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Authors: Lidija T. Izrael Živković, Ljiljana S. Živković, Vladimir P. Beškoski, Kristina R. Gopčević, Bojan M. Jokić, Dragoslav S. Radosavljević, Ivanka M. Karadžić

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The *Candida rugosa* lipase adsorbed onto titania as nano biocatalyst with improved thermostability and reuse potential in aqueous and organic media

Author names and affiliations
Lidija T. Izrael Živković[a*], Ljiljana S. Živković[b], Vladimir P. Beškoski[c], Kristina R. Gopčević[a], Bojan M. Jokić[d], Dragoslav S. Radosavljević[e], Ivanka M. Karadžić[a]

[a] University of Belgrade, Faculty of Medicine, Institute of Chemistry in Medicine, Visegradska 26, 11000 Belgrade, Serbia;
[b] University of Belgrade, The Vinča Institute of Nuclear Sciences, 11000 Belgrade, Serbia;
[c] University of Belgrade, Faculty of Chemistry, Studentski trg 12-16, 11000 Belgrade, Serbia
[d] University of Belgrade, Faculty of Technology and Metallurgy, Karnegijeva 4, 11001 Belgrade, Serbia
[e] University of Belgrade, Faculty of Medicine, Center for Communication and IT

Lidija Izrael Živković and Ljiljana Živković contributed equally to this article.

Corresponding author
Lidija Izrael Živković,
Phone+381-11-69-1898-007
Email: lidija.izrael-zivkovic@mfub.bg.ac.rs and lidijajob@yahoo.com

Abstract
The immobilization of *Candida rugosa* lipase by adsorption was performed onto commercial titania powder (Degussa P25). The change of titania particles surface was diagnosed by means of FTIR and FESEM analysis, as well as by shift of zeta potential value towards that of lipase. A detailed study of the effect of immobilization on enzyme kinetic, temperature stability, as well as on potential for its reuse in aqueous organic media was undertaken. Immobilization of lipase altered enzyme affinity toward substrates with different length of carbon chain in hydrolytic reaction. The Vmax value decreased 2-8-fold, where major constraint was registered for the ester containing the longest carbon chain. Thermostability of lipase improved more than 7-fold at 60 ^{o}C. Significant potential for reuse in water solutions was also found after immobilization. In cyclohexane immobilized lipase catalyzed synthesis of amyl octanoate by ping-pong bi-bi mechanism with inhibition by amyl alcohol. Obtained kinetic constants were Vmax=26.4 μmol/min, K_{Ac}=0.52 mol/l, K_{Al}=0.2 mol/l and $K_{i,Al}$=0.644 mol/l. Esterification activity remained 60 % after 5 reuse cycles in cyclohexane indicating moderate reuse stability.

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1. Introduction

Enzyme catalyzed reactions in aqueous medium have been extensively exploited for a wide range of applications in biotechnology, such as production of food, detergents, leather, pharmaceuticals. In spite of a broad implementation of enzymes in different fields, some constraints referred to their cost and process stability still exist. To overcome a limit related to short catalytic lifetime of enzymes in process conditions, a spectrum of immobilization methods have been extensively studied to increase stability and enhance reuse, offer easier separation, making production economically viable [1]. Properties of a vast number of enzymes were significantly improved when immobilized onto different supports [2]. The characteristics of support are of paramount importance in determining the performance of the immobilized enzyme system [3]. Frequently used supports are polymers and inorganic materials [4]. Apart from being affordable, an ideal matrix must encompass characteristics like inertness, physical strength, ability to increase enzyme stability and activity, reduce microbial contamination [5]. Lately, nanoscale supports in combination with enzymes have emerged a new field of nanobiocatalysis which achieved a great success in enzyme stabilization [6]. Actually, nanobiocatalytic systems, compared with enzymes immobilized on non-nanostructured materials, have advantagies related to improvement of enzyme stability, more efficient loading, prolonged lifetime and larger number of reuse cycles [7].

Having a long history, enzyme reactions in water conditions implied a general attitude that the enzymes can work only in water solutions, while in organic solvents they denature. Nowadays, however is well known that in spite of enzyme instability in organic solvents, a significant number of reactions in organic chemistry can be catalyzed by enzymes in non-water conditions (to avoid by-products and to obtain desired product of high purity). Although the transformations in reduced water conditions are relatively new challenge in enzymology [8], merits of this concept are recognized. Possibility to shift thermodynamic equilibrium in favor of synthesis over hydrolysis, which allows for highly specific organic synthesis, e.g. of enantioselective drugs [9], is undoubtedly one of the most important advantages. Aside of that, there are several more merits of catalysis in non-water media: increased solubility of non-polar substrates, such as e.g. lipase substrates, insolubility of enzymes which allows their reuse and recovery [10], enzyme stabilization [11], and elimination of microbial contamination in reaction mixtures [12]. Although enzymatic conversions in water restricted environments open a possibility to perform complex organic synthesis, enzymes being proteins, generally easily lose their activity in such conditions [13]. Lipases produced by microorganisms isolated from water reduced environment and active in organic solvents are rare [14,15]. In that respect, it has became necessary to develop strategies to improve their lifespan in the presence of organic solvents. The use of novel nanomaterials for immobilization has been considered as promising route [16].

Lipases are an intriguing group of enzymes which catalyze the hydrolysis of carboxylic esters in water and the reverse reaction of ester synthesis in organic solvents. In water solutions, lipases transform water insoluble substrates. Possessing a mobile lid, which covers substrate binding site, lipases structure is specifically adjusted to non-polar substrates. Interestingly, a molecular dynamic study showed that an organic solvent promotes and stabilizes open lid structure of lipases [17]. Enzyme conversions of hydrophobic substrates can be facilitated by addition of non-polar organic solvents, however, loss of enzymatic activity promoted by solvent becomes factor of limitation in such conditions. On the other side, to be active in non-water environments appropriate for substrate solubilization, a certain amount of water is necessary for conformational mobility of enzymes. Delicacy of experimental conditions to achieve a certain

lipase reaction demands a good balance of nature/properties and amount of organic solvent, amount of water and stability and amount of lipase. It is expected that immobilization of enzymes /lipases on nanostructured materials would improve their stability and protect its necessary conformational mobility in transformations of water non-soluble substrates in organic solvents.

Lipase form *Candida rugosa* (*C. rugosa*) is well established in water solutions and its stabilization on several inorganic materials has been documented in literature, the findings of our team included [18,19]. The current study focuses on the use of commercial titania, a biosafe nanostructured material with excellent physico-chemical stability, as support for lipase immobilization. Degussa P25 is widely applied model powder for various applications of TiO_2. Interestingly, only one study deals with immobilization of lipase onto Degussa titania [20]. Hereby, it was selected for its morphology and surface properties. In such a manner, immobilization process was performed under the same electrostatic conditions as done for SBA15, zirconia and alumina [18,19] enabling evaluation of support morphology impact on enzyme percent adsorption. Effect of lipase immobilization on enzyme kinetic, temperature stability, as well as on potential for its reuse in water solution is investigated. Aside of behavior of nanobiocatalyst in water solution, in this study, potential of immobilized lipase to act in organic solvent was evaluated too.

