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Immobilization of lipase from *Enterobacter aerogenes E13* on modified lignins to obtain a novel biocatalyst

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Lipase of *Enterobacter aerogenes E13* was immobilized by adsorption on lignins – wastes from lignocellulosic ethanol production. It was shown that the prehistory of the chosen lignins contributes to lipase immobilization: lignin obtained in the process of wood enzymatic hydrolysis for fuel ethanol production (SHF lignin) is a good solid support for lipase, which provides the highest activity yield and thermal stability (up to 50 °C) of immobilized lipase. Lipase immobilized on SHF lignin retained over 50% of its original activity after 10 cycles of reuse. After three months of storage, immobilized lipase retained 80–97% of its initial activity. The catalytic activity of the novel biocatalyst in the hydrolysis of triacylglycerols and the transesterification of biodiesel and rapeseed oil by octyl alcohol was close to that of the known commercial product Lipozyme RM IM.

Introduction

Lipases (triacylglycerol acylhydrolase, E.C. 3.1.1.3) are enzymes that catalyze the reversible hydrolysis of glycerol ester bond. Lipases have been successfully used in low-water environments as an excellent tool for the transformation of commercial triglycerides to esters, including that on an industrial scale. At present, transesterification of vegetable oils using enzymes, normally lipases, as biocatalysts are on the top of corresponding researches [1]. The use of lipases has much to offer from the Green Chemistry point of view: mild reaction conditions, high catalytic activity, high specificity and economic viability [2]. However, the stability of lipases in various environments is relatively low. Furthermore, lipases are expensive and cannot be reused because of their high solubility in water. Immobilization of lipases has several advantages for industrial applications. It helps in their reuse and enables their easy separation from reaction media and allows operating under conditions (temperature, pH, presence of organic solvents) that are extreme for lipases [3].

A variety of techniques have been applied for immobilization of lipases, among them the solid support-binding method. Immobilization by ionic or physical adsorption is the easiest and least expensive technique for preparing solid-support biocatalysts [4]. The non-covalent linkages (mainly van der Waals, hydrogen bonds and hydrophobic interactions) between the enzyme and the solid support provides for minimal changes in enzyme conformation, which is very important for lipase activity, although the adsorbed enzyme is rather susceptible to desorption from the support [2]. Hydrophobic interactions are especially efficient for lipase immobilization [5]. Most lipases display an increase in activity when adsorbed on hydrophobic supports, which is ascribed to both conformational micro-tuning and selective adsorption during the immobilization process. Lipases recognize such supports as their natural substrates.

Natural and synthetic fibers, ion exchange resins, plastics, organogels etc. were proposed as insoluble supports for lipase immobilization [2, 6, 7]. However, most of these supports have a limited capacity to be reused, therefore, problems of the disposal of hard to biodegrade synthetic organic materials arise [4]. Inorganic carriers are more expensive than organic ones [8]. At present, the application of insoluble lignocellulosic materials as prospective matrices for biosorbents is considered due to such advantages as their porous structure (the presence of large meso- and macropores), ecological safety and low cost [9, 10]. In particular, recently a solid residue after acidic pretreatment of wood biomass, containing 35% of lignin and 65% of cellulose (so-called cellulignin) has been tested as a solid support for lipase (*Candida rugosa*) immobilization [11].

There are data in the literature [11] that lignin – phenylpropanoid wood polymer – shows affinity towards enzymes including lipase. Soluble lignins (lignosulphonates, kraft lignin, oxylignins) have been found to be very efficient agents for improving the storage stability of enzymes (proteases, amylases, lipases, cellulases and their composition).

The high cost of the enzymes often makes the enzymatic processes economically unattractive [13]. The use of crude lipase from unpurified culture liquid allows avoiding additional expenses. It has been shown that salts

present in the culture liquid may enhance enzyme adsorption onto a hydrophobic support.

The present work was focused on the immobilization of lipase from a microbial source, *Enterobacter aerogenes E13*, onto lignocellulosic materials. The objective of this study was to contribute to the search of an inexpensive and available biocatalyst. Lignin-based lipase preparations can be used successfully in typical biotransformation of fat and oils (including environmental bioremediation). We directed our attempt to applying an immobilized enzyme for conversion of vegetable oils by hydrolysis and for the synthesis of oil alkyl esters by transesterification.

