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Research Article

Engineering aspects of immobilized lipases on esterification: A special emphasis of crowding, confinement and diffusion effects

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Cross-linked enzyme crystal (CLEC) and sol-gel entrapped *Pseudomonas* sp. lipase were investigated for the esterification of lauric acid with ethanol by considering the effects of reaction conditions on reaction rate. The activation energy for the reaction was estimated to be 1097.58 J/mol and 181.75 J/mol for sol-gel and CLEC entrapped lipase respectively. CLEC lipase exhibited a marginal internal diffusion effect on reaction rate over sol-gel lipases and found to be interesting. The overall reaction mechanism was found to conform to the Ping Pong Bi Bi mechanism. The higher efficiency of sol-gel lipases over CLEC lipases in esterification reaction is mainly due to the combined effects of crowding, confinement and diffusional limitations.

Keywords: CLEC lipase / Confinement / Crowding / Diffusion / Ethyl laurate / Sol-gel lipase

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1 Introduction

Retaining the primary, secondary, tertiary and quaternary structures are prerequisite for enzymes to maintain the catalytic activity under harsh industrial process conditions (high temperature, extreme pH, operation parameters) by devoiding the substrate/ product inhibition. Cross-linked enzyme crystal (CLEC) and sol-gel immobilizations are a choice of interest in protein stabilization techniques and find practical importance in biocatalysis domain [1, 2]. Sol-gel entrapped lipases on a thin film of inert support facilitate reuse of enzymes by overcoming diffusion limitations and also the structural aspect makes them an easy usage in enzymatic bioreactors [3–5]. The sol-gel entrapped lipases also avoid the problems incurred during covalent immobilization techniques where strong binding affect the catalytic triad residues or desorption (van der Waals, hydrogen or ionic binding), which usually encountered during conventional immobilization techniques [6]. As entrapped in a thin film of inert support, sol-gel lipases also overcome the activity inhibition by reaction components such as alcohols, water-miscible solvents, high temperatures and pressures sensitivities of lipases. In case of CLEC, the high catalytic activity of purified lipase is immobilized by crosslinking with a suitable crosslinker such as glutaraldehyde [1].

Once lipase interacts with inert support, the conformation will be changed due to the folding/unfolding phenomenon in a crowded and/or confined environment. The free volume space of the lipase is limited either by the dense surrounding biomolecules, or by the small confinements which eventually affect the protein stability in terms of thermal stability, and chemical reactivity [7, 8]. Hence, the knowledge of crowding and confinement of proteins on immobilization drive the researcher for better understanding the folding and its stability in crowding and/or confinement conditions. The profound effects of crowding and confinement on the dynamic and functional properties of enzymes and subsequently on the dependent bioprocess have been started to acknowledge by the researchers [9, 10]. Several studies have been carried out using these immobilized benign catalysts in the esterification and transesterification reactions [11–17]. These studies are mainly targeting the selection/ optimization of the process conditions, reaction mechanism and reusability studies by devoiding the crowding and confinement effects on the enzyme stability and subsequent bioprocess. Hence, in this paper, we have attempted the relative assessment of CLEC and sol-gel entrapped lipases for esterification of lauric acid with ethanol in terms of crowding and confinement effect on reaction rates along with the selection of process conditions.

2 Materials and methods

2.1 Materials

Lipase from *Pseudomonas* sp. L9518 containing ≥ 15 U/mg (Sigma), Lauric acid, ethanol, solvents of analytical grade were

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Abbreviations: CLEC, cross-linked enzyme crystal; PAMS, poly (ethoxymethyl) siloxane; PHOMS, poly (hydroxymethyl) siloxane

from CDH Pvt. Ltd. Mumbai, India. Ethyl Laurate was purchased from fluka, Bombay, India. Poly (dimethylsiloxane-co-methylhydroxiloxane, 96% wt/wt, MeHSiO) supplied by Sigma-Aldrich, USA.

2.2 Methods

2.2.1 Preparation of sol-gel entrapped lipase

PHOMS (polyhydroxymethyl siloxane) support was synthesized by adding poly(dimethylsiloxane-co-methylhydroxiloxane (6.4 g, 96% wt/wt, MeHSiO) to a dry ethanol (10 mL) and Et-ONa (2 mL, 0.1 M) mixture. The mixture was then stirred for 1 and 2 h at ambient temperature and 50°C, respectively to form Poly (ethoxymethylsiloxane) (PAMS). The formed PAMS were then diluted with aqueous ethanol (20 mL, 50%) and NaOH (25 mL, 5 M) and the resultant mixture was stirred for 1.5 h. The obtained silonate was diluted with deionized water (150 mL) and adjusted the pH to 7.0 with HCl (5 M) in a stirrer equipped beaker. The formed precipitate of PHOMS (snow white) was filtered through buchner funnel with repeated washings with deionized water till attainment of chloride ions. The resultant immobilization matrix was further stored in the refrigerator for further immobilization [18].