2. Experimental

2.1. Materials

Lipase from *C. rugosa* (lyophilized powder, Type VII, 746 U/mg), *p*-nitrophenyl palmitate (*p*-NPP), *p*-nitrophenyl laurate (*p*-NPL), *p*-nitrophenyl caprylate (*p*-NPC) and *p*-nitrophenyl butirate (*p*-NPB) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). All other chemicals were of analytical grade.

Support material, titania (TiO_2) (Degussa P25), purchased from Degussa Chemicals (Hanau, Germany) was used as received. It had the following characteristics: phase composition of 80 % anatase and 20% rutile, primary particle size of 27 nm and specific surface area of 57.4 m^2g^{-1} with a mesopore range 13-23 nm [21].

2.2. Lipase immobilization

Titania was prepared for immobilization as follows: suspensions containing 4 mg solid/ml in 10 mM Tris-HCl buffer pH 7.6 were de-agglomerated for 10 min by sonication. Lipase solution (2 mg/ml in the same buffer), was added to previously de-agglomerated support and immobilization was carried out for 1 h at room temperature under mild shaking (100 rpm). Final concentration of support was 2 mg/ml, final concentration of lipase was 1 mg/ml. Immobilized lipase was precipitated by centrifugation at 10000 rpm for 10 min, using micro-centrifuge (Denver Instruments, USA). After removing supernatant, immobilized enzyme was washed 2 times (in order to remove excess of unbounded enzyme), dried for 1 h at room temperature and after that used for an assay.

Kinetics of the adsorption process was studied for 180 min using 10 mM Tris-HCl buffer pH 7.6 and the fixed lipase/support ratio (w/w) 1/2.

The efficiency of immobilization was evaluated in term of enzyme coupling yields, η_{enz}, calculated as follows:

$\eta_{enz}(\%) = P_1/P_0 \times 100$

P_o is the initial concentration of the enzyme and P_1 is the concentration of immobilized enzyme. P_1 was calculated as a difference between initial lipase concentration and concentration in supernatant after immobilization.

The efficiency of immobilization was also evaluated in term of activity coupling yields η_{act}.

$$\eta_{act}(\%) = SA_2/SA_1 \times 100$$

SA_2 is the specific activity of immobilized lipase and SA_1 is the specific activity of free lipase [22].

2.3. Methods for material characterization

2.3.1. Zeta potential determination

Zeta potential measurements were performed by Zetasizer Nano ZS (Malvern, UK). The change of ζ-potential with pH was firstly recorded in NaCl (0.01 M) electrolyte to determine the isoelectric point (IEP) of titania as support. Prior to titration, the powder dispersion (1 mg/1mL) was equilibrated for 24 h under mild shaking at room temperature. The ζ-potential of lipase and that of support (before and after enzyme adsorption) was also determined in Tris-HCl buffer, pH7.6

2.3.2. Fourier transform infrared spectroscopy (FTIR)

FTIR was used to examine the support surfaces before and after immobilization of lipase. The spectra were recorded using FTIR Nicolet 6700 (Thermo Scientific, USA) in transmission mode between 400 and 4000 cm^{-1}. The platinum-ATR (Attenuated Total Reflectance) sampling module with diamond crystal was used.

2.3.3. Field emission scanning electron microscopy (FESEM)

Field emission scanning electron microscopy (FESEM), using a Tescan Mira3 XMU (Czech Republic) at 20 kV was used to compare the morphological features of support particles before and after immobilization of *C. rugose* lipase. Prior to FESEM analysis, the samples were coated with Au alloy using a spatter coater.

2.3.4. Determination of organic content in commercial lipase preparation

The content of ash was determined according to the standard test method - ASTM E1755-01 [23].

2.3.5. Elemental analysis

The C, H, S, and N contents of the commercial *Candida rugosa* lipase (Sigma L-1754) were determined by a Vario EL III CHNS/O Elemental Analyzer (Vario EL III, Elemental Analysis System GmbH, Germany).

2.4. Characterization of immobilized enzyme in water solution

2.4.1. Determination of lipase activity

Activity of free lipase was measured spectrophotometrically by using an assay based on the hydrolysis of *p*-nitrophenyl palmitate (*p*-NPP).The *p*-NPP solution was prepared as follows: 30 mg of *p*-NPP in 10 ml of 2-propanol was added to 90 ml of 0.05 M phosphate buffer (pH 8.0) supplemented with 200 mg of Na-deoxycholate and 100 mg of gum arabic. The absorbance was measured at 410 nm for the first 3 min of reaction at 25°C. One unit (1 U) is defined as that

quantity of enzyme which under test conditions liberates 1 μmol of *p*-nitrophenol per min. The reaction mixture was composed of 900 μl of *p*-NPP solution and 100 μl of lipase solution [8]. Activity of immobilized lipase was determined using 10 mg of immobilized preparation supplemented with 2 ml of *p*-NPP solution. Reaction was carried out at 25ºC for 3 min and it was stopped by addition of 0.5 M sodium carbonate solution. Precipitate was removed by centrifugation at 10000 rpm for 10 min. Supernatant was diluted 10-fold with deionized water and activity was measured at 410 nm. Activity was calculated as U per g of support material.

2.4.2. Thermal stability

Reaction mixtures containing free or immobilized enzyme were incubated at 50 and 60 ºC for various periods of time (20 - 120 min) and quickly cooled. Enzymatic activity was measured according to the standard protocol for free and immobilized enzyme with *p*-NPP as substrate. Lipase activity without previous incubation was determined as 100%.
Half life ($t_{1/2}$) of enzyme with kd as the decay constant was calculated as:
$t_{1/2} = \ln 2/kd$ (1)

2.4.3. Determination of kinetic parameters

Michaelis –Menten kinetics was used to describe the dependence of enzyme activity on substrate concentration for free and immobilized lipase. V_{max} is the highest possible specific lipase activity (U per mg of protein) and K_m is the Michaelis constant determined from substrate concentration that gives a specific activity of half of V_{max}. The two kinetic parameters V_{max} and K_m were calculated from experimental data using Lineweaver-Burk equation:
$1/v = 1/V_{max} + K_m/V_{max} \times 1/S$ (2)
The y-axis intercept is $1/v_{max}$, and the slope is K_m/v_{max}. [24]
The kinetic parameters of free and immobilized lipase were determined using solutions of *p*-NP esters: *p*-NP palmitate (*p*-NPP), *p*-NP laurate (*p*-NPL), *p*-NP caprylate (*p*-NPC) , *p*-NP butyrate (*p*-NPB) at following concentrations: 0.2, 0.4, 0.8, 1.2, 1.6, 2.0 mM. The lipase activity was measured as described in section 2.4.1. Measurements were conducted in triplicate.