With this aim, two lignocellulosic wastes (hydrolysis lignins) obtained after wood hydrolysis oriented on bioethanol production were used as material for solid support. Owing to the high yield of hydrolysis lignins (40–45% from wood raw material), their conversion into valuable products will be profitable for the lignocellulosic fuel ethanol production industry intensively developing all over the world [14]. The high potential of hydrolysis lignins as a resource for production of bio-based materials, including sorbents, composites, etc., is now recognized worldwide [10, 15].

It was proposed that the prehistory of lignins would contribute to lipase immobilization. In the course of the enzymatic hydrolysis process, lignin was in contact with the cellolytic enzymes that had been partly adsorbed by lignin. Such a way of lignin surface modification could open new opportunities for lipase immobilization [16, 17]. The choice of Si-modified hydrolysis lignin (further Si-HL) as a matrix for lipase immobilization was based on data indicating its rather high porosity and sorption properties towards proteins in comparison with the parent lignin [9, 18].

Materials and methods

MATERIALS. Lipase from *Enterobacter aerogenes E13* strain was used for immobilization. Supernatant (obtained after centrifugation at 4000 rpm for 15 min) of culture liquid of *Enterobacter aerogenes E13* strain grown in a complex nutrient medium containing sunflower oil as an inductor was used as a crude lipase solution. The crude lipase solution had pH 7.3 and lipase activity of 13 500 ±3 500 Units ml^{-1}, which was determined by hydrolysis of p-nitrophenyl butyrate (as described below). The commercial product Lipozyme RM IM (*Rhizomucor miehei* lipase, Novozyme, Denmark) with the activity of 1000 Units mg^{-1} was used as a reference sample in tests on assessment of immobilized lipase catalytic properties.

Isopropanol, 4-nitrophenyl butyrate, *p*-nitrophenol, buffer components, glyceryl trioleate and *n*-octanol were from Sigma Aldrich. Rapeseed oil and biodiesel (methyl ester of rapeseed oil fatty acids) were purchased from local manufacturers and used without further purification. Methanol was purchased from Chempur, isooctane and hexane from Lachner, sodium sulfate from Lachema, diethyl ether and acetic acid from Penta, silica gel aluminum plates for thin-layer chromatography (TLC) from Merck. The reagents used were all of reagent grade and chemically pure.

SUPPORTS. Non-hydrolyzed rich-in-lignin residue after enzymatic hydrolysis of softwood for fuel ethanol production (further SHF lignin) was kindly supplied by the Ornsholdsvik pilot plant (Sweden) as a brown water-insoluble powder with a moisture content of 65%. Siliceous lignin was obtained with Si content of 5% as described elsewhere [19] on the basis of the commercial acid hydrolysis lignin (Kėdainiai, Lithuania).

Before lipase immobilization, SHF and Si-lignins were dried in vacuum at 30 °C.

Klason lignin was determined according to the procedure described in detail in [20]; C, H, N, S contents were determined using an ELEMENTAR (Vario MACRO) analyzer.

The parameters of the porous structure (BET specific surface, pore size) were established from the nitrogen adsorption-desorption isotherms obtained with the *Kelvin 1042 sorptometer* (Costech).

The surface hydrophobicity of lignin supports was assessed in terms of the amount of water regain in swollen material as described in [21] and wetting the contact angle. Static contact angle measurements were conducted using a computer-controlled videobased *Dataphysics Contact Angle System OCA20* instrument. The contact angle was measured at least on 5 different locations on each tabletted sample and averaged to yield the contact angles and their standard deviation. All measurements were performed at 20 °C and about 50–60% relative humidity.

IMMOBILIZATION PROCEDURE. Immobilization of lipase was performed by adsorption on the lignins using batch process (equilibrium conditions) and column technique (dynamic conditions). The lignins had been kept in 0.2 M phosphate buffer for 1 hour to bring pH to 7.3 (pH of the culture liquid).