Immobilization was carried out by addition of aqueous lipase solution (15 mL) to a stirred mixture of ice cold, wet PHOMS (5 g, 30% solid wt/wt, in 13 mL of 2.05 M hexameta phosphate buffer) and isopropanol (2 mL). After 15 min, add another portion of ice cold hexametaphosphate buffer (10 mL, 0.25 M) and allow the mixture to stir for 2 h at 0°C. The formed lipase – PHOMS adsorbate was separated by filtration with suction and washed successively with ice-cold 0.25 M buffer, acetone, and pentane. The adsorbate was stored in a desiccator over a molecular sieve. The amount of the enzyme adsorbed on PHOMS was determined as the difference between UV absorbance of the starting and the final aqueous filtrate.

2.2.2 Preparation of CLEC lipase

CLEC immobilized lipase was prepared by addition of celite 545 (2.0 g) to pseudomonas sp. lipase (5 mL) with constant stirring for 1 h with a magnetic stirrer at room temperature. To the mixture, chilled acetone (20 mL) was added and the resultant suspension was filtered off. The filtered CLEC lipase was further washed twice with acetone (20 mL) and then dried for 4 h in a vacuum desiccator [11].

2.3 Esterification reaction and its kinetics

The esterification reaction was carried out by mixing the reaction mass in a round bottom flask (100 mL) using a magnetic stirrer at a speed of 200 rpm by maintaining the constant reaction temperature of 30°C. The substrate and immobilized lipase (Sol-gel and CLEC) concentrations were maintained between 10 and 60 mM and 0.75 mg/mL respectively. All kinetic experiments were carried out in n-hexane which was found to exhibit better reaction environment in dispersed systems and constant water concentration (water activity) was maintained by mea-

suring with a Karl Fischer Titrator (Spectralab MA-101-B, alfa instruments, New Delhi). All experiments were carried out in triplicates and the all the values were represented as $\pm$ SD values of three replicates. The reaction samples were collected in 30-min intervals and analysed by HPLC (Waters 510, injector: U6K, Column: varian supplied SP- C-18, Particle size 4.5 micrometer, dimension: 4.0 mm $\times$ 150 mm s.s.) using water (5%) and acetonitrile (95%) as solvent in Isocratic mode (flow rate of 0.5 mL/min, using UV detector at 254 nm). The results were presented in terms of initial reaction rates which were calculated from concentration of limiting substrate i.e. lauric acid conversion versus time profiles corresponding to the first 10% conversion where the profiles were found to be linear i.e.

$$R_2 = V \Delta C \Delta V t \quad (1)$$

Where V is the volume of reactant mixture, C is a concentration of limiting substrate and t is temperature. The parameters studied were enzyme and ethanol concentration. The temperature effect was also studied to obtain the activation energy of the reaction.

2.4 Effect of diffusion on CLEC and sol-gel immobilized enzymes

The impact of diffusion was analyzed based on the classical theory of reaction diffusion in heterogeneous catalytic systems by considering spherical particles of the immobilized enzyme [19]. As the catalyst follows a first-order reaction, the theory leads to a conclusion in the form of

$$\eta = \frac{3}{\phi^2} (\phi \cot \phi - 1) \quad (2)$$

Where η = Catalytic effectiveness factor and ϕ = Thiele modulus

Considering internal diffusion effect theory [20] in heterogeneous catalysis, catalytic effectiveness factor (η) can be written as

$$\eta = \frac{3}{r} \left(\frac{D_e}{k_1 \rho A_g} \right)^{1/2} \quad (3)$$

And

$$\phi_0 = r \left(\frac{\rho A_g V_m}{D_e [S]} \right) \quad (4)$$

Where D_e is diffusivity (cm^2/s), ρ is particle density (g/cm^3), r denotes the particle radius (cm), A_g symbolizes the particle surface area (cm^2), k_1 is rate constant (s^{-1}), and $[S]$ denotes the poly(allylamine) concentration (mM). The values of D_e can be estimated from the Wilke–Chang correlation [21], implying marginal internal diffusion effect. As

$$D_e = \frac{D_m \epsilon_p \sigma_e}{\tau} \quad (5)$$

Where D_m is molecular diffusivity, ϵ_p porosity of catalyst particle, σ_e constriction factor, and τ tortuosity factor.

According to Hanes-Woolf equation, the modified form of the Michaelis–Menten equation,

$$\frac{S}{V} = \frac{S}{V_{max}} + \frac{K_m}{V_{max}} \quad (6)$$

Figure 1. Effect of enzyme concentration on initial rate. The reaction mixture consists of lauric acid 20 mM: ethanol 20 mM.

Where S is the concentration substrate, V denotes reaction rate, $V_{\max}$ is the maximum rate and K_m is the Michaelis constant. Effectiveness factor gives information on the role of diffusion in the reaction.

$$\eta = \frac{\text{Rate (immobilised enzyme)}}{\text{Rate (free enzyme)}} \quad (7)$$

3 Results

3.1 Effect of enzyme concentration

The effect of enzyme concentration on initial reaction rate was shown in Fig. 1, the initial rate increases with a lower range of lipase concentrations, and latter on slow gradual increase in initial rate and reaches an asymptote at a certain lipase concentration. The higher reaction rates with immobilized lipases are mainly due to the amino acids active form those have been adsorbed in the immobilized media [22]. Similar results were also be reported with the crosslinking with glutaraldehyde [9,23]. Presence of active lipase at lower concentrations throughout the reaction denotes the kinetic control of the reaction at lower immobilized concentrations and comply with the reported reactions of lipase mediated esterification and transesterification [24]. The relative slowness of initial rate at higher lipase concentrations has been attributed to the lipase saturation with the substrate [25].