2.4.4. Reuse stability

After immobilisation of lipase, its potential for reuse was determined by the following procedure: 25 mg of immobilised enzyme packed in a filter paper bag was used for hydrolysis of *p*-NPP solution in 5 ml phosphate buffer pH 8.0, for 30 min at room temperature, under mild stirring (90 rpm). After each batch reaction, the immobilised lipase was washed three times in order to remove the product, *p*-NP, and dried for 1h (one cycle). Five cycles were performed during one day, and the immobilised lipase was stored at 4 ºC overnight. The lipase activity was measured as described in section 2.4.1. Activity of the first cycle was determined as 100%. In order to follow the loss of enzyme i.e., desorption process, after each of 12 reuses, the amount of proteins was determined in the batch solution. Measurements were conducted in triplicate.

2.4.5. Protein concentration

The concentration of protein was determined by the Bradford`s method using bovine serum albumin as a standard [25].

2.5. Characterization of immobilized enzyme in organic solvent
2.5.1. Determination of lipase activity

Solution of amyl alcohol, octanoic acid, and 0.1% of water in 5 ml cyclohexane were incubated with 10 mg of immobilized lipase at 40 °C with mechanical stirring at 150 rpm on rotary shaker (Kuhner, Switzerland). The rate of esterification was determine as the residual acid content by titration with sodium hydroxide, with phenophtalein as an indicator and ethanol and diethyl ether mixture as a quenching agent. The quantity of ester formed was calculated as being equivalent to acid consumed [26]. This was confirmedby determination of ester concentration using gas chromatography–mass spectrometry (GC-MS) performed with a GCMS (QP2010 Ultra, Shimadzu, Kyoto, Japan). A Rtx®-1 (RESTEK, Crossbond® 100 % dimethyl polysiloxane, 30 m × 0.25 mm I.D., 0.25-μm film thickness) column was used, with Helium as the carrier gas. Injector temperature was constant: 280 °C. The temperature program used was: initial temperature 60 °C, then 4 °C per min until 310 °C, and isothermal at 310 °C for 7.5 min. The GC-MS data was collected and analyzed (automated mass spectral deconvolution and identification) with GCMS Solution software (Shimadzu). Spectrum analysis was performed using NIST11 and Wiley8 database libraries, and relative ratios of components were calculated from the corresponding peak areas.

2.5.2. Determination of kinetic parameters in organic solvent

The kinetic parameters of immobilized lipase were determined in cyclohexane using solutions of amyl alcohol and octanoic acid in a range of concentrations: 0.05 to 0.6 M for alcohol and 0.05 to 1 M for acid. The concentrations of alcohol and acid were varied one at a time keeping the other constant. The reaction mixture volume was 5 ml with 10 mg of immobilized lipase. One unit (1 U) is defined as that quantity of enzyme which under test conditions synthesizes 1 μmol of amyl-octanoate per min.

The experimentally obtained data were fitted with model for bisubstrate ping-pong bi-bi model with alcohol inhibition using Matlab software [26]. The rate equation describing this model is given by:

$$v = \frac{V_{max}[Ac]\,[Al]}{[Al][Ac] + K_{al}[Ac] + K_{ac}[Al] + \left(\frac{K_{ac}}{K_i},al\right)[Al]^2} \tag{3}$$

where v is the initial reaction rate, V_{max} is the maximum reaction rate, [Ac] and [Al] are the concentrations of octanoic acid and amyl alcohol, K_{Al} and K_{Ac} are Michaelis constants for amyl alcohol and octanoic acid, and $K_{i,al}$ is the amyl alcohol inhibition constant [26].

Kinetic constants (V_{max}, K_{Al}, K_{Ac}, $K_{i,al}$) were calculated using a non-linear regression fit of the 48 experimental points. Parameters were optimized using MatLab software. For the kinetic model applied, goodness of fit, R^2 and Root Mean Squared Error, was determined.

2.5.3. Synthesis of methyl esters in solvent free system

10 mg of immobilised enzyme was incubated in 5 mL of methanol with palmitic, lauric and octanoic acid with concentration of 0.6 mol/L. The rate of methyl esters synthesis was determine as the residual acid content by titration with sodium hydroxide, with phenophtalein as an indicator. The quantity of ester formed was calculated as being equivalent to acid consumed Synthesis of methyl caprilate was confirmed by GC-MS analysis as described (2.5.1).

2.5.4. Reuse stability

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After immobilisation of lipase, its potential for reuse in organic solvent was determined by the following procedure: 10 mg of immobilised enzyme was used for amyl-octanoate synthesis in 5 mL of cyclohexane. After each batch reaction, the solvent was decanted, immobilised lipase was washed and dried overnight (one cycle). Six cycles were performed. The lipase activity was measured as described (2.5.1.). Activity of the first cycle was determined as 100%. In order to follow loss of enzyme i.e., desorption process, after each of 6 reuses, decanted cyclohexane was evaporated, residues were dissolved in water and proteins content determined. All measurements were conducted in triplicate.

3. Results and discussion
3.1. Conditions for lipase immobilization

Lipase from *C. rugosa* is a globular glycoprotein with molecular mass of 57 kDa, with 31 acidic and 18 basic amino acids exposed on the surface,[18] its IEP is located at pH 4.65. Above this pH, the lipase acquires negative charge. Protein and organic compound content of commercial *C. rugosa* lipase were determined and it was found that the preparation comprised of 13 % protein and 83 % organic matter. Result corroborates the literature data where the main organic impurity in commercial lipase was lactose, which acted as water reservoir [27]. In spite of high content of lactose in the preparation, it did not disturb the process of lipase adsorption.

In this study, titania Degussa P25 was selected as support material for its well defined morphology: nanometer-sized solid particles of spherical shape. Moreover, its isoelectric point value permitted the Tris-HCl buffer at pH 7.6 to be used for immobilization, with an aim to perform the adsorption of lipase under the same (repulsive) electrostatic conditions (i.e. the same surface charge of the interacting partners) as previously done for SBA15, zirconia and alumina supports.

Zeta potential values were collected over the pH range 3≈9, using NaCl as indifferent electrolyte [28, 29]. The dependence presented in Figure 1, indicated the isoelectric point at pH 6.7 (at IEP ζ-potential equals to zero), in good accordance with literature [29].

Fig. 1.

Being amphoteric, titania particles develop positive and negative charge below and above pH 6.7, respectively, due to dissociation of the surface hydroxyl groups. Lipase has an acidic IEP (4.6) and is negatively charged above this pH [18]. Accordingly, the adsorption partners should carry a negative surface charge in the pre-selected immobilization conditions. Indeed, negative ζ-potentials were measured, namely: -14.3 mV for lipase and -12.2 mV for support. Immobilized lipase shifted the ζ value of titania to -19.4 mV. The change in zeta potential ascertained adsorption of lipase onto support, in accordance with previous findings [18, 19, 30] that ζ-potential is useful tool for qualitative confirmation of adsorption.