The immobilization in equilibrium conditions. 50 ml of lipase solution prepared by mixing a crude lipase solution and phosphate buffer was added under low stirring (150 rpm) to lignin sample (1 g) and kept under stirring at 20, 40 or 60 °C for a time needed to reach the sorption equilibrium. The effect of enzyme loading on the lipase immobilization on lignin was investigated at 20 °C. Lipase activity loading varied from 100 to 800 Units mg^{-1} support.

The time course of lipase sorption by lignin was followed at 20 °C and lipase loading of 600 Units mg^{-1} support. Aliquots of the enzyme solution were taken out every 30 min, centrifuged for 5 min, and the supernatant was analyzed for enzyme activity and protein content. The protein content of supernatants was determined spectrophotometrically in accordance with Lowry's method [22] using bovine serum albumin as the standard.

Immobilization in dynamic conditions. In accordance with the procedure described in [16], the crude lipase

solution was put through the column (diameter 2 cm, length 5 cm) filled with 1 g of lignin at rate of 1 ml min^{-1}. The aliquots were taken out for analysis from every 20 ml of lipase solution putting out the column. The treatment lasted until the input and output lipase solutions showed the same activity.

The samples of immobilized lipase obtained by both techniques were rinsed with 0.02 M phosphate buffer (pH 7.0) to remove soluble lipase until no lipase activity was detected in the rinsing water. Immobilized lipase samples were dried under vacuum.

DETERMINATION OF LIPASE ACTIVITY. Lipase activity was measured using *p*-nitrophenyl butyrate (*p*-NPB). This assay was performed spectrophotometrically (Spectrophotometer Helios λ, USA) by measuring the increase in the absorbance at 400 nm caused by the *p*-nitrophenol released during 3 min of the *p*-NPB hydrolysis in the Sorensen phosphate buffer (pH 8.0) at 40 °C. For hydrolysis realization, 0.02 ml of 25 mM *p*-NPB solution in isopropanol was mixed with 1.8 ml of buffer. Thus, the system consisted of 50 mM of the buffer, and 0.5 mM *p*-NPB was pre-equilibrated at 40 °C and either 0.2 ml of free lipase solution of a chosen concentration or a dose by weight of the immobilized lipase sample was added. The reaction rate was calculated from the slope of the absorbance curve versus time, using the molar extinction coefficient of $16 \cdot 10^6$ $cm^2 mol^{-1}$ for *p*-nitrophenol [11]. One lipase unit corresponded to the amount of enzyme that liberated 1 μmol of *p*-nitrophenol per minute under the assay conditions.

The effect of pH on free and immobilized lipase activity towards *p*-NPB was determined by a standard assay using phosphate (pH 6.5–8.0) and tetraborate (pH 8.5–9.5) buffers at 40 °C.

The effect of temperature on lipase activity was assessed varying the temperature of the *p*-NPB assay in the range of 25–55 °C at a constant pH of 8.0. The maximal enzyme activity was taken as 100%.

ASSESSMENT OF THE EFFICIENCY OF IMMOBILIZATION TECHNIQUES. The efficiency of lipase immobilization, η, was determined according to [23] as follows:

$$\eta = \left[\frac{E_o V_o - E_f V_f}{E_o V_o} \right] \cdot 100\,(\%),$$

where E_0 and E_f are free lipase activities in the solution before and after immobilization, and V_0 *and* V_f are the volumes of lipase solution before and after immobilization, respectively.

The lipase sorption value (*C*) was calculated using the equation [17]:

$$C = \left[\frac{(E_o - E_f) * V_o}{m} \right] \text{(Units mg}^{-1}\text{)},$$

where *m* is the mass of the lignin support, mg.

Activity yield was determined as a ratio (%) of immobilized lipase activity and activity of free lipase used for immobilization [24, 25].

INVESTIGATION OF STORAGE STABILITY AND REUSABILITY OF IMMOBILIZED LIPASE. The storage stability of immobilized lipase was evaluated by measuring its activity (towards *p*-NPB) after 1, 2 and 3 months of storage at 4 °C.

