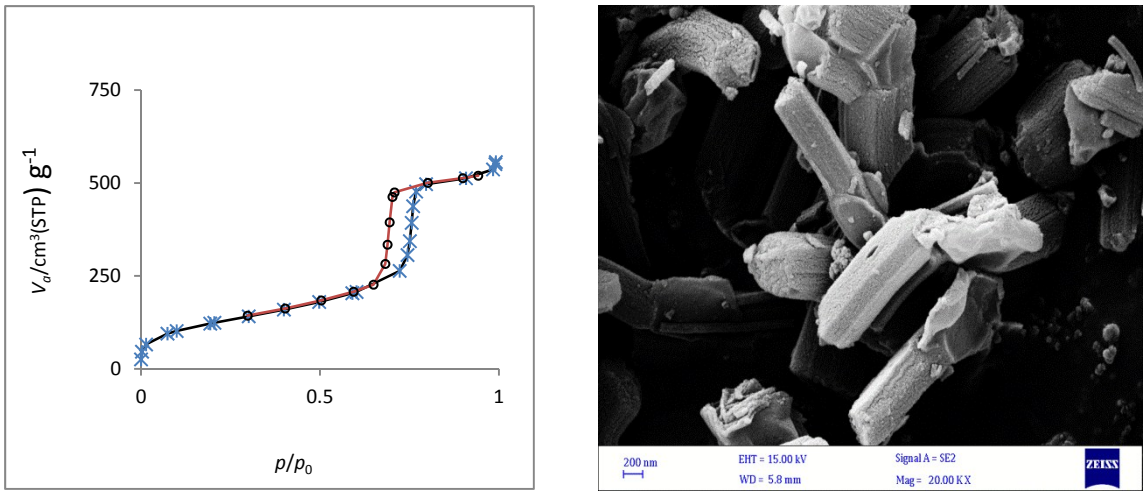
Large-pore SBA-15 was synthesized according to our previously published procedure [15]. The textural properties of the modified and unmodified SBA-15 were assessed by N_2 adsorption–desorption experiments (Fig. 1). The pore size distribution and the pore volume for SBA-15 material is 10.2 nm and 1.21 cm^3/g, respectively and the surface area of SBA-15 is 952 m^2/g calculated by the BET method. After functionalization the surface area, pore volume and the pore size were 615 m2/g, 0.91 cm^3/g and 8.1nm respectively.



(a)



(b)

Fig. 1. Nitrogen adsorption isotherms and scanning electron micrographs for a) SBA-15 and (b) SBA-epoxy-RML.

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Obviously, the decrease in surface area, pore volume and pore diameter of the functionalized SBA-15 is due to the presence of organic groups on the surface of this material. The epoxy functionalization of silica and SBA-15 was carried out using 3-glycidoxypropyltrimethoxylsilane. Functionalization of the supports was confirmed by using FT-IR spectroscopy and TGA analysis (Fig. S1 and S2). For unmodified silica and SBA-15, the broad band between 3200 cm^{-1} and 3600 cm^{-1} could be ascribed to the O–H stretching frequency of silanol groups. Remarkable decrease in intensity of this band after functionalization together with appearance of aliphatic C–H stretch band at 2855-2940 cm^{-1} clearly demonstrate successful functionalization of the supports. The functionalization of silica particles was further confirmed by thermogravimetric analysis (TGA). While the un-functionalized silica and SBA-15 only showed a minor mass loss at 350 °C, the mass loss of 7.7% for silica-epoxy and 11.9% for SBA-epoxy at the same temperature correspond to epoxy residues bound to the supports surface. Quantification of oxirane groups on these supports was performed by titration of the released hydroxide ion from the reaction between epoxy groups on the support and sodium thiosulphate. The results revealed that the amount of epoxy groups on the surface of silica and SBA-15 was about 277 and 805 μmol per gram of each support, respectively. High degree of functionalization of SBA-15 compared to silica can be attributed to more surface area of this support. From the SEM images (Fig. 1) and XRD patterns (Fig. S3) of SBA-15 before and after functionalization, it can also be concluded that the modification still preserving the textural properties of the parent SBA-15.

Immobilization of RML and CALB on silica-epoxy

A novel approach was used to covalent immobilization of RML on epoxy-functionalized supports via an isocyanide-based multicomponent reaction. In this three-component reaction the enzyme supplies carboxylic acid groups and the support supplies epoxy group and isocyanide is added to the reaction medium as third component. This coupling reaction was

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carried out in water at 25 °C, in which high immobilization yield was achieved. Similar to our previous report, negligible immobilization of RML and CALB was observed in sodium phosphate buffer solution even at low ionic strength (5 mM) after long time of incubation. Further investigation is needed to find the reasons of negative effect of impurities on enzyme immobilization in this procedure. As Table 1 shows immobilization of 10 mg of RML on 1 g of silica was performed in 100% yield and produced 3.0 U/mg specific activity. This high immobilization yield was obtained very quickly after only 30 minutes incubation of RML in the presence of the support.