3.2. Kinetic of adsorption process

Adsorption of proteins on solid surfaces is common but very complex phenomenon [31]. The effect of immobilization time on the *C. rugosa* lipase adsorption onto titania surface was studied, as shown in Fig. 2. The adsorption of protein onto inorganic supports is relatively fast process in suitable conditions of stirring or mixing [32].

Fig. 2.

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The adsorption of lipase proceeds in two kinetic regimes: fast one followed by slower kinetics. Simple models can provide useful insight into adsorption process. Protein adsorption onto surface can be divided in two classes: I-random sequential adsorption and ballistic deposition, and II- diffusional model [20]. In this work the adsorption process of seems to follow random sequential mode where the lipase aggregates sequentially occupy the support surface [33, 34]. In aqueous media, aggregation of proteins is possible to occur at the hydrated oxide surface; therefore, adsorption of the lipase aggregates onto TiO_2 is more realistic scenario than adsorption of isolated molecules [20].

Despite repulsive electrostatic conditions, very good result was achieved already after 1h: adsorption plateau with 65.5 % adsorbed lipase. As longer incubation time (two or three hours) did not significantly improve the immobilization efficiency, the chosen contact time was 60 min. The obtained results of adsorption are summarized in Table 1.

Table 1.

Although this procedure enabled the coupling of a higher amount of enzyme, the loss in activity was inevitable and the activity coupling yield was 21%. It seems to be the result of conformational changes and movement restrictions of lipase and barrier formation after adsorption. Determined specific activity of immobilized lipase was 64.7 U/mg of protein.

3.3. FTIR characterization

The adsorption of lipase onto titania was also analyzed by FTIR. Figure 3. presents the spectra of titania before and after adsorption process and spectrum of lipase for comparison. In titania spectrum (curve a), the peak at 400–700 cm^{-1} corresponds to Ti-O stretching and Ti-O-Ti bridging stretching modes. The broad band at 3400 and the peak at 1650 cm^{-1} are assigned to the surface-adsorbed water and OH^- group [33]. The bands characteristic for functional groups of lipase (curve b), i.e. at 1530 cm^{-1} (amine groups), at 1650 cm^{-1} (C-O stretching), together with a sharp peak in the region 1200–900 cm^{-1} representing carbohydrate band (the lipase is glycoprotein), are clearly visible [34]. After immobilization process, titania (curve c) exhibited three new bands: at 1530, 1650 and in the region 1200–900 cm^{-1}, which is a verification of successful adsorption of lipase.

Fig. 3.

3.4. FESEM characterization

Adsorption of lipase was also followed by FESEM, comparing morphological features of bare titania and titania with adsorbed lipase (Fig. 4.).

Typical microphotograph of P25 revealed aggregated powder, composed of nanometric (cca 30 nm) spherical particles (Fig. 4a.). After adsorption of lipase, the extent of particle aggregation evidently increased (Fig. 4b.).

It is known that due to high surface/volume ratio, ultrafine powders tend to stick together forming aggregates, or secondary particles. A pore volume, made up of gaps between the particles, is created, which is often comparable in size and related in shape to the primary particles [35]. Indeed, with a mesoporosity range 13-25 nm [36], Degussa P25 can perfectly accommodate single lipase molecules (5 x 4.2 x 3.3 nm), or small aggregates of several

molecules. Lipase thus easily entered the internal voids, which resulted in formation of numerous clusters, due to bonding occurring between enzyme and support particles (Fig. 4b).

Fig. 4.

As stated, 65.5 % adsorption occurred onto titania within 1 h. For the sake of comparison, the percent adsorption determined for other materials used as supports for *C. rugosa* lipase in our previous studies: SBA15, zirconia and alumina is summarized in Table 2 [18,19].

Table 2.

As seen, the greatest amount of bound lipase was registered onto SBA15, followed by titania, and smaller ones using zirconia and alumina as support materials. Morphological features of previously used supports are provided in supplementary material (Fig. A. 1), to facilitate visual comparison.
SBA-15 exhibited fiber-like morphology, made up of peanut-shaped domains, organized in wheat macrostructures (supplementary Fig. A.1.). Its internal mesoporosity (ca half of the total surface area), based on ordered hexagonal channels with small openings (2-25 nm radius), was suitable for lipase to assess.
Sub-micrometer macroporous zirconia had much smaller Sp value. Its irregular broken sphere morphology, with undeveloped geometry afforded twice smaller amount of lipase.
Even smaller percent adsorption was registered in the case of alumina. Composed of irregular sub-micrometric solid particles with smooth surfaces, quite unsuitable for lipase to adhere, alumina provided just a moderate adsorption of lipase.
In summary, it can be concluded that geometry and surface texture of particles are of paramount importance in adsorption process of lipase. Its influence overrules largely that of electrostatics. Owing to its patchwork surface charge, *(In silico* data [18]) made up of randomly distributed negative (Glu, Asp) and positive residues (Lys, Arg, Hys), the lipase always establishes favorable localized electrostatic conditions, leading to a successful adsorption in overall either repulsive or attractive conditions. Therefore, it is a morphology of support particles that challenges the lipase to approach, establish mutual bonds and adhere.

3.5. Effect of immobilization on enzyme properties in water solution
3.5.1. Kinetic parameters
The kinetic behavior of free and immobilized lipase was investigated using *p*-NP esters with different length of carbon chain. Although it is known that lipase catalytic action is much more complex than described by the Michaelis-Menten mechanism, where it is assumed that the enzyme and substrate are parts of the same phase, it is commonly used as simplified model where the trend of modification of catalytic property can be compared.
Free lipase and lipase immobilized onto titania obeyed Michaelis-Menten kinetics on *p*-NP esters. Two parameters, Km and Vmax, were calculated from experimental data using Lineweaver-Burk equation [24] and they are presented in Table 3.

Table 3.

The affinity for the substrates (*p*-NP esters) of both forms of enzyme, free and immobilized, depended on the carbon chain length of the acid in the ester hydrolysed.

Free lipase showed similar affinity to substrates containing butyric, caprylic and lauric acid, Km ≈ 0.24 mM, however for palmitic acid, Km was considerably higher (0.4 mM), meaning lower affinity. The hydrolytic reactions catalyzed by free lipase were the most rapid when lauric acid (C-12) ester was hydrolysed, Vmax was more than 1200 U/mg of protein. When the substrates were palmitic, caprylic and butyric acid esters, the reaction was 3.1, 2.3 and 1.9 times slower respectively.

After immobilization onto titania, the affinity of lipase toward *p*-NPB and *p*-NPC was markedly lower: 8.5 and 6.9 times, respectively. Change of the affinity of immobilized lipase was negligible toward *p*-NPL (Km decrease for less than 17%), but the affinity toward *p*-NPP doubled.