3.2 Role of substrate concentration on initial rate

The effect of ethanol concentration on the initial rate of esterification was shown in Fig. 2 by keeping the lipase and lauric acid

concentrations as constant. Higher reaction rates were observed with the higher ethanol concentrations with a maximum reaction rate at a lauric acid to ethanol molar ratio of 1:2 beyond this molar ratio there is no further increase in rate. The observed pattern may be attributed to the passive to the inhibition effect which was induced at higher concentrations of ethanol.

3.3 Effect of temperature on initial rate

The effect of temperature (30–60°C) on the reaction was depicted under Fig. 3 and found that the initial rate almost linearly enhances with the lower temperatures up to a certain point and later on decreases with the increasing temperature.

In the case of a soluble/free lipases, the initial rate decreases more pronouncedly than the sol-gel and CLEC lipases due to the intrinsic lower activation energies [11]. Once the lipase immobilized, the enzyme diffusion may become rate limiting step and the reaction switch from kinetic reaction to diffusion controlled system which results in a apparent activation energy change (E_a) Fig. 4. Accordingly, the experimentally determined the activation energy (E_{app}) for this immobilized lipase system may be different from true E_t on value as a result of the so-called distinguished kinetics [26,27]. True E_t value was obtained from the following relation, $E_t = 2E_{app}$. However in our system the particle size is small (less than 0.5 mm) and the nature of immobilized media is such that a rigid structure with lower porosity may be expected [22,27] implying that

$$E_t = E_{app} \quad (8)$$

Where E_t is the theoretical activation energy and E_{app} is the experimental activation energy

Figure 2. Effect of ethanol and lauric acid concentration on initial rate. Lauric acid concentrations were 20, 40, and 60 mM.

Based on the Arrhenius rate equation, the activation energy (E_a) was determined using

$$k = Ae^{-E_a/RT} \quad (9)$$

Where k is the reaction rate constant (s^{-1}); A denotes the Arrhenius pre-exponential factor; R is the gas constant (8.3144 J/(K mol)); T denotes the absolute temperature. The Arrhenius plots for sol-gel and CLEC catalyzed esterification reactions were shown in Fig. 4. The calculated activation energies (E_{app}) were found to be 1097.58 J/mol, which denotes the immobilized lipase's higher reactivity with enhanced stability. The true E_t may be estimated as 2195.16 J/mol. Moreover, the esterification reaction follows the Ping Pong Bi Bi mechanism, where one substrate is bound to the lipase at any time and results in the formation of substrate-enzyme complex. After product formation and subsequent release, the other substrate binds to the modified lipase to form the second product [14].

3.4 Stability of sol-gel and CLEC lipases

The stability of sol-gel and CLEC lipases were tested by incubating the immobilized lipases for different time intervals. The initial rates of reaction with the subsequent incubation time for the immobilized lipases (sol-gel and CLEC) were shown in Fig. 5, and it appears that the enzyme retains its activity up to the six days of incubation time. To test the reusability of immobilized lipases, immobilized lipases were recovered from the reaction mixture by adjusting the pH to its isoelectric point. The immobilized lipases were filtered off and washed with distilled water and reused in three successive experimental runs, and found

that the immobilized lipases activity was unaffected even after the third usage (data was not shown).

3.5 Effect of diffusion on CLEC and sol-gel immobilized enzymes

The kinetic parameters of sol-gel and CLEC lipases were calculated through Hanes–Wolf plot and the results are summarized in Table 1 [1]. Usually, in case of homogeneous reactions with free enzymes, where complete diffusion conditions are prevalent, the obtained η value will be around 1. Enzyme immobilization facilitates the enzymes to use in a heterogeneous environment where diffusion effects are predominant and whose extent is usually drawn from the measuring η (Table 1).

4 Discussion

Usually, the sol-gel lipases exhibit enhanced thermal and chemical denaturation resistance along with the improved storage and operational stability due to the controlled porous structure of sol-gel polymers [1,28–30]. Among different hydrophobic polymers, Ormosils such as alkyl-alkoxysilanes shown better results than the free enzyme counterparts. This behavior of lipases is mainly due to the unique 'lid' structure which can be opened while exposing to any hydrophobic interface which facilitates the full expose of the binding site to the substrate [31–33]. Moreover, the hydrophilic/hydrophobic balance of neighboring surfaces plays an important role in protein structure and biological activity sensitivity and subsequently towards improved structural rigidity. The hydrophobic organic ligands of silane play

Figure 3. Effect of temperature on initial rate by CLEC and sol-gel immobilized enzyme. (Lipase 0.2 g/mL, ethanol 20 mM and lauric acid 20 mM).