The reusability of immobilized lipase was determined in a set of 30-min cycles [6, 23] by enzyme hydrolytic activity towards glyceryl trioleate (2,3-bis[[(Z)-octadec-9-enoyl]oxy]propyl (Z)-octadec-9-enoate) as a substrate. For each cycle, the glyceryl trioleate and phosphate buffer (pH 7.0) mixed at a 1 / 1 (v / v) ratio was vigorously stirred for 5 min to form a water emulsion; 10 ml of the emulsion was mixed with 0.5 g of the immobilized lipase and incubated at 37 °C for 30 min under stirring (200 rpm). After each cycle, the immobilized lipase was filtered off, rinsed with 10 ml water and 10 ml isooctane and then reused for the next cycle in the same manner. The total amount of free fatty acids was determined in the filtrate by titration with 0.05 M NaOH. One unit of lipase activity was defined as the amount of enzyme that liberates 1 μmol of free fatty acids per 1 minute at 37 °C.

CATALYTIC PROPERTIES OF IMMOBILIZED LIPASE. Lipase-catalyzed oil hydrolysis was performed by adding 0.3 g of an immobilized lipase preparation to the mixture containing 5 g of rapeseed oil and 0.5 g of distilled water. The mixture was incubated at a temperature of 37 °C during 5 h under continuous stirring. Every hour, an aliquot of the reaction mixture (0.1 ml) was taken and analyzed by thin-layer chromatography (TLC). For this purpose, 0.9 ml of hexane / diethyl ether mixture (1 / 1, v / v) was added to the 0.1 ml of reaction mixture in order to extract the reaction products. The extracts were transferred onto aluminium plates, and the system of solvents (hexane / diethyl ether / acetic acid 70 / 30 / 2, v / v / v) was used for the elution. The plates were dried and treated by iodine vapour to reveal the analytic signals.

For the transesterification of rapeseed oil with long-chain alcohol, a mixture of 5 g of the product of rapeseed oil 5-hour hydrolysis, 0.3 g of immobilized lipase sample and 0.86 g *n*-octanol were incubated at a temperature of 37 °C under normal pressure for 1 hour. The further transesterification was carried out under vacuum (pressure 50 mbar) for 1 hour [26].

Transesterification of rapeseed oil with methanol was carried out at their molar ratio 1 : 3.5 with 20% (w / w) of an immobilized lipase sample (or 10% of Lipozyme RM IM) and 5% of water during 72 hours under normal pressure and a temperature of 40 °C. The yields of methyl ester obtained in the presence of commercial immobilized lipase (Lipozyme RM IM) and lipase immobilized on lignin were used for the evaluation of catalytic efficiency of the latter.

The catalytic properties of immobilized lipase were determined using the transesterification reaction of biodiesel (0.6 g) in hexane (2 ml) with *n*-octanol (1.2 g) in

the presence of immobilized enzyme (0.3 g) at 40 °C for 16 hours.

The transesterification processes were monitored by TLC and gas chromatography (GC). For GC analysis, 5 ml of the mixture obtained after transesterification was centrifuged at 4000 rpm for 20 min and then 0.1 ml of supernatant was added to 14.9 ml of hexane. The obtained extract was dried by treatment with water-free sodium sulphate and put through a column with aluminum oxide and then analyzed using a *GC Dani 1000* chromatograph (Italy) equipped with a silica-fused capillary column (OPTIMA K-102, 25 m, id 0.32 mm, 0.25 μm film thickness; Macherey-Nagel, Germany), and the identification and quantification of the products was made using the *Clarity Chromatographic Station* software.

Results and discussion

BASIC CHARACTERISTICS OF LIGNINS AS ENZYME SUPPORTS. It is widely accepted that many factors including specific surface (BET), pore size, hydrophobicity and surface functional groups of solid support influence enzyme immobilization and the properties of the final products. Our data (Table 1) show that the values of specific surface and pore size of the lignins under study are rather close, however, SHF lignin differs from Si-lignin by a higher hydrophobicity: the wetting contact angle on the air–water–SHF lignin interface is 65° versus 45° for Si-HL, and the water regain index for SHF lignin is 1.6 times lower than for Si-lignin. The higher hydrophobicity of SHF lignin is obviously connected with the enhanced content of a phenylpropanoid polymeric wood component – lignin (Table 1, Klason lignin), which shows a more hydrophobic behavior than carbohydrate wood components.