After immobilization, the Vmax for hydrolytic reaction was influenced in the same manner for all investigated substrates. Lowering of Vmax is considered to be a consequence of increased rigidity of enzyme. Conformational constraint and steric hindrance is well known to decrease lipase activity [37]. In particular, *p*-NPP (with larger particle dimensions) hydrolysis slowed 8 times after immobilization. Besides transport, which depends on particle size, the steric hindrance is likely to represent major constraint for the ester containing an acid with the longest carbon chain (C16). Rate of hydrolysis of esters containing acids with a shorter carbon chain (12, 8 and 4) was also lower (i.e. decreased 2-3.6 folds).

3.5.2. Thermal stability

One of advantages of enzyme immobilization is improvement in thermal stability. Therefore, the stability of lipase immobilized onto titania was determined at 50 and 60 °C, Fig. 5. After 2 h of incubation at 50 °C, the remaining activity of free lipase and lipase adsorbed onto titania was 31 and 64 %, respectively. In terms of half-life, $t_{1/2}$ of free lipase was 55 min, while after immobilization, $t_{1/2}$ increased to 180 min. The thermostability of lipase was increased more than 3-fold after immobilization. After 1 h of incubation at 60 °C, the free lipase was inactive, while the remaining activity of immobilized enzyme was close to 60%. Even after 2 h, the activity remained 40%, or in terms of half-life, increased more than 7-fold.

A significant improvement in thermal stability of immobilized lipase seems to be result of restrict movements of protein after adsorption, preventing conformational changes and unfolding. Similar improvements in temperature stability of *C. rugosa* lipase were obtained after its immobilization onto other inorganic supports [18, 19].

Fig. 5.

3.6. Properties of immobilized enzyme in organic solvent

3.6.1. Kinetic parameters in organic solvent

It is important that kinetic measurements can be used to predict the optimum kinetic behaviour of a particular biocatalyst [38]. Based on those predictions, optimisation of biocatalytic reactions, as well as process design to improve productivity and reduce the cost of various processes can be performed [39].

Most of the kinetic studies of lipase catalyzed esterification reaction in organic solvent assume ping-pong model with inhibition by alcohol [26, 40, 41]. In order to find kinetic model of amyl caprylate synthesis, catalyzed with *C. rugosa* lipase immobilized onto titania, series of

experiments were performed at determined conditions. Values of the equation parameters in Eq. (1) obtained after processing experimental data are given in Table 4.

The proposed model Eq. (1) was validated by fitting the model to the set of experimental data. For kinetic ping-pong model with inhibition by alcohol l fitting, goodness of fit was $R^2 = 0.835$, Adjusted $R^2 = 0.819$ and Root Mean Squared Error = 1.95. A good agreement for this kinetic model was obtained.

Table 4.

Fig. 6. presents dependence of initial rate of amyl octanoate synthesis on concentrations of both substrates. The increase of concentration of octanoic acid increases the rate of esterification achieving the maximum rate of 26.36 µmol min^{-1} at 1 mol/L of acid and 0.3 mol/L of alcohol. On the other hand, the increase of concentration of amyl alcohol confirmed hypothesis of substrate inhibition as presented in Fig.6 b.

Fig. 6.

3.6.2. Synthesis of methyl esters in solvent free system

Fatty acid methyl esters were the first fatty acid esters to be introduced for use as biodiesel [42]. Biodiesel appears to be an attractive energy resource hence it is a renewable with several favourable environmental properties. It contains no aromatic compounds and other chemical substances which are harmful to the environment [43]. Considering the importance of biodiesel, a possibility of synthesizing methyl esters of palmitic, lauric and octanoic acid in solvent free system, using lipase immobilized onto titania, was tested. Synthesis of methyl octanoate was confirmed by GCMS analysis and titrimetry, with low reaction yield of 9% while esters of methanol with lauric and palmitic acid were barely detected. This result is in a agreement with previously published observation in which higher yield of methyl esters was obtained with heptanoic and octanoic acids [44].

3.7. Reuse stability

The reusability of immobilized enzyme is one of the most important advantages for application. Adsorption, as an immobilization method, is usually considered as a method with poor reuse potential, as the linkages established are usually weak and the enzyme could be easily desorbed. Reuse stability of lipase immobilized onto titania in water solution and in organic solvent is shown in Fig. 7. As seen, the remaining lipase activity in water was about 90% after nine reuses. The activity decreased continuously from 9 to 20 reuse cycles; however, it remained ca. 40%. Protein desorption process was followed in parallel for 12 reuses. Although protein desorption was 40% after 4 uses lipase activity remained almost the same.

Easily desorbed proteins, according to Table 1. (specific activity of 64.7 U/mg of protein) were probably protein impurities. If any amount of lipase was desorbed, those were enzyme molecules positioned at the surface of aggregates (section 3.2.) [20]. In return, the former inner lipases became exposed and active towards the substrate, resulting in almost unaffected enzyme activity.

Fig 7.

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On the other side, immobilized enzyme in cyclohexane shows lower reusability. As presented in Fig.7., the second application of biocatalyst in synthesis of amyl octanoate was as good as the first one, but after the third and the fourth reuse cycles the activity decreased to 80% and 77%, respectively. The synthetic activity of lipase remained 60% after 5 reuse cycles, but dropped to 10% after the following cycle. Desorption process was followed for 5 cycles but protein leaching was not registered. Denaturation of lipase as a consequence of damaged water layer around lipase molecule could result in decreased activity. Although reuse stability in organic solvent was not as good as in water, presented results point to the potential for reuse of lipase after immobilization in cyclohexane.

4. Conclusions

C rugosa lipase was immobilized onto titania powder (Degussa P25) as support in the Tris-HCl buffer, pH 7.6. High percent adsorption was found (65.5%) owing to mesoporous structure of the nanometric sized powder. Determined specific activity of immobilized lipase was 64.7 U/mg of protein. After immobilization, lipase affinity decreased toward *p*-NPB and *p*-NPC, but increased toward *p*-NPP. Vmax for hydrolytic reaction decreased from 2 to 8 fold. The highest reduction of activity was toward the largest substrate *p*-NPP. A significant improvement in thermal stability was achieved. Excellent reuse stability in water solution was revealed.
Lipase immobilised onto titania was stable and active in organic solvent and could be used for synthesis of esters of octanoic acid in organic solvents, and methyl esters in solvent free system. Synthesis of amyl octanoate in cyclohexane was well described with ping-pong bi-bi mechanism with inhibition by amyl alcohol. Reuse stability in cyclohexane was confirmed.
Based on the findings of current and our earlier studies, the morphology of powder used as a support was ascertained as more important adsorption parameter than electrostatics.