Figure 4. Activation energy curve, based on effect of temperature on initial rate.

a major role in gel matrix characterization (porosity, rigidity and matrix hydrophilicity) that is crucial for catalytic properties of lipase [1, 34]. As an interface-active enzyme, the presence of surface hydrophobicity of the sol-gel will be helpful in maintaining molecular conformation which enhances the lipase activity too [1].

The enhanced stability of sol-gel lipases may also be attributed to the restricted global movement of the rigid polymer cages [35, 36]. Sol-gel lipases of *Candida rugosa* exhibited enhanced activity and enantioselectivity in hydrolyzing the *p*-NPP and racemic Naproxen methyl ester [37]. The additive capability of calixarene derivatives in sol-gel technology for hydrolysis

Figure 5. Effect of storage time (days) on initial rate.

Table 1. Kinetic parameters of CLEC and sol-gel immobilized lipase-mediated transesterification

Enzyme mode	K_m (mmol/mL)	V_{max} (mmol/mL/s)	Effectiveness factor (η)
Free	0.28594	11.64923	
CLEC 60 mmol	0.00452	0.564972	0.048499
Sol-gel 20 mm	0.252874	11.49425	0.986696

reaction of (R, S)-naproxen methyl ester results in higher conversion and enantioselectivity than the free lipase [38].

Enhanced pH stability with sol-gel lipases is mainly due to the existing hydrophilic and hydrophobic interactions between the lipase and immobilization support [39]. The more rigid structure of sol-gel lipases is the primary attribute for the enhanced catalytic activities of sol-gel lipases at higher temperatures. The enhanced catalytic activities of sol-gel lipases are further attributed to the conformational limitations of lipase movements with the existing hydrophobic interactions between the lipase and sol-gel support or improved thermal denaturation of lipase [40]. Presence of lower diffusional limitations at higher temperatures also contributes a little extent for enhanced catalytic activities with the increased temperature. The enhanced thermo stability of sol-gel lipase is mainly due to the strong confinement of the lipase in the sol-gel matrix [41]. Distortion of the protein conformation is the main attributing phenomenon for reported higher enantioselectivities with sol-gel lipases in the presence of additives [42, 43]. Lipase immobilized in silica aerogels reinforced with silica quartz fiber felts in the presence of organic solvent shown enhanced activities in biodiesel production compared to free lipase [44].

The highly retained efficacy of sol-gel lipases is mainly due to the incurred conformational changes upon immobilization, which facilitates lipase's fixation in a favorable conformation and to the hybrid matrix structure which facilitates reducing the diffusional problems for the transport of the hydrophobic

substrate to the active sites [45]. Sol-gel entrapped lipases of *Candida antarctica* B exhibited enhanced enantio-selectivity and activity while using the 1-octyl-3-methyl-imidazolium tetrafluoroborate as an additive. The sol-gel lipase also exerts excellent operational stability with unchanged enantioselectivity till 15 recycles [46]. The sol-gel encapsulated lipase in highly ordered mesoporous matrix exhibited six times higher productivity than the free counter-part in transesterification reaction of tri-olein with methanol. The sol-gel lipase preserves the mobility of the enzyme and allows increasing its stability [47, 48]. Sol-gel lipase of *C. rugosa* lipase B in the presence of an alkyl surfactant (octyl-d-glucopyranoside) exhibited a 3.3-fold rate enhancement over the free lipase [49].

The beneficial use of enzymes for synthesis of industrial organic chemicals provides impetus to further developments in technological perspectives. The immobilized enzyme is gaining more importance due to their advantageous feature favoring wide acceptability for the economic benefit of process technologies [50].

Sol-gel matrices have a controllable surface area with an average pore size with equal pore size distribution, fractal dimension and thermal stability. In sol-gel form, the lipases orient in such a way that their active sites are amenable to the pore network of the immobilization matrix. The unique feature of the sol-gel system lies in the tuning of process parameters (precursors nature, usage of surfactant) for attaining the tailored host-guest interactions. Thus an analysis of the chemical effect of molecular sol-gel entrapment could perhaps strongly suggest that the sol-gel phenomena with enormous control on fine tuning the host guest interaction can provide scope for a wide range of novel chemical process in sol-gel matrix [51–53]. Reusability feature of sol-gel lipases facilitates the cost-advantageous and easy separation of the lipase from the lipase-catalyzed reactions [11, 54–56].

Crowding and confinement play a vital role in enzyme immobilization techniques by affecting the conformation and native structure of the enzyme. In the study by Daryl and Joan, 2000 [54] and Reetz et al. 1996 [1], the encapsulated lysozyme

and lipases retain reported the enhanced stability and activity. These findings reveal the unaffected crowding and confinement phenomenon on Sol-gel lipases which accounts for the higher reaction rates associated with the sol-gel immobilized lipases while using in esterification/transesterification reactions [57].