Table 1. Characteristics of the lignin supports

Support	Klason lignin content, %	Element content, %				Porous structure indices		Hydrophobicity indices	
		C	H	N	S	Pores diameter, nm	S_{BET}, $m^2 g^{-1}$	Water regain, $g g^{-1}$	Contact angle, °
SHF lignin	74.6	61.70	5.91	1.37	0.21	1.5–25	14.3	1.4	65 ±2.5
Si-HL	61.7	56.62	5.32	0.17	0.04	4–20	14.0	2.2	45 ±1.0

Data of elemental analysis show a more than tenfold higher nitrogen content of SHF lignin in comparison with Si-HL (Table 1) and raw material (wood) used for bioethanol production (nitrogen content 0.2%). This indicates the presence of proteins on the SHF lignin surface which has sorbed enzymes (other than lipase) used for wood hydrolysis.

IMMOBILIZATION OF LIPASE USING THE BATCH PROCESS. The time courses of lipase sorption by lignins under study (Fig. 1) are rather similar, although the enzyme sorption value for SHF lignin is higher than that for Si-lignin over the time of all the experiments. Based on the obtained results, the time needed for achieving adsorption equilibrium was 120 min for both lignin supports.

The sorption capacity of the Si-HL and SHF supports is different. For the Si-HL support sorption, the highest activity of immobilized lipase and the maximum activity yield (Table 2) were observed when the enzyme was immobilized at the lipase activity loading 600 Units mg^{-1} support. The analysis of literature data show that when the loading of the adsorbed enzyme is increased, less area becomes available for the lipase molecules to spread themselves on the solid support surface and more of them retain an active conformation; correspondingly, the activity of immobilized enzyme increases [24]. For SHF lignin, the saturation in terms of both lipase sorption value and immobilized lipase activity were not reached even when more than 800 Units of lipase per 1 mg of support had been loaded. Obviously, the lower activity of lipase immobilized at 20 °C on SHF in comparison with that of the enzyme immobilized on Si-HL is related to the more spread conformation of lipase molecules provided either by the multi-point attachment of lipase on SHF surface or by a larger the SHF-specific surface available for enzyme adsorption. Far away from the point of sorbent saturation there is a large excess of surface area available for the lipase molecules, and the enzyme attempts to maximize its contact with the support surface; this results in a decreasing activity [24].

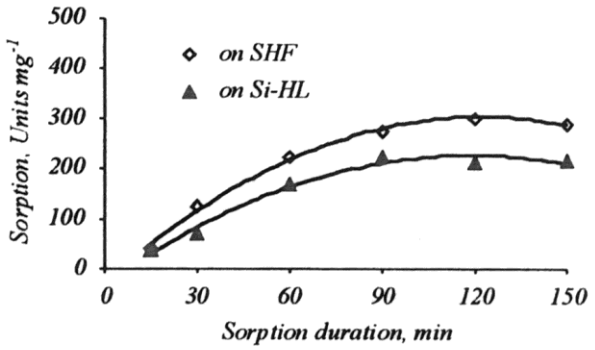

Fig. 1. Lipase sorption on lignin supports, realized by the batch technique. Duration course of lipase sorption on lignin supports (20 °C, at lipase loading 600 Units mg^{-1} support)

Table 2. Characteristics of immobilization process realized by the batch technique (at 20 °C and lipase loading of 600 Units mg^{-1} lignin) and by the column technique

Immobilization technique	Support	Immobilization efficiency, %	Sorption, Units mg^{-1}	Activity of immobilized lipase, Units mg^{-1}	Activity yield, %
Batch	SHF	42	252.5	10.3 ±0.88	2
	Si-HL	35	214.6	22.3 ±1.14	4
Column	SHF	90	1280.3	267.6 ±16.49	14
	Si-HL	25	228.5	22.1 ±2.03	2

The temperature of immobilization is an important parameter in the case of using SHF lignin as a support (Table 3). With increasing the temperature, lipase protein sorption by SHF lignin also increased, and the activity of lipase immobilized on SHF increased 8 times when the immobilization temperature was risen from 20 up to 40 °C. For Si-HL, these parameters practically did not change with increasing the immobilization temperature from 20 up to 40 °C. For both supports, the further increase of temperature (from 40 to 60 °C) led to inactivation of immobilized lipase, probably due to its denaturation.