Acknowledgements

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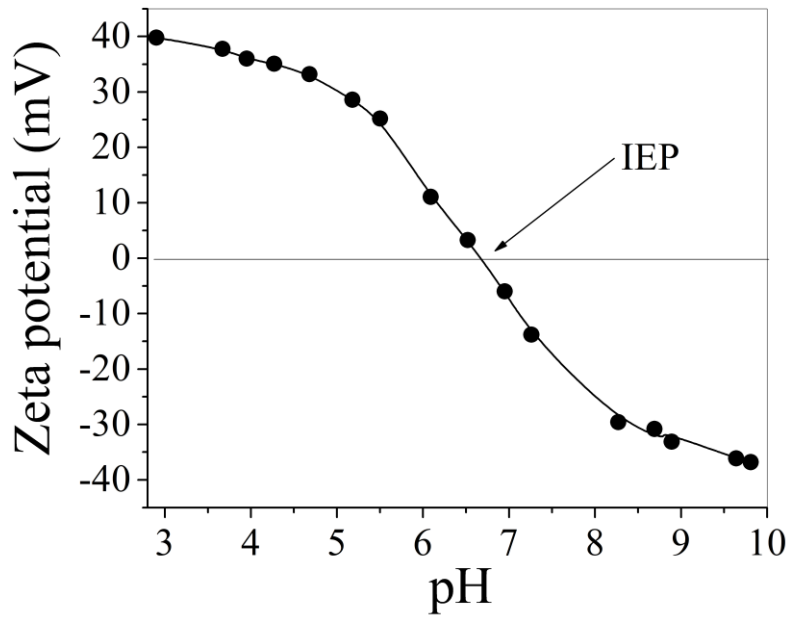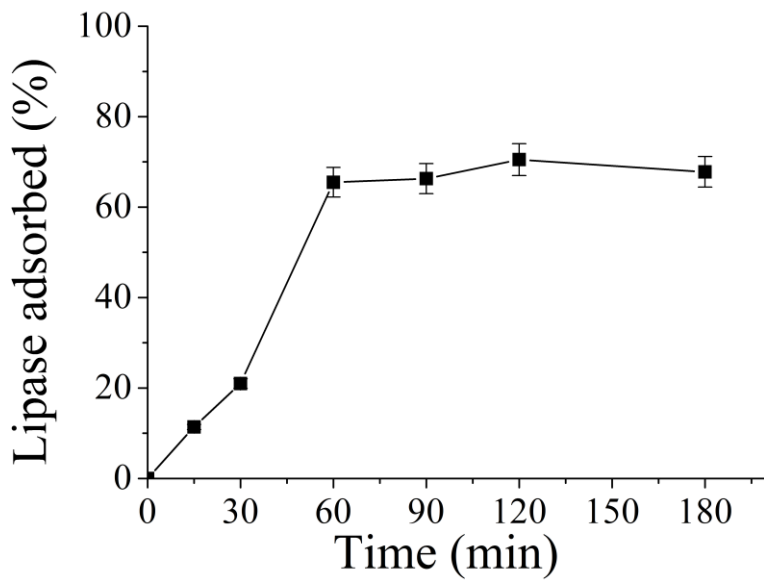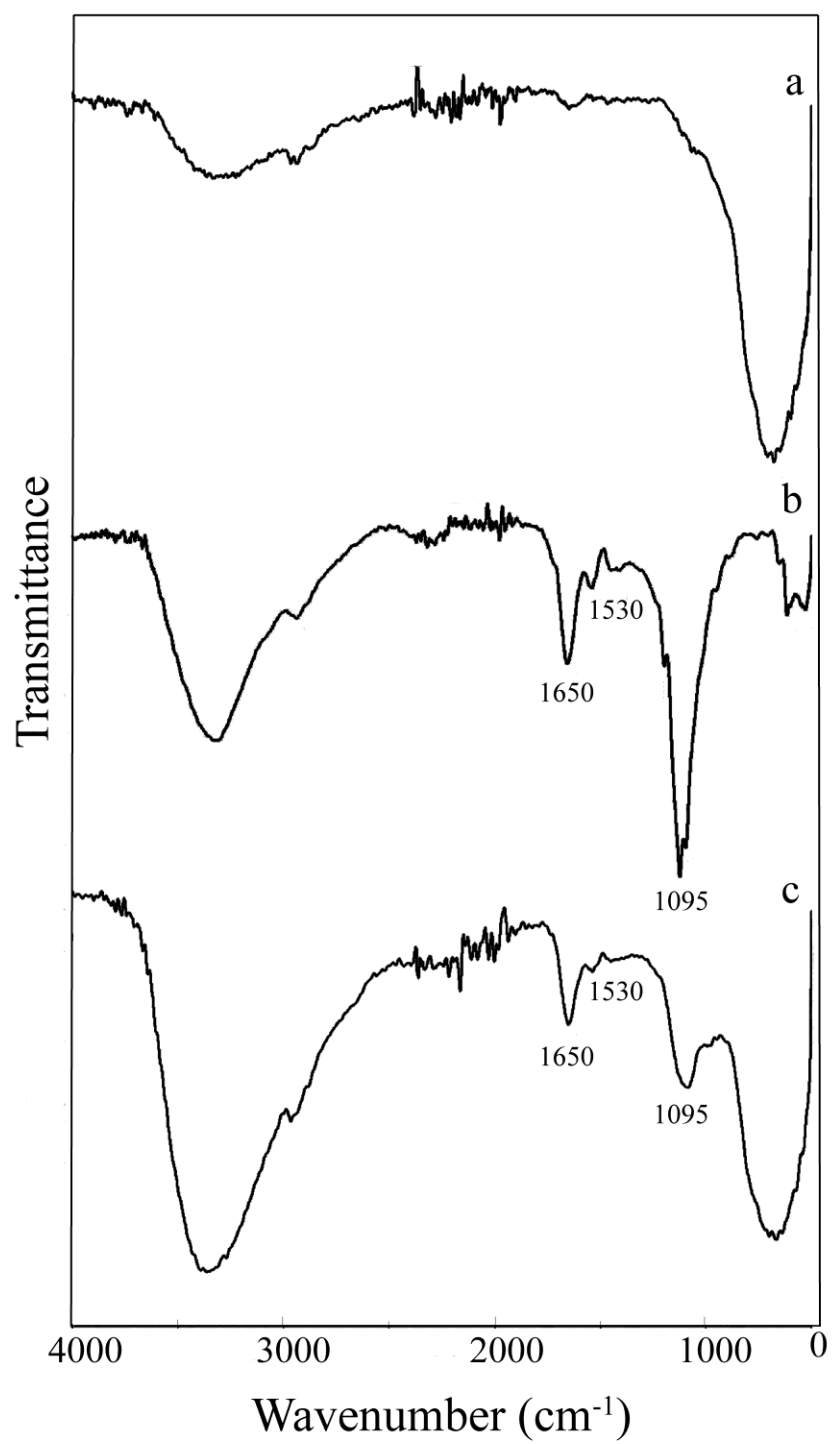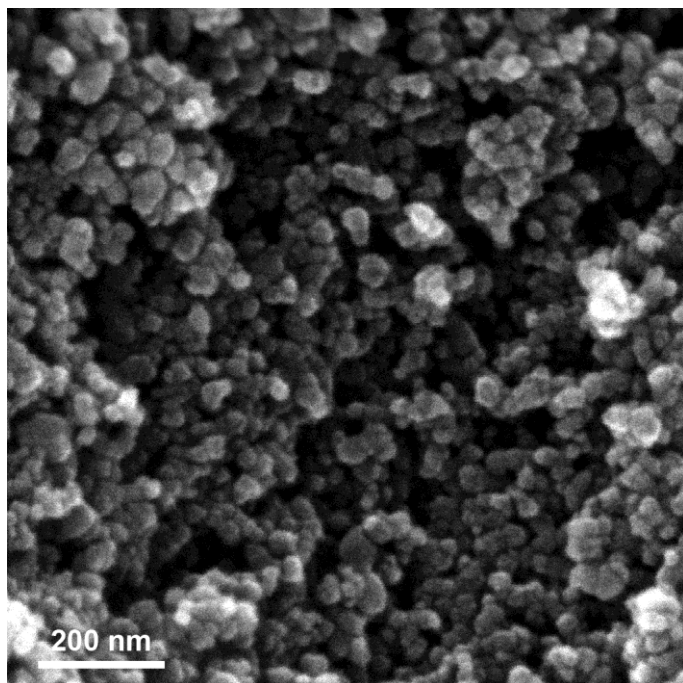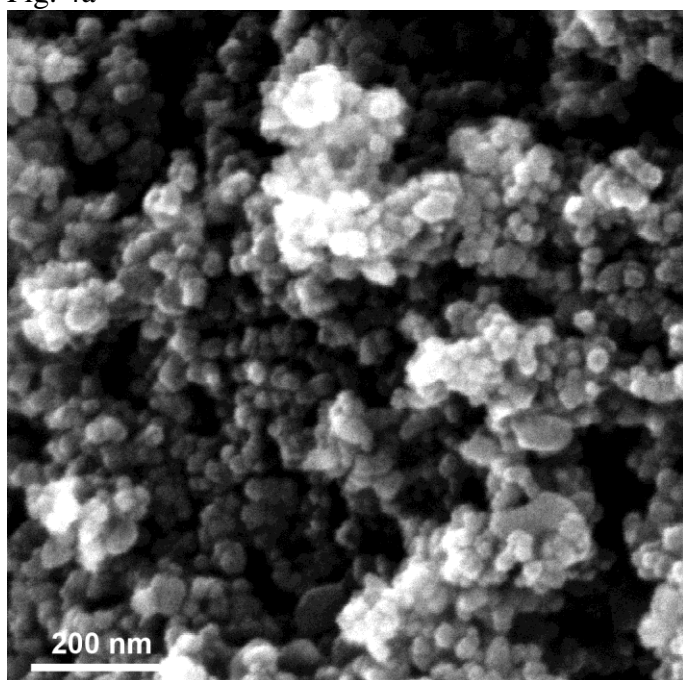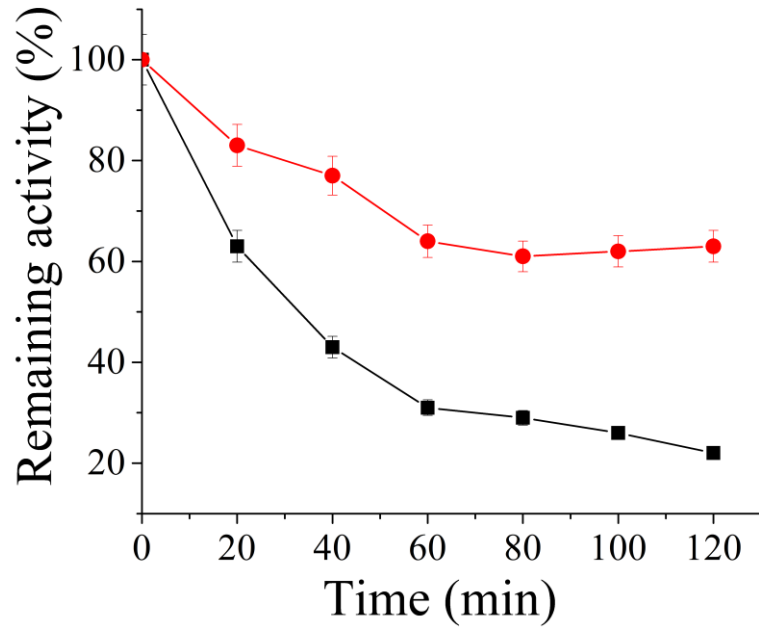
Fig 1