Table 1. Immobilization parameters of CALB and RML

Enzyme derivative	Immoilization time (min)	Immobilization yield[b] (%)	U/mg enzyme[d]
Free RML	---	---	3.3
Free CALB	---	---	6.5
Silica-RML	30	100	3.0
SBA-RML	30	100	3.1
SBA-CALB	200	100	5.1
Silica-CALB	60	100	5.6

[a]Immobilizations were performed as described in the experimental section. 10 mg and 60 mg of the enzymes were offered to 1 g of silica and SBA-15, respectively
[b]Yield is defined as the percentage of the soluble enzyme that becomes attached to the support.
[c]Experimental condition for activity measurement: 1.25 ml of 0.3 mM *p*NPB and 0.05-0.2 ml of lipase solution in 25 mM sodium phosphate buffer at pH 7.0 and 25°C.
[d]Specific activity (U/mg lipase) is expressed as micromole of substrate hydrolyzed per minute per mg of each lipase.

Our previous investigation on the use of traditional nucleophilic method for immobilization of RML on the same support showed 95% immobilization yield after 24h of incubation [26]. The produced specific activity for immobilized RML in this method was about 15% lower than the specific activity of the free enzyme, while by the use of three component reaction for immobilization of RML, almost 9% decrease in specific activity of the enzyme is observed. Immobilization of the same amount of CALB on 1g of silica was also performed in 100%

immobilization yield after 60 minutes. Almost 14% decrease in specific activity was observed for this derivative compared to that specific activity of free CALB. As 100% of the offered enzymes (CALB, RML) were attached to the surface of silica, further investigation was performed to study the influence of isocyanide-based immobilization strategy on loading capacity of this support. Various amounts of RML and CALB ranging from 10-100 mg were used for immobilization on 1g of epoxy-functionalized silica. As the results show (Table 2), the immobilization percentages varied from 77-100% for RML and 75-100% for CALB. In both cases, increasing the amount of offered enzyme causes to decrease in immobilization yield. The maximum loading capacity of silica was determined as 75 mg for RML and 77 mg for CALB.

Table 2. Immobilization of the lipases on silica-epoxy

	Time of immobilization (min)		**Yield[a] (%)**		**mg enzyme/gr support**		**Desorption[b] (%)**	
Offered enzyme (mg/g)	RML	CALB	RML	CALB	RML	CALB	RML	CALB
10	30	60	100	100	10	10	nd	nd
20	45	90	98	100	20	20	nd	nd
30	45	120	97	97	29	29	nd	nd
40	60	300	94	89	38	36	2	2
50	60	360	92	85	46	42	1	1
60	120	360	93	77	56	46	1	1
70	180	1440	88	81	62	57	1	2
100	360	1440	75	77	75	77	1	2

[b]Yield is defined as the percentage of the soluble enzyme that becomes attached to the support.
[b]Desorption was performed in a solution containing 1 M of NaCl and 0.5% of sucrose laurate

Up to date different loading capacities for silica have been reported in literature. For example we have reported the loading of 55 mg of RML on 1 g of aldehyde-functionalized silica.

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Hakob et al. have also reported covalent immobilization of β-glucosidase (55 mg/g) on amino modified silica gel [37]. The obtained loading capacity for CALB and RML in this study is definitely amongst the highest reported capacity of silica for covalent immobilization of enzymes. Protein immobilization on epoxy-functionalized silica usually assumed to be due to the covalent linkages. To propose a conclusive evidence to show that the proteins were covalently conjugated onto the silica support, the leaching experiment was performed in a solution containing 1 M of NaCl and 0.5% of sucrose laurate with vigorous magnetic stirring for 24 h. The results show negligible leakage of the enzymes in the reaction medium clearly confirming the covalent nature of the enzyme attachment.

Immobilization of RML and CALB on SBA-epoxy

Investigation on covalent immobilization of RML and CALB on epoxy-functionalized SBA-15 was also performed in water at 25 °C. Bearing in mind the large surface area of the mesoporous silica nanoparticles, the different amounts of the enzymes ranging from 40-200 mg were offered to 1 g of this support. As can be seen from Table 3, 40 mg of RML was attached to the surface of the support (1g) quickly after 30 minutes of incubation producing 100% immobilization yield and preserving almost 94% of its specific activity. The maximum loading capacity was obtained by offering 200 mg of RML to 1g of the support. In that way, 158 mg of the enzyme was immobilized on the surface of the support and produced 79% of immobilization yield. On the other side by utilizing traditional nucleophilic immobilization, the maximum capacity of 57 mg of RML per gram of epoxy-functionalized SBA after 24 h of incubation has been reported [15]. The leaching experiment also showed the release of 0-5% of RML, proving that 95-100% of the enzyme is covalently attached on the support. Similar to the results obtained from immobilization of CALB on silica, the rate of immobilization of CALB on SBA-15 is also lower than the binding rate of RML at the same condition. However, almost the same loading capacity was observed for the attachment of CALB (144

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mg) compared to RML (158 mg) most likely because of the same molecular dimension and relative mass of these enzymes. Desorption experiment also clearly proved that by performing the isocyanide based protocol; CALB is completely immobilized on the support via strict covalent bonds.