Enzyme stability also influenced by macromolecular crowders such as dextran, poly(ethylene glycol) (PEG), Ficoll, proteins and DNA and bioprotective osmolytes, like sugars, polyols, methylamines. These help in stabilization of the native conformation of globular proteins under external stresses [58]. Radhakrishna et al. 2013 [59] reported the influence of crowding and confinement conditions for higher activities associated with the immobilized alcohol dehydrogenase (ADH) in SBA-15. However in case of CLEC, the enzyme stability is mainly dependent on the crosslinking efficiency, internal mass-transfer resistance and crowding and confinement environment created by modification of essential amino groups by the cross linking agents such as glutaraldehyde [60]. In our study sol-gel lipases shown greater activity than CLEC in esterification reaction. The lower reaction rates of CLEC are attributed to the crowding effect atmosphere created by modification of amino acid residues. The similar result has also been postulated in case of CLEC *Candida antarctica* lipase, where modification of lysine residues by cross linking agent responsible for crowding environment and imparted negative effect on the enzyme activity [61]. In our study sol-gel lipases shown higher activity and stability than CLEC lipases in the esterification reaction and this may be due to profound effect of crowding and confinement on CLEC lipases than the sol-gel lipases.

The kinetics of sol-gel and CLEC lipases can be affected by either through external diffusion (transport of substrate and products from the bulk solution to the outer surface of the enzyme particle) or internal diffusion (internal transport of these species inside the porous system) [62]. Since enzymes caged in pores of sol-gel matrix, the dominant effect in case of sol-gel lipases is mainly through external diffusion resistance. The η value of sol-gel catalyzed esterification was close to 1 suggesting the absence of diffusional effects in sol-gel systems. However, in case of CLEC reaction takes place on the surface of the crystal even though value of η is half of the free enzyme which may be due to inactivation of enzyme active site during crosslinking. The K_m and V_{max} values of sol-gel lipases are very close to that of free lipases which suggesting the efficacy retaining of sol-gel lipase with native confirmation only. A lower K_m and V_{max} values of CLEC lipases than the free lipase proposing the decreased efficacy of CLEC lipase upon immobilization.

Increase the pore size of sol-gel matrix is the one approach (using d-glucose as template agent) to overcome the loss of catalytic activity due to diffusion limitation of substrate molecules for poor accessibility of enzymes inside the matrices [63]. Introduction of the organic groups to the Glucoamylase immobilized in TEOS and PhTES / TEOS systems results in a slight decrease in the pore volume [64]. *Candida cylindracea* lipase immobilized in MTEOS/TEOS in 5:1 molar ratio with, NaF as catalyst and polyethylene glycol as additive shown highest activity in transesterification reaction [65]. In another Khalameida et al., 2014 [66], pore diameter, specific pore volume, skeletal density

and porosity of APM/SiO₂ sol-gels increase linearly with CaO concentration [67].

The esterification reaction of ethyl laurate synthesis catalyzed by sol-gel and CLEC lipases was found to be an effect of reaction conditions (reaction temperature, substrate concentration, lipase concentration) and the reaction mechanism conform to the Ping Pong Bi Bi mechanism. The activation energy for the reaction was estimated to be 1097.58 J/mol and the true value is 2195.16 J/mol for the reaction. The effect of internal diffusion on observed rate was found to be marginal. The activity of immobilized lipase remains same up to the third cycle.

The higher activation energy and K_m of the sol-gel immobilized lipases over CLES lipases are mainly attributed to the better crystal structure and the crystal packing information in the sol-gel systems over CLES [68]. In case of CLES, lipases are cross-linked in three-dimensional lattices which facilitate the less hindered diffusion effects due to the presence of cross linked 'rinds' on the surfaces of CLES which determines the passage of the substrate to reach the enzyme active site [69]. Moreover, the enzyme loading in CLES will be only around 0.1–10% w/w of the total, hence more amount of CLES is needed to achieve the higher reaction rates such as sol-gel systems [70].

Overall, based on the present study we have concluded the crowding and confinement phenomenon has profound role in case of CLEC lipases than the sol-gel lipases in the esterification reaction.

Practical application

The present study emphasizes the role of immobilized enzyme engineering aspects namely crowding, diffusion and confinement on the total performance of CLEC and sol-gel lipases. CLEC and sol-gel lipases studied in esterification reaction of lauric acid and ethanol. CLEC lipase exhibited a marginal internal diffusion effect on reaction rate over sol-gel lipase. The efficiency of sol-gel over CLEC lipases attributed to the crowding and confinement effects. The results of this study showcasing the importance of crowding, diffusion and confinement effects with the immobilized enzymes which in turn depicts the probable solutions for lower reaction rates associated immobilized enzymes.

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5 References

- [1] Reetz, M. T., Zonta, A., Simpelkamp, J., Efficient immobilization of lipases by entrapment in hydrophobic sol-gel materials. *Biotechnol. Bioeng.* 1996, 49, 527–534.