Table 3. Effect of immobilization temperature on activity of immobilized lipase and protein adsorption (batch technique, lipase loading 600 Units mg^{-1} support)

Support	Activity of immobilized lipase, Units mg^{-1}			Proteins, mg g^{-1}		
	20 °C	40 °C	60 °C	20 °C	40 °C	60 °C
SHF	10.3 ±0.88	96.6 ±1.40	1.0 ±0.10	28.4 ±2.89	36.4 ±0.61	37.2 ±1.14
Si-HL	22.3 ±1.14	19.8 ±0.72	1.6 ±0.79	19.2 ±1.48	20.9 ±1.20	22.2 ±1.25

The increase in lipase sorption by SHF lignin support with the temperature indicates an important role of hydrophobic interactions [5], in particular lipase protein, i.e. an enzymatic protein previously sorbed by lignin during wood enzymatic hydrolysis for bioethanol production. Therefore, immobilization of lipase on SHF lignin can be rather easily controlled by temperature variation.

Due to the lower hydrophobicity of the Si-HL support (Table 1), the impact of hydrophobic interactions on lipase sorption on this lignin are neutralized by other sorption forces, which are weakened by heating; as a result, the enzyme sorption does not depend on temperature.

THE COLUMN TECHNIQUE OF IMMOBILIZATION. The usage of the column technique for lipase immobilization provided for a much higher activity of the enzyme immobilized on SHF lignin as compared with samples obtained by the batch process, whereas activity of lipase immobilized on Si-HL was practically the same for both immobilization techniques (Table 2). The sorption saturation of Si-HL under dynamic conditions was observed at lipase loading of 600 Units mg^{-1} when the value of immobilization efficiency was ~25%. For SHF lignin, no sorption saturation was achieved even at the lipase loading of 2000 Units mg^{-1}, and the maximal immobilization efficiency was 90% (Table 2 and Fig. 2).

In the case of lipase immobilization on SHF lignin by the column technique, the efficiency of immobilization and activity yield reached higher values in comparison with these indices for lipase immobilized using the batch process, and *vice versa,* in the case of Si-HL the column technique was less efficient than the equilibrium method of sorption immobilization (Table 2).

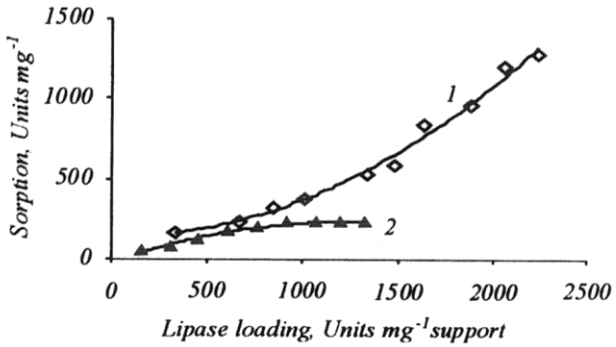

Fig. 2. Effect of lipase loading on enzyme sorption realized by the column technique (20 °C): *1 – SHF; 2 – Si-HL*

IMMOBILIZED LIPASE: EFFECT OF PH AND TEMPERATURE ON ITS ACTIVITY. The estimation of the activity of free and immobilized lipase samples at different pH values revealed no changes in optimum pH (pH 9) for the lipase immobilized on Si-HL in comparison with free lipase (Fig. 3, a). The lipase immobilized on SHF lignin displayed a broader optimal pH range (pH 8–9).