Table 3. Immobilization of the lipases on SBA-epoxy

	Time of immobilization (min)		**Yield[a] (%)**		**mg enzyme/gr support**		**Desorption[b] (%)**	
Offered enzyme (mg/g)	RML	CALB	RML	CALB	RML	CALB	RML	CALB
40	30	200	100	100	40	40	nd	nd
60	75	200	98	95	59	57	nd	nd
80	180	258	92	91	74	73	5	nd
100	180	360	92	90	92	90	3	2
120	210	360	87	87	104	104	3	1
200	1440	1440	79	72	158	144	2	2

[b]Yield is defined as the percentage of the soluble enzyme that becomes attached to the support.
[b]Desorption was performed in a solution containing 1 M of NaCl and 0.5% of sucrose laurate

Investigation on mechanism of immobilization

The enzymes surface is usually covered with various functional groups from the amino acid main and side chains. Amino and carboxylic acid groups are the most abundant nucleophilic residues on the outer surface of enzymes and can be involved in covalent immobilization of enzymes via three-component approach. To investigate the real mechanism of the immobilization two experiments were separately arranged (Fig. 2). At the first experiment the carboxylic acid groups of CALB were blocked by ethyl amine in presence of EDC as a coupling agent. The carboxylic acid-blocked CALB was then subjected to immobilize on silica-epoxy in presence of cyclohexyl isocyanide. No immobilization was observed after 24

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of incubation, proving that the presence of free carboxylic acid residues on the surface of the enzyme is necessary for covalent immobilization.

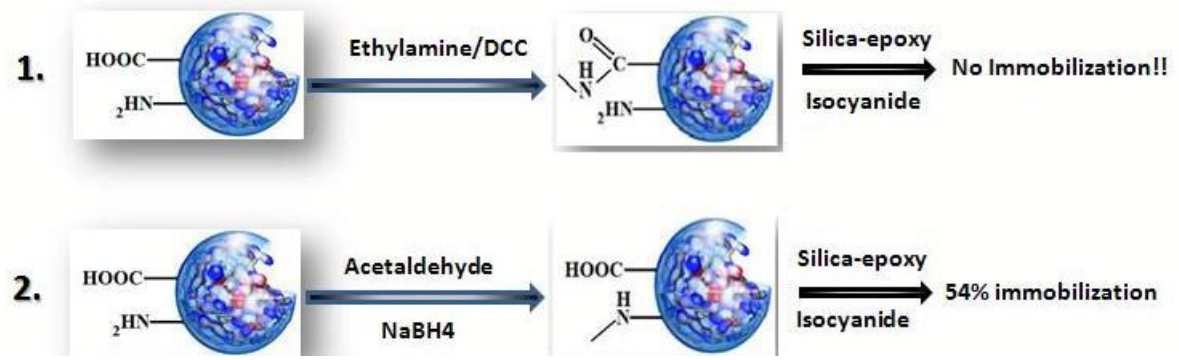

Fig. 2. Investigation on mechanism of the immobilization

In the second experiment the amino groups of CALB were blocked by the reaction with acetaldehyde at pH 10 followed by subsequent reduction of unstable iminium bonds by using sodium borohydride. More than 50% of the modified enzyme is immobilized on silica-epoxy, confirming that the amino groups of the enzyme surface are not involved in the immobilization process. Therefore in view of the above observations we propose the participation of epoxy, isocyanide and carboxylic acid in a three-component reaction and most likely according to the reaction mechanism reported by Oliver et al. [27]

The positive effect of immobilization on thermal stability of enzymes compared to the corresponding free enzyme is well documented.

Stability and optimal activity of the free and immobilized derivatives

Thermal stability is one of the most important criteria for long term and commercial application of enzymes in chemical reactions. The stability of CALB and RML in their free and immobilized forms was investigated after 2h incubation at 45 °C, 50 °C, 55 °C and 60 °C (Fig. 3). The results show that the soluble RML is completely stable at 45 °C and retains 100% of its initial activity. Increasing temperature to 55°C causes to 70% inactivation of the enzyme after 2h of incubation. Further increasing (5°C) also leads to fully inactivation of the

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enzyme at 60°C. On the other side, when compared to the free lipase, the immobilized lipase exhibited a better thermal stability at all temperatures tested.