- [2] Chen, J. P., Lin, W. S., Sol-gel powders and supported sol-gel polymers for immobilization of lipase in ester synthesis. *Enzyme Microb. Technol.* 2003, 32, 801–811.
- [3] Brányik, T., Kuncová, G., Páca, J., The use of silica gel prepared by sol-gel method and polyurethane foam as microbial carriers in the continuous degradation of phenol. *Appl. Microbiol. Biotechnol.* 2000, 54, 168–172.
- [4] Pogorilyi, R. P., Siletskaya, E. Y., Goncharik, V. P., Kozhara, L. I. et al., Immobilization of urease on the silica gel surface by sol-gel method. *Russ. J. Appl. Chem.* 2007, 80, 330–334.
- [5] Sharma, R., Chisti, Y., Banerjee, U. C., Production, purification, characterization, and applications of lipases. *Biotechnol. Adv.* 2001, 19, 627–662.
- [6] Jin, W., Brennan, J. D., Properties and applications of proteins encapsulated within sol-gel derived materials. *Anal. Chim. Acta.* 2002, 461, 1–36.
- [7] Liu, J., Peng, J., Shen, S., Jin, Q. et al., Enzyme entrapped in polymer-modified nanopores: the effects of macromolecular crowding and surface hydrophobicity. *Chemistry* 2013, 19, 2711–2719.
- [8] Pioselli, B., Bettati, S., Mozzarelli, A., Confinement and crowding effects on tryptophan synthase $\alpha_2\beta_2$ complex. *FEBS Lett.* 2005, 579, 2197–2202.
- [9] Lee, C. F., Bird, S., Shaw, M., Jean, L. et al., Combined effects of agitation, macromolecular crowding, interfaces on amyloidogenesis. *J. Biol. Chem.* 2012, 287, 38006–38019.
- [10] Eggers, D. K., Valentine, J. S., Molecular confinement influences protein structure and enhances thermal protein stability. *Protein Sci.* 2001, 10, 250–261.
- [11] Gogoi, S., Hazarika, S., Rao, P. G., Dutta, N. N., Esterification of lauric acid with lauryl alcohol using cross-linked enzyme crystals: Solvent effect and kinetic study. *Biocatal. Biotransformation.* 2006, 24, 343–351.
- [12] Hazarika, S., Goswami, P., Dutta, N. N., Hazarika, A. K., Ethyl oleate synthesis by Porcine pancreatic lipase in organic solvents. *Chem. Eng. J.* 2002, 85, 61–68.
- [13] Hazarika, S., Goswami, P., Dutta, N. N., Lipase catalysed transesterification of 2-o-benzylglycerol with vinyl acetate: solvent effect. *Chem. Eng. J.* 2003, 94, 1–10.
- [14] Mahapatra, P., Kumari, A., Garlapati, V. K., Banerjee, R. et al., Kinetics of solvent-free geranyl acetate synthesis by *Rhizopus oligosporus* NRRL 5905 lipase immobilized on to cross-linked silica. *Biocatal. Biotransf.* 2009, 27, 124–130.
- [15] Barbosa, O., Torres, R., Ortiz, C., Fernandez-lafuente, R., Versatility of glutaraldehyde to immobilize lipases: effect of the immobilization protocol on the properties of lipase B from *Candida antarctica*. *Process Biochem.* 2012, 47, 1220–1227.
- [16] Kumari, A., Mahapatra, P., Garlapati, V. K., Banerjee, R. et al., Lipase mediated isoamyl acetate synthesis in solvent-free system using vinyl acetate as acyl donor. *Food Technol. Biotechnol.* 2009, 47, 13–18.
- [17] Brabcová, J., Zarevúcka, M., Macková, M., Differences in hydrolytic abilities of two crude lipases from *Geotrichum candidum* 4013. *Yeast* 2010, 27, 1029–1038.
- [18] Hettflejš, J., Kuncová, G., Sabata, S., Blechta, V. et al., Alternative synthesis of poly(hydroxymethylsiloxane) for lipase immobilization and use of the adsorbates as esterification biocatalysts. *J. Sol-Gel Sci. Technol.* 2006, 38, 121–131.