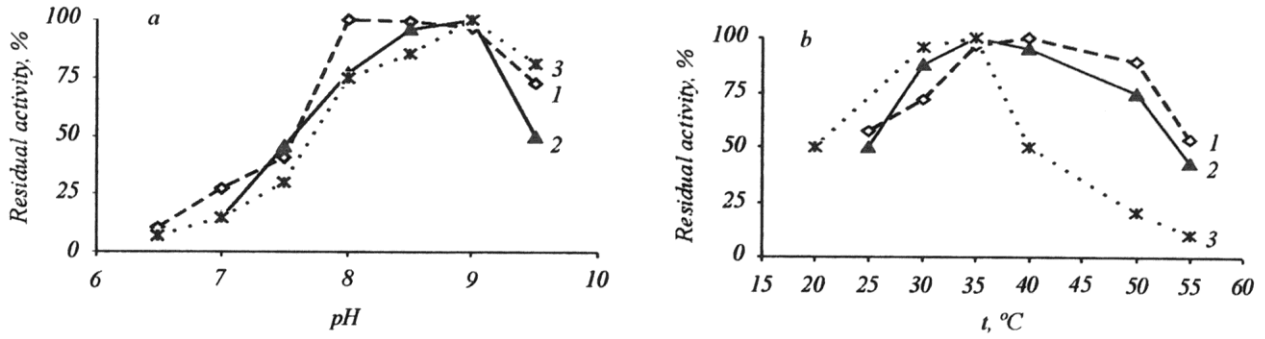

Fig. 3. Effect of pH (*a*) and reaction temperature (*b*) on the activity of lipase samples: *1 – E13-SHF; 2 – E13-Si-HL; 3 – free E13 lipase*

The optimal temperature for activity of free lipase was 35 °C; at 40 °C its activity sharply reduced (Fig. 3, b). For lipase immobilized on both lignin supports, the optimum temperature shifted to 40 °C; this was more characteristic of SHF lignin. The residual activity at 50 °C of the immobilized enzyme remained 89% and 75%, respectively for SHF lignin and Si-HL, versus 18% for free lipase. Even at 55 °C, the immobilized lipase preserved the residual activity at about 53% and 43%, respectively for SHF lignin and Si-HL supports, whereas free lipase revealed only an 11% residual activity (Fig. 3, b). Based on the literature data [6, 13, 27], these results can be explained by providing a more rigid external backbone for lipase molecules through immobilization. Consequently, the disturbing effect of the increasing temperature on the catalytically active lipase structure became less prominent.

IMMOBILIZED LIPASE: REUSABILITY AND STORAGE STABILITY. The reusability of immobilized lipase is among the most appropriate ways to evaluate the competitiveness of the immobilization procedure [23]. As is shown by the obtained data (Fig. 4, a), the half-life of the lipase immobilized on SHF lignin is at least 10 cycles of reuse, whereas for Si-HL the half-life is three cycles only. The activity of lipase immobilized on SHF lignin after five cycles retained over 75% of its initial value, whereas for lipase immobilized on Si-HL, activity of the sample after five cycles sharply reduced (to 25%) (Fig. 4, a).

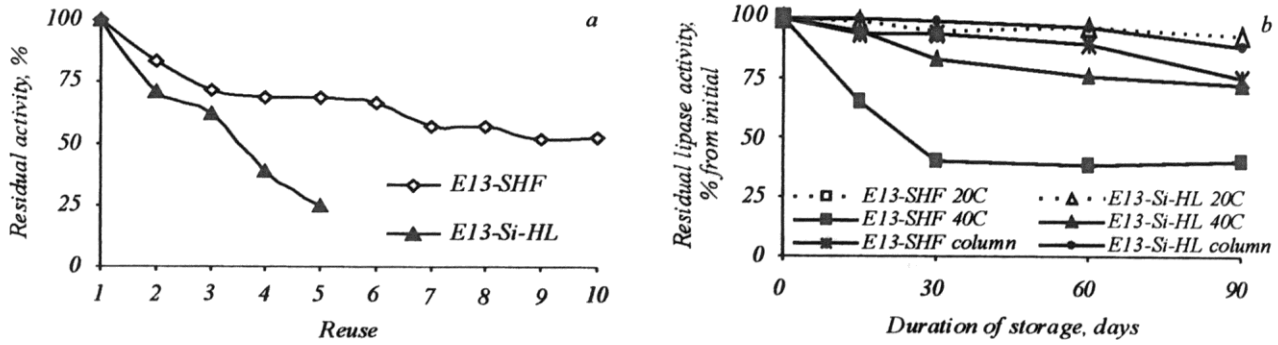

Fig. 4. Effect of reuse on the activity of immobilized lipase samples (*a*) and storage stability of immobilized lipase samples (*b*)