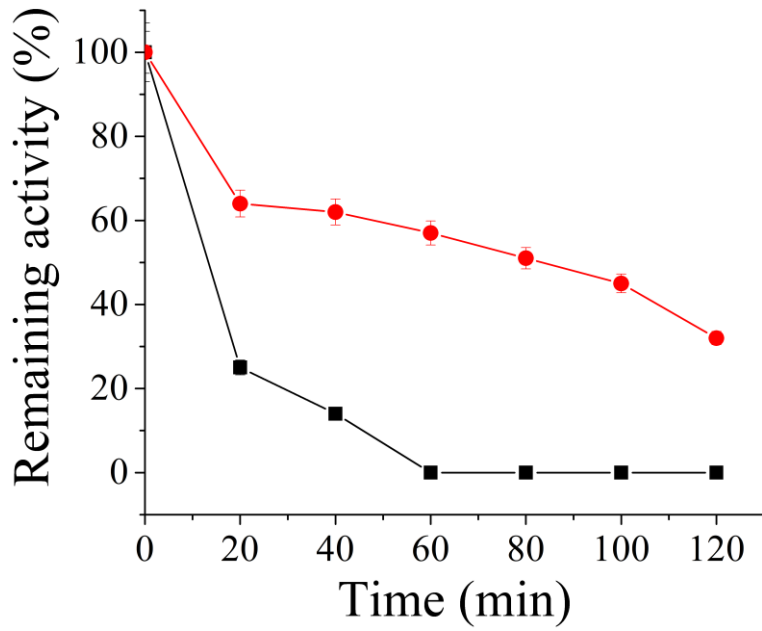Fig 2
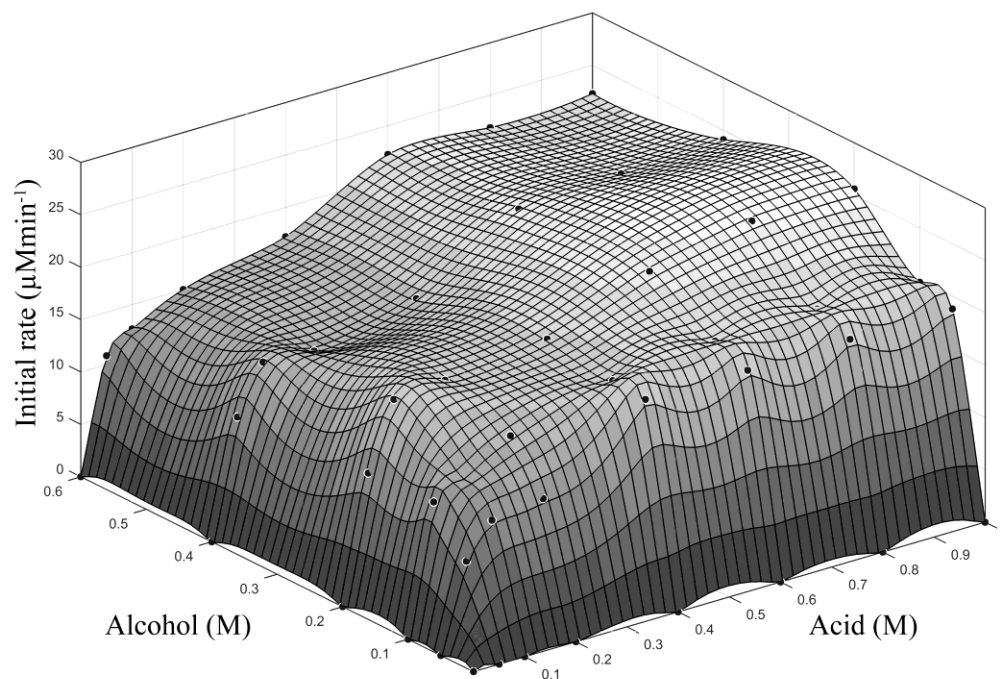
Fig. 3.