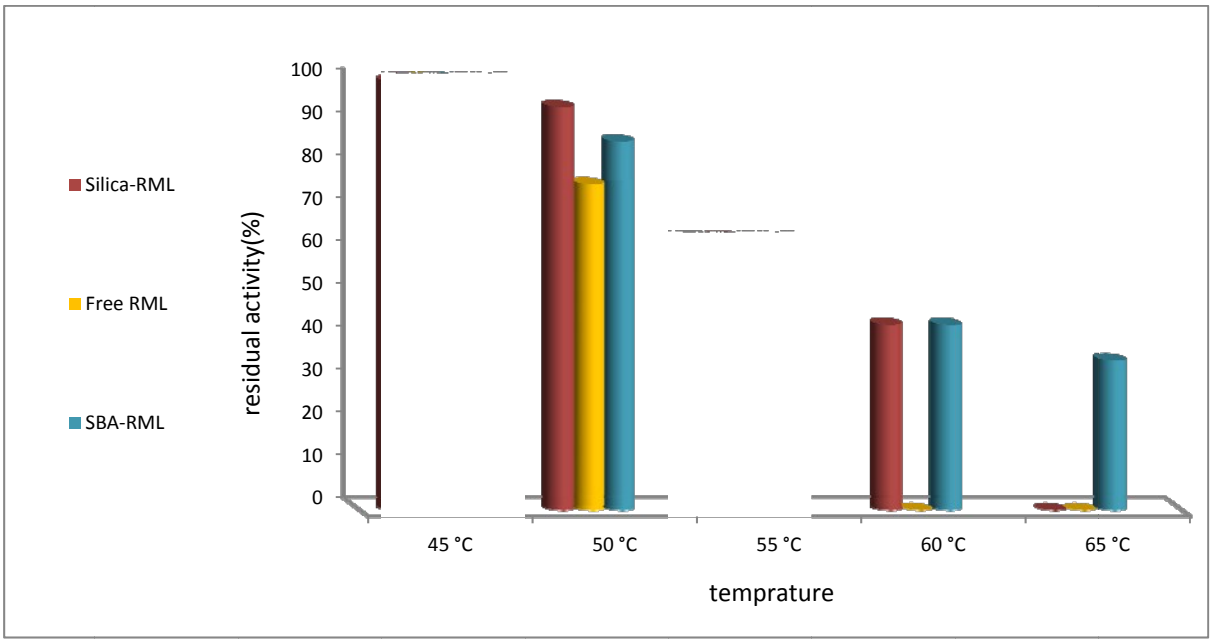

Fig. 3. Thermal stability of free and immobilized preparations of RML at 45°C, 50 °C, 55 °C, 60 °C, 65 °C. Experimental condition: Increasing certain amount of each biocatalyst in 1mL of sodium phosphate buffer 25 mM (pH 7.0) and incubation at different temperatures for 2h. Initial activity of RML and each immobilized derivatives was determined in 1 mL of sodium phosphate buffer 25 mM (pH 7.0) at 25 °C and set as 100%.

Silica-RML and SBA-RML are almost quite stable at 50°C, keeping 90% of activity after 2h of incubation at the same condition. At 60 °C both derivatives shows similar thermal stabilities, retaining 43% of their initial activities. The obtained results for silica-RML are almost same with the results reported for immobilization of RML on silica-epoxy by means of traditional nucleophilic method [26]. In the case of SBA-RML diminished thermal stability is observed compared to thermal stability of the immobilized derivative of RML obtained from nucleophilic immobilization [38]. The results of investigation on thermal stability of immobilized derivatives of CALB showed also interesting results. As can be seen from Fig. 4, the soluble CALB and its immobilized forms remain completely active at 45 °C. At 50 °C,

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while the free enzyme loses 12% of its initial activity, the immobilized preparations are quite stable without decrease in activity. Also upon incubation for 2 h at 55 °C, the free lipase retains the activity about 20% while the immobilized preparations display a residual activity of 75% and 65% for silica-CALB and SBA-CALB, respectively.

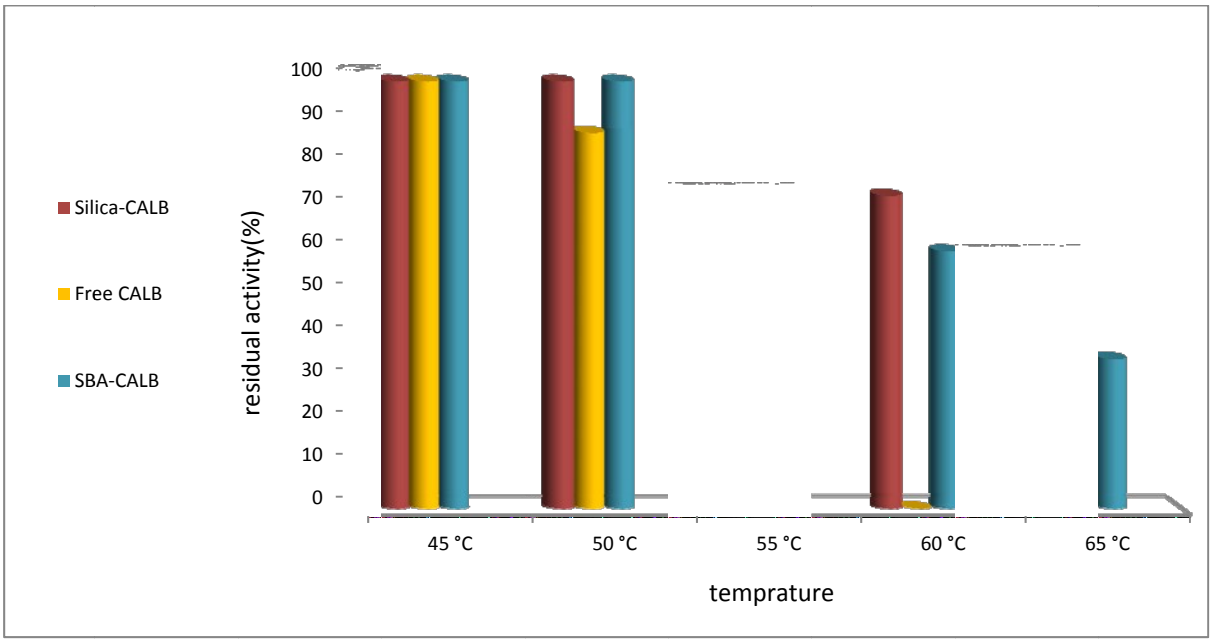

Fig. 4. Thermal stability of free and immobilized preparations of CALB at 45°C, 50 °C, 55 °C, 60 °C, 65 °C. Experimental condition: Increasing certain amount of each biocatalyst in 1mL of sodium phosphate buffer 25 mM (pH 7.0) and incubation at different temperatures for 2h. Initial activity of CALB and each immobilized derivatives was determined in 1 mL of sodium phosphate buffer 25 mM (pH 7.0) at 25 °C and set as 100%.