The storage stability was determined for lipase immobilized at 20 and 40 °C (the batch process) and 20 °C (the column procedure) on lignin supports. The storage stability of lipase immobilized on both lignin supports at 20 °C was rather high (Fig. 4, b). After three months of storage, lipase immobilized on SHF lignin lost 11% (column technique) and 15% (batch process) of the initial activity. For lipase immobilized on Si-HL, the loss of activity was about 3% for both techniques used for immobilization. Lipase immobilization on SHF lignin support at 40 °C, in spite of it, provides for a strongly enhanced immobilized lipase activity and has a considerable disadvantage connected with a significant decrease of enzyme activity at storage – as much as 60% after 30 days (Fig. 4, b).

THE ACTIVITY OF IMMOBILIZED LIPASE IN HYDROLYSIS AND TRANSESTERIFICATION PROCESSES. Obtaining long-chain alkyl esters by lipase-catalyzed transesterification is industrially important for manufacturing a variety of products, including biodiesel and lubricants [28]. Lipase-catalyzed transesterification is possible to realize in an organic solvent-free medium. It is especially important because such a system allows to avoid the problems connected with the separation, toxicity, and flammability of organic solvents [1]. Transesterification of vegetable oils is an essential reaction to be optimized for enzymatic production of biodiesel. The reaction can be performed directly or after the hydrolysis of oils in a two-step process for an efficient conversion of

free fatty acids and acylglycerols into fatty acid alcohol esters [29].

Samples of lipase immobilized using the column technique on SHF and Si-HL lignins (E13-SHF and E13-Si-HL) were tested for esterification activity in the transesterification of methyl-ester of rapeseed oil fatty acids (biodiesel) with *n*-octanol in non-aqueous media. The E13-SHF sample showed the results close to commercial lipase product Lipozyme RM IM immobilized on an anionic resin and higher than the E13-Si-HL sample (Fig. 5 and Table 4).

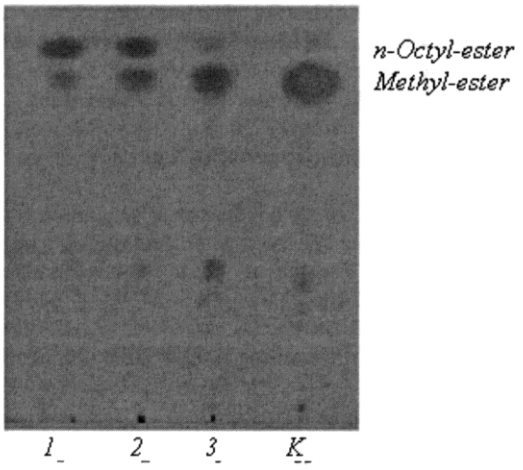

Fig. 5. Transesterification of biodiesel with *n*-octanol catalyzed by different lipases. TLC chromatogram of the products of the reaction carried out in the presence of: *1* – Lipozyme RM IM (1000 Units mg^{-1} towards *p*-NPB), *2* – E13-SHF (60 Units mg^{-1} towards *p*-NPB), *3* – E13-Si-HL (13.5 Units mg^{-1} towards *p*-NPB), *K* – control biodiesel (methyl ester of rapeseed oil fatty acids)

Table 4. Biodiesel conversion in the presence of different immobilized lipase samples (by GC data)

Preparation	Conversion of methyl ester, %
Lipozyme RM IM	92.5
E13-SHF	77.5
E13-Si-HL	11.4

Therefore, further investigations of the catalytic activity of lipase immobilized on lignin supports were carried out with E13-SHF.

Rapeseed oil was hydrolyzed in the presence of lipase immobilized on the SHF to diglycerols, monoglycerols, and free fatty acids. The composition of the mixture of hydrolysis products was stabilized in 4 hours (Fig. 6, a). The TLC chromatogram of rapeseed oil in the absence of lipase did not change during the whole experiment (5 hours).