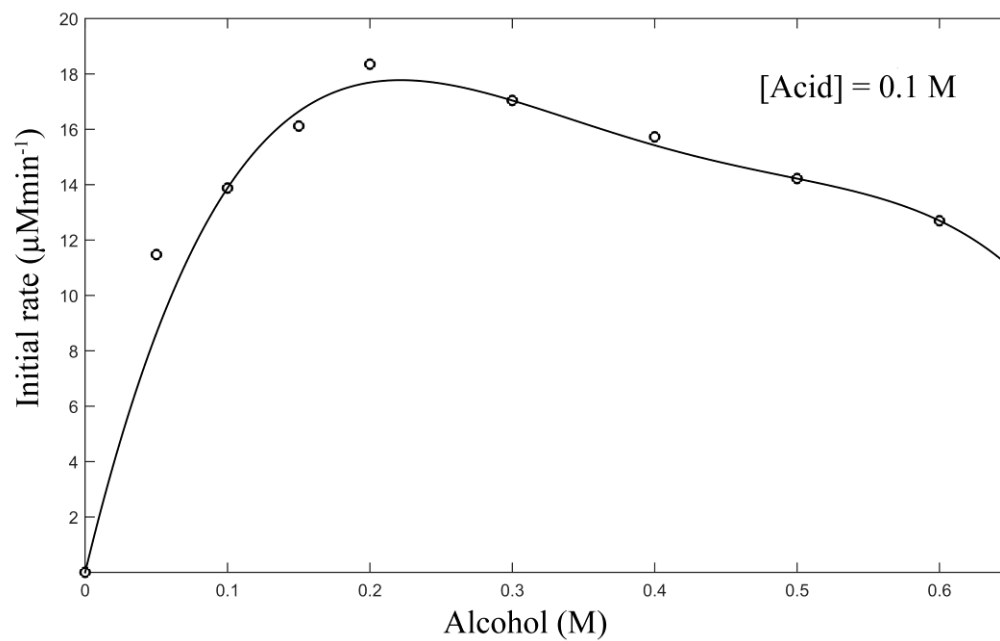Fig. 4a

Fig. 4b

Fig 5a

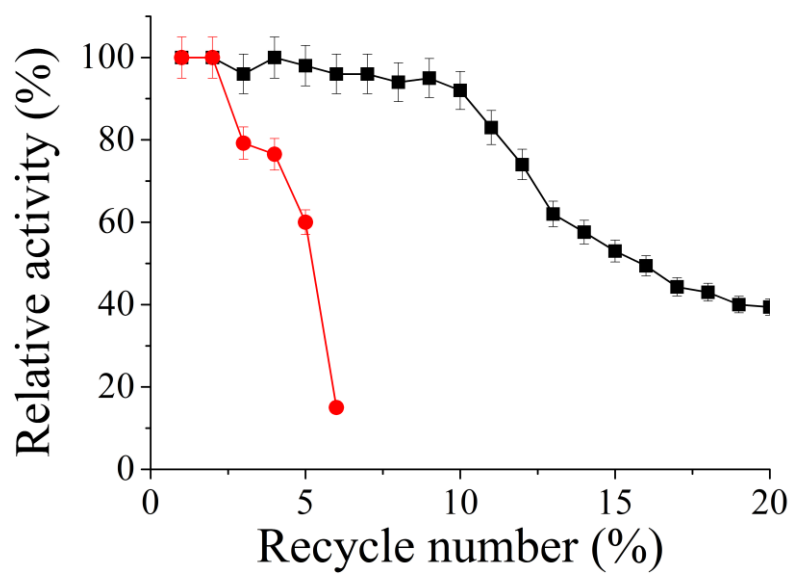Fig 5b

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Fig 6.a

Fig 6.b

Fig 7

Table 1.
Efficiency of immobilization in terms of enzyme coupling yields, η_{enz} and activity coupling yields η_{act}

Crude lipase loading (mg/g support)	Enzyme loading (mg protein/g support)	η_{enz} (%)	Free lipase specific activity (U/mg of protein)	Imobilised lipase specific activity (U/mg of protein)	η_{act}(%)
500	65	65.5	308	64,7	21

Table 2.
Efficiency of immobilization (η_{enz} and η_{act}) of *C. rugosa* lipase on different supports after 1 h

Support material	η_{enz} (%)	Sp^a ($m^2 g^{-1}$)	η_{act}
SBA 15 [18]	80	641	29
Titania	65.5	57.4	21
Zirconia [18]	42	14	18
Alumina [19]	32	7	24

[a]specific surface area

Table 3.
Kinetic constants of *C. rugosa* lipase before and after immobilization determined by hydrolysis of *p*-NP esters

Supstrate	Free lipase		Immobilized lipase	
	Vmax (U/mg)	Km (mM)	Vmax (U/mg)	Km (mM)
p-NPP (C16)	410.6	0.405	51	0.208
p-NPL (C12)	1279	0.243	354	0.202
p-NPC (C8)	557	0.237	271	1.644
p-NPB (C4)	683	0.247	238	2.012

Table 4.
Values of kinetic constants from ping-pong bi-bi model with alcohol inhibition

Parameter	Value
Vmax (μmol $min^{-1}g^{-1}$)	26.36
K_{Al} ($moldm^{-3}$)	0.200
K_{Ac} ($moldm^{-3}$)	0.522
$K_{i,Al}$ ($moldm^{-3}$)	0.644