Increasing temperature to 60 °C clearly unraveled the difference in the stability of the free enzyme and immobilized preparations. At this condition CALB is rapidly deactivated while silica-CALB and SBA-CALB loses only 27% and 40% of their original activities. These data apparently confirm the positive effect of immobilization on stability of both lipases.

Co-solvent stability of RML and CALB in free and immobilized forms was also investigated through incubation in presence of three organic solvents (1-propanol, 2-propanol and dioxane) simulating the conditions of an enzymatic reaction. The presence of organic solvents in enzymatic reactions can improve substrate solubility in water. However, the

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enzyme structure may be affected by organic solvents leading to undesired changes in its catalytic performance. This is because of the fact that the presence of a few amounts of water molecules is essential for enzymatic function. Organic solvents with log P values below 2 tend to strip this essential water and decrease the catalytic activity of enzyme [39]. Three above-mentioned solvents with log P < 2 were used to evaluate the effect of immobilization on co-solvent stability of RML and CALB. As Fig. 5 shows in presence of 20% 2-propanol, RML, silica-RML and SBA-RML present a similar stability, retaining almost 40% of their initial activities. Higher stabilities for immobilized derivatives of RML compared to the free RML is also observed in presence of dioxane and 1-propanol. For example while the soluble enzyme loses almost 90% of its activity, SBA-RML and silica RML remain 75% and 52% active at the same condition.

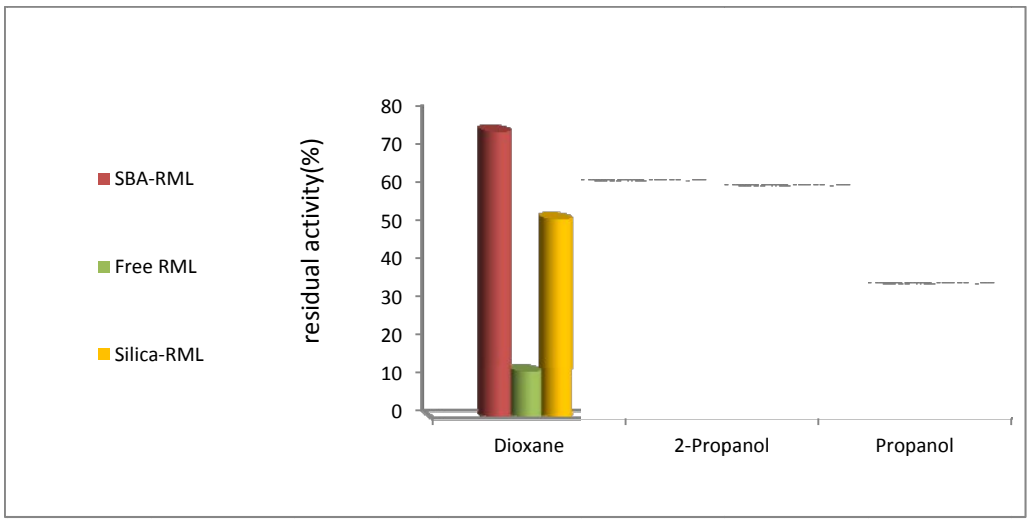

Fig. 5. Co-solvent stability of free RML and immobilized preparations in the presence of 20 % of organic solvents. Experimental condition: Incubation of each biocatalyst in 1 mL solution containing 25 mM sodium phosphate buffer (pH 7.0) and 20% of three organic solvents at 25°C. Initial activity of RML and each immobilized derivatives was determined in 1 mL of sodium phosphate buffer 25 mM (pH 7.0) at 25 °C and set as 100%.

Immobilization of CALB also causes to significant improvement in its stability in the presence of 1-propanol and dioxane in particular (Fig. 6). However it seems that the method

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used for immobilization has no impact on co-solvent stability of the immobilized derivatives in 1-propanol.