- [19] Calef, D. F., Deutch, J. M., Diffusion-controlled reactions. *Ann. Rev. Phys. Chem.* 1983, 34, 493–524.
- [20] Lapidus, L., Amundson, N. R., *Chemical Reactor Theory: A Review*, Prentice Hall publishers, New Jersey 1977.
- [21] Young, M. E., Carrood, P. A., Bell, R. L., Estimation of diffusion coefficients of proteins. *Biotechnol. Bioeng.* 1980, 22, 947–955.
- [22] Janssen, A. E., Van der Padt, A., Riet, K. V., Solvent effects on lipase-catalyzed esterification of glycerol and fatty acids. *Biotechnol. Bioeng.* 1993, 42, 953–962.
- [23] Gogoi, P., Hazarika, S., Dutta, N. N., Rao, P. G., Laccase catalysed conjugation of catechin with poly(allylamine): Solvent effect. *Chem. Eng. J.* 2009, 155, 810–815.
- [24] Govardhan, C. P., Crosslinking of enzymes for improved stability and performance. *Curr. Opin. Biotechnol.* 1999, 10, 331–335.
- [25] Roy, J. J., Abraham, T. E., Preparation and characterization of cross-linked enzyme crystals of laccase. *J. Mol. Catal. B Enzym.* 2006, 38, 31–36.
- [26] Roy, J. J., Abraham, T. E., Strategies in making cross-linked enzyme crystals. *Chem. Rev.* 2004, 104, 3705–3721.
- [27] Hazarika, S., Dutta, N. N., Transesterification of 2-o-Benzylglycerol with Vinyl Acetate by Immobilized Lipase: Study of Reaction and Deactivation Kinetics. *Org. Process Res. Dev.* 2004, 8, 7200–7205.
- [28] Gill, I., Ballesteros, A., Bioencapsulation within synthetic polymers (Part 1): Sol-gel encapsulated biologicals. *Trends Biotechnol.* 2000, 18, 282–296.
- [29] Reetz, M. T., Zonta, A., Simpelkamp, J., Efficient heterogeneous biocatalysts by entrapment of lipases in hydrophobic sol-gel materials. *Angew. Chemie Int. Ed. English.* 1995, 34, 301–303.
- [30] Reetz, M. T., Entrapment of biocatalysts in hydrophobic sol-gel materials for use in organic chemistry. *Adv. Mater.* 1997, 9, 943–954.
- [31] Brzozowski, M., Derewenda, U., Derewenda, Z. S., Dodson, G. G. et al., A model for interfacial activation in lipases from the structure of a fungal lipase-inhibitor complex. *Nature* 1991, 351, 491–494.
- [32] Louwrier, A., Drtina, G. J., Klibanov, A. M., On the issue of interfacial activation of lipase in nonaqueous media. *Biotechnol. Bioeng.* 1996, 50, 1–5.
- [33] Mena, B., Herrero, M., Rives, V., Lavrenko, M. et al., Favourable influence of hydrophobic surfaces on protein structure in porous organically-modified silica glasses. *Biomaterials* 2008, 29, 2710–2718.
- [34] Soares, C. M. F., Dos Santos, O. A., Olivo, J. E., De Castro, H. F. et al., Influence of the alkyl-substituted silane precursor on sol-gel encapsulated lipase activity. *J. Mol. Catal. B Enzym.* 2004, 29, 69–79.
- [35] Edmiston, P. L., Wambolt, C. L., Smith, M. K., Saavedra, S. S., Spectroscopic characterization of albuminmyoglobin entrapped in bulk sol-gel glasses. *J. Colloid Interface Sci.* 1994, 163, 395–406.
- [36] Gottfried, D. S., Kagan, A., Hoffman, B. M., Friedman, J. M., Impeded rotation of a protein in a sol-gel matrix. *J. Phys. Chem.* 1999, 103, 2803–2807.
- [37] Uyanik, A., Sen, N., Yilmaz, M., Improvement of catalytic activity of lipase from *Candida rugosa* via sol-gel encapsulation in the presence of calix(aza)crown. *Bioresour. Technol.* 2011, 102, 4313–4318.