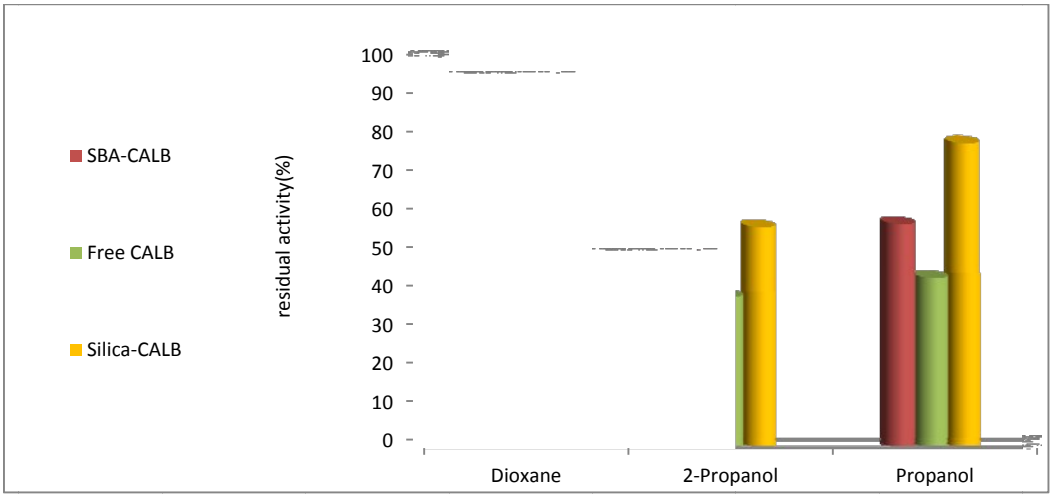

Fig. 6. Co-solvent stability of free CALB and immobilized preparations in the presence of 20 % of organic solvents. Experimental condition: Incubation of each biocatalyst in 1 mL solution containing 25 mM sodium phosphate buffer (pH 7.0) and 20% of three organic solvents at 25°C. Initial activity of CALB and each immobilized derivatives was determined in 1 mL of sodium phosphate buffer 25 mM (pH 7.0) at 25 °C and set as 100%.

As previously reported depending on both enzyme and matrix properties, shifts in optimum pH of up to 2 units for immobilized enzyme activities compared to the free enzyme can occur. The effect of pH on the activity of the free and immobilized enzymes was also examined by incubation of certain amounts of biocatalysts in the solutions with different pH values ranging from 5 to 9 at 25°C. In Fig. 7 and 8 the activities of the soluble and immobilized RML and CALB were plotted versus the pH. The results show the optimum activity for free CALB at pH 7.5, while the immobilization alters the optimum pH of the immobilized enzymes to the pH values 7 and 8 for silica-CALB and SBA-CALB, respectively. However in the case of silica-CALB, remarkable activities (90-100%) obtained from the incubation of this biocatalyst within the broad range of pH values (5.5-8) showing a great degree of adaptability in a wider range of pH, when compared with that of CALB and SBA-CALB.

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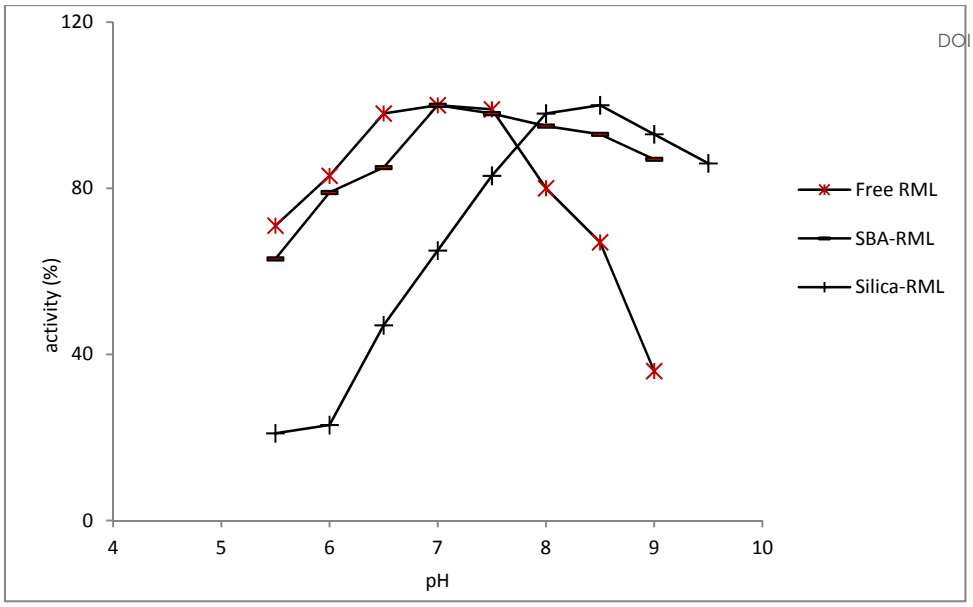

Fig. 7. The effect of pH on the activity of the free and immobilized preparations of RML.

Investigation on pH dependence of RML and its immobilized derivatives activities also showed the optimum activity of RML and SBA-RML at pH 7, while immobilization of RML on silica changes the optimum activity of the obtained derivative to pH 8.5.

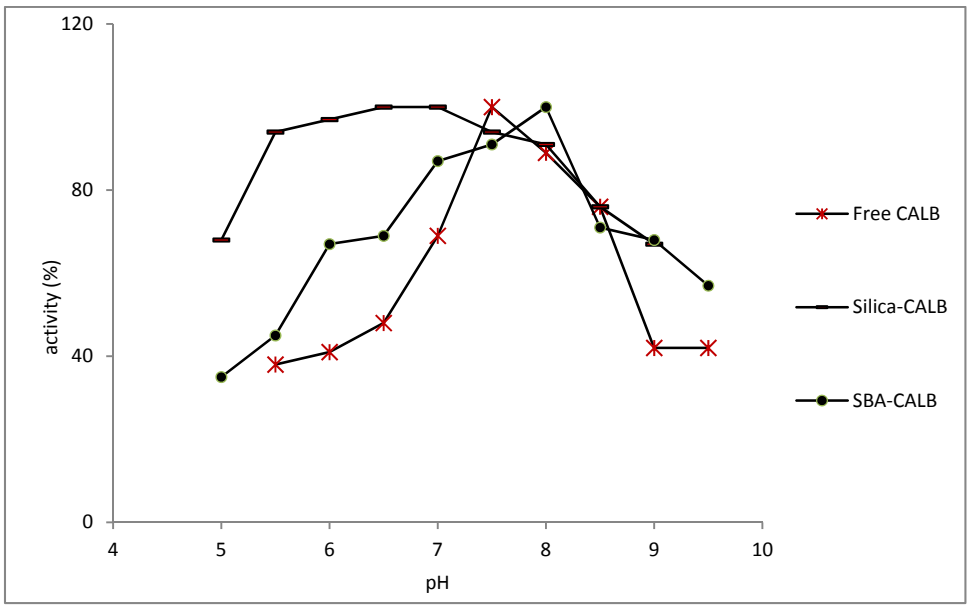

Fig. 8. The effect of pH on the activity of the free and immobilized preparations of CALB

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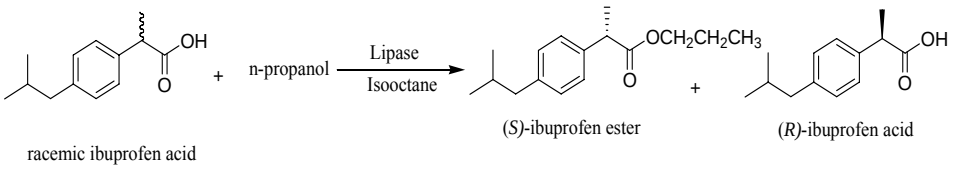
Enantioselective esterification for resolution of racemic ibuprofen

To evaluate the enantioselectivity and catalytic performance of the immobilized preparations, enzymatic esterification of racemic ibuprofen with 1-propanol in anhydrous isooctane was carried out in different conditions (scheme 1). The results showed that the immobilized preparations of both CALB and RML discriminate between (R)- and (S)-ibuprofen in favor of the S enanthiomer.



Scheme 1. Enantioselective esterification of racemic ibuprofen with 1-propanol in anhydrous isooctane

In order to investigate the effect of temperature on the performance of the immobilized preparations, three different temperatures (0 °C, 25 °C and 30 °C) were applied. Furthermore various amounts of immobilized preparations were examined in order to evaluate its influence on the reaction parameters. The conversion degrees (c), the enantiomeric excess (ee) of remaining acid and produced ester and the E-values are shown in Table 4-7. However the reported results are only the best results in terms of E-value obtained for silica and SBA in Tables 4-5 and 6-7, respectively.

Kinetic resolution by using immobilized preparations of RML

Under the tested conditions for RML, the results show that with increasing the temperature the E-values and enantiomeric excess of the product decrease. Although the rate of the reaction improves with the higher amounts of both biocatalysts producing higher values of conversion the E-values decrease with increasing amount of both silica-RML and SBA-RM.

Table 4. Kinetic resolution of (*R*,*S*)-ibuprofen catalyzed by silica-RML

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Biocatalyst (mg)	Reaction time (h)	Temperature	% ee_p	% ee_s	% C	E
5	24	0 ˚C	91.6	28.4	23.7	29.9
5	24	25 ˚C	79.4	8.5	9.7	9.5
5	4	30 ˚C	86.6	21.0	19.5	17.1
10	6	0 ˚C	88.8	19.2	17.8	20.2
10	2	25 ˚C	82.2	13.1	13.7	11.7
10	4	30 ˚C	80.1	9.0	10.1	9.9
20	4	0 ˚C	81.1	19.5	19.4	11.6
20	0.5	25 ˚C	79.1	14.8	15.7	9.9
20	0.5	30 ˚C	60.2	9.0	13.1	4.4

The results also show that silica-RML produces higher E-values compared to SBA-RML. For example, with using 5 mg of the immobilized enzyme on silica in 0 °C, conversion was reached 23.7% with E-value of 29.9 after 24 h of the reaction time (Table 4) whilst with the same amount of immobilized enzyme on SBA-15, the conversion was reached 35.5% with E-value of 4.1 after 12 h (Table 5). With increasing the amount of enzyme to 10 mg in 0 ˚C, the reaction reached 17.8% of conversion degree with 19.9 % of enantiomeric excess for (S)-ibuprofen resulting in E-value of 20.1 (Table 4). When the reaction was heated up to 25˚C, the reaction was reached 15.7% of conversion degree (esterification activity) within 30 minutes (E= 9.9, ee_p = 79.1%) by using 20 mg of the RML immobilized on silica support. The same result was also achieved after 1 h of reaction time in 25˚C with using 5 mg of RML immobilized on SBA support (C=20.9%, ee_p = 76.7% and E=9.2).

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Table 5. Kinetic resolution of (*R,S*)-ibuprofen catalyzed by SBA-RML

Biocatalyst (mg)	Reaction time (h)	Temperature	% ee_p	% ee_s	% C	E
5mg	4	0 ˚C	84.3	14.4	14.6	13.5
5mg	1	25 ˚C	76.7	20.3	20.9	9.2
5mg	1	30 ˚C	72.8	11.7	13.8	7.1
8 mg	2	0 ˚C	74.8	13.7	15.5	7.9
8 mg	0.5	25 ˚C	66.9	12.1	15.3	5.7
8 mg	0.5	30 ˚C	66.6	11.4	14.6	5.6

Kinetic resolution by using immobilized preparations of CALB

The esterification reaction was also performed with CALB immobilized on silica and SBA-15. With 20 mg of CALB immobilized on silica, the conversion was 18.6% with E-value of 21.5. The S-acid was converted to its ester form, leaving the (R)-ibuprofen (ee = 89.3%) after 48 h of reaction time in 0 ˚C (Table 6).