- [38] Sahin, O., Erdemir, S., Uyanik, A., Yilmaz, M., Enantioselective hydrolysis of (R/S)-Naproxen methyl ester with sol-gel encapsulated lipase in presence of calix[n]arene derivatives. *Appl. Catal. A Gen.* 2009, 369, 36–41.
- [39] Puthli, M. S., Rathod, V. K., Pandit, A. B., Enzymatic hydrolysis of castor oil: process intensification studies. *Biochem. Eng. J.* 2006, 31, 31–41.
- [40] Mateos, J. C., Ruiz, K., Rodriguez, J. A., Cordova, J. et al., Mapping substrate selectivity of lipases from thermophilic fungi. *J. Mol. Catal. B Enzym.* 2007, 49, 104–112.
- [41] Yilmaz, E., Sezgin, M., Yilmaz, M., Enantioselective hydrolysis of racemic naproxen methyl ester with sol-gel encapsulated lipase in the presence of sporopollenin. *J. Mol. Catal. B Enzym.* 2010, 62, 162–168.
- [42] Reetz, M. T., Tielmann, P., Wiesenhofer, W., Konen, W. et al., Second generation sol-gel encapsulated lipases: robust heterogeneous biocatalysts. *Adv. Synth. Catal.* 2003, 345, 717–728.
- [43] Fernández-Lorente, G., Terreni, M., Mateo, C., Bastida, A. et al., Modulation of lipase properties in macro-aqueous systems by controlled enzyme immobilization: enantioselective hydrolysis of a chiral ester by immobilized *Pseudomonas* lipase. *Enzyme Microb. Technol.* 2001, 28, 389–396.
- [44] Orcaire, O., Buisson, P., Pierre, A. C., Application of silica aerogel encapsulated lipases in the synthesis of biodiesel by transesterification reactions. *J. Mol. Catal. B Enzym.* 2006, 42, 106–113.
- [45] Nouredini, H., Gao, X., Characterization of sol-gel immobilized lipases. *J. Sol-Gel Sci. Technol.* 2007, 41, 31–41.
- [46] Ursioiu, A., Paul, C., Kurtán, T., Péter, F., Sol-gel Entrapped *Candida antarctica* lipase B — A biocatalyst with excellent stability for kinetic resolution of secondary alcohols. *Molecules* 2012, 17, 13045.
- [47] Macario, A., Moliner, M., Corma, A., Giordano, G., Increasing stability and productivity of lipase enzyme by encapsulation in a porous organic–inorganic system. *Microporous Mesoporous Mater.* 2009, 118, 334–340.
- [48] Chamorro, S., Sánchez-Montero, J. M., Alcántara, A. R., Sinisterra, J. V., Treatment of *Candida Rugosa* lipase with short-chain polar organic solvents enhances its hydrolytic synthetic activities. *Biotechnol. Lett.* 1998, 20, 499–505.
- [49] Mingarro, I., Abad, C., Braco, L., Interfacial activation-based molecular bioimprinting of lipolytic enzymes. *Proc. Natl. Acad. Sci. U. S. A.* 1995, 92, 3308–3312.
- [50] Sheldon, R. A., Enzyme immobilization: the quest for optimum performance. *Adv. Synth. Catal.* 2007, 349, 1289–1307.
- [51] Fidalgo, A., Ciriminna, R., Ilharco, L. M., Pagliaro, M., Role of the alkyl - alkoxide precursor on the structure catalytic properties of hybrid sol - gel catalysts. *Chem. Mat.* 2005, 17, 6686–6694.
- [52] Pagliaro, M., Ciriminna, R., Palmisano, G., The chemical effects of molecular sol-gel entrapment. *Chem. Soc. Rev.* 2007, 36, 932–940.
- [53] Sajja, H. K., Divakar, S., Prapulla, S. G., Karanth, N. G., Enzymatic synthesis of isoamyl acetate using immobilized lipase from *Rhizomucor miehei*. *J. Biotechnol.* 2001, 87, 193–201.
- [54] Sajja, H. K., Karanth, N. G., Lipase-catalyzed synthesis of isoamyl butyrate: a kinetic study. *Biochim. Biophys. Acta - Protein Struct. Mol. Enzymol.* 2001, 1547, 262–267.
- [55] Zaks, A., Klivanov, A. M., Enzyme-catalyzed processes in organic solvents. *Proc. Natl. Acad. Sci. U. S. A.* 1985, 82, 3192–3196.
- [56] Van Tol, J. B. A., Stevens, R. M. M., Veldhuizen, W. J., Jongejan, J. A. et al., Do organic solvents affect the catalytic properties of lipase? Intrinsic kinetic parameters of lipases in ester hydrolysis and formation in various organic solvents. *Biotechnol. Bioeng.* 1995, 47, 71–81.
- [57] Perham, M., Stagg, L., Wittung-stafshede, P., Macromolecular crowding increases structural content of folded proteins. *FEBS Lett.* 2007, 581, 5065–5069.
- [58] Senske, M., Törk, L., Born, B., Havenith, M. et al., Protein stabilization by macromolecular crowding through enthalpy rather than entropy. *J. Am. Chem. Soc.* 2014, 36, 9036–9041.
- [59] Radhakrishna, M., Grimaldi, J., Belfort, G., Kumar, S. K., Stability of proteins inside a hydrophobic cavity. *Langmuir* 2013, 29, 8922–8928.
- [60] Cui, J. D., Jia, S. R., Optimization protocols and improved strategies of cross-linked enzyme aggregates technology: current development and future challenges. *Crit. Rev. Biotechnol.* 2015, 35, 15–28.
- [61] Adlercreutz, P., Immobilisation and application of lipases in organic media. *Chem. Soc. Rev.* 2013, 42, 6406–6436.
- [62] Siso, M. I. G., Graber, M., Condoret, J. S., Combes, D., Effect of diffusional resistance on the action pattern of immobilized α -amylase. *J. Chem. Technol. Biotechnol.* 1990, 48, 185–200.
- [63] Vlad-Oros, B., Bizerea-Spiridon, O., Preda, G., Chiriac, A., Studies regarding the influence of the enzyme immobilization methods on the electrodic surface upon the performances of the glucose biosensor. *Rev. Chim. (Bucureşti)*. 2009, 60, 693–698.
- [64] Vlad-Oros, B., Preda, G., Dudas, Z., Dragomirescu, M. et al., Entrapment of glucoamylase by sol-gel technique in PhTES/TEOS hybrid matrixes. *Process. Appl. Cera.* 2007, 1, 63–67.
- [65] Szocs-Biro, E., Kiss, C., Zarcu, C., Olteanu, A. et al., Sol-Gel immobilized enzymes activity in esterification and transesterification reactions. *Ann. Fac. Eng. Hune.* 2003, 1, 3.
- [66] Khalameida, S. V., Sydorchuk, V., Skubiszewska-Zikeba, J., Lebeda, R. et al., Sol-gel synthesis and properties of compositions containing heteropoly compounds in porous silica matrix. *Glas. Phys. Chem.* 2014, 40, 8–16.
- [67] Coleman, N. J., Bellantone, M., Nicholson, J. W., Mendham, A. P., Textural and Structural properties of bioactive glasses in the system CaO–SiO₂. *Ceramics – Silikáty* 2007, 51, 1–8.
- [68] Margolin, A. L., Novel crystalline catalysts. *Trends Biotechnol.* 1996, 14, 223–230.
- [69] Margolin, A. L., Navia, M. A., Protein crystals as novel catalytic materials. *Angew. Chem., Int. Ed.* 2001, 12, 2204–2222.
- [70] Govardhan, C. P., Crosslinking of enzymes for improved stability and performance. *Curr. Opin. Biotechnol.* 1999, 10, 331–335.