Table 6. Kinetic resolution of (*R,S*)-ibuprofen catalyzed by silica-CALB

Biocatalyst (mg)	Reaction time (h)	Temperature	% ee_p	% ee_s	% C	E
20 mg	48	0 ˚C	89.3	20.4	18.6	21.5
20 mg	24	25 ˚C	81.2	9.6	10.6	10.6
20 mg	6	30 ˚C	79.3	8.5	9.7	9.4
50 mg	48	0 ˚C	88.0	17.6	16.7	18.7
50 mg	6	25 ˚C	85.3	13.4	13.6	14.3
50 mg	2	30 ˚C	82.8	10.9	11.6	11.8
70 mg	6	0 ˚C	86.1	14.5	14.4	15.4
70 mg	1	25 ˚C	81.5	9.9	10.8	10.8
70 mg	1	30 ˚C	82.5	10.7	11.5	11.6

Comparison of the data from the performed reactions by CALB immobilized on silica and SBA revealed that CALB immobilized on silica worked better than CALB immobilized on

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SBA in terms of esterification activity and enantiomeric excess as a function of reaction time. Among all the performed reactions by CALB immobilized on SBA, the best result was obtained after 24 h by using 5 mg of immobilized preparation in 0 ˚C (C=12.7%, ee_p = 83.4% and E=12.5). With the same amount of enzyme, with heating up the reaction to 25 ˚C, the reaction time was significantly decreased (from 24 h to 2 h) (Table 7).

Table 7. Kinetic resolution of (*R*,*S*)-ibuprofen catalyzed by SBA- CALB

Biocatalyst (mg)	Reaction time (h)	Temperature	% ee_p	% ee_s	% C	E
5mg	24	0 ˚C	83.4	12.1	12.7	12.5
5mg	2	25 ˚C	78.9	8.2	9.5	9.2
5mg	2	30 ˚C	77.8	7.7	9.0	8.6
10 mg	24	0 ˚C	75.4	12.0	13.8	8.0
10 mg	1	25 ˚C	78.1	7.8	9.1	8.8
10 mg	1	30 ˚C	60.0	9.1	13.1	4.4

Result showed that with increasing the amount of the immobilized preparations and also temperature, the E-values of the reaction and the also the enantiomeric excess of (S)-ibuprofen dropped which can be caused by the rate of the reaction. According to the results, the best temperature for the enantioselective esterification is 0 ˚C in terms of E-values. It is worthwhile to note that with increasing the incubation time of the esterification reaction, the rate of the ibuprofen ester formation worsened, most likely due to the gradual formation of water that favors the hydrolysis of the product.

In 2008, da Silva et al. reported using of lipase from *Aspergillus niger* immobilized on Celite in the esterification of racemic ibuprofen with 1-propanol in isooctane. The conversion degree (esterification activity) after 72 h of reaction time was 26%, which was almost two-

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fold higher than that of free lipase (14.6%). However, the enantiomeric excess of the substrate was only 6% after 72 h and the E-value was significantly decreased (E = 1.5) [40]. They also reported that the immobilized lipase from *Aspergillus oryzae* was active in the esterification of (R,S)-ibuprofen (3.7%), but not in the free form. Lipases from *A. terreus* and *A. flavus* demonstrated lower performances, attaining maximum esterification activities of 4.8% and 13.4%, respectively, after immobilization [40]. Chang et al. reported using of Carica papaya lipase for the enantioselective esterification of racemic 2-methylhexanoic acids with n-butanol in isooctane at 35˚C with maximum enantioselectivity of E = 24.3 [41].

Conclusion

In this paper, application of an isocyanide-based multicomponent reaction was investigated in the covalent immobilization of RML and CALB on epoxy functionalized supports including silica and SBA-15 in the mild conditions for the first time. This protocol can be used not only for the covalent immobilization of enzymes, but also as a very interesting and simple method for rapid attachment of other biomolecules on epoxy-functionalized supports at extremely mild conditions. Evaluation on mechanism of the immobilization reaction confirmed the participation of three functional groups including carboxylic acid groups from the enzyme surface, epoxy groups from the support and isocyanide from the reaction medium. Investigation on thermal/co-solvent stability of the immobilized preparations showed considerable improvement in stabilities compared to the free enzymes. The immobilized preparations were also used in enantioselective esterification of (*R*, *S*)-ibuprofen with 1-propanol in anhydrous isooctane at three temperatures (0 ˚C, 25˚C and 30 ˚C). The best result was achieved by using of 5 mg of the silica-epoxy-RML in 0 ˚C, 23.7% of conversion with the *E*-value of 29.9. Regarding the fact that in the most of the reported results on the

resolution of ibuprofen performed by various lipases, the long time of the reaction and poor enantioselectivity are the main problems, the lower reaction times and suitable enanthioselectivity of the immobilized preparations in the present study are of considerable interest.

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Graphical Abstract:

One pot three-component reaction was used for covalent immobilization of CALB and RML on epoxy-functionalized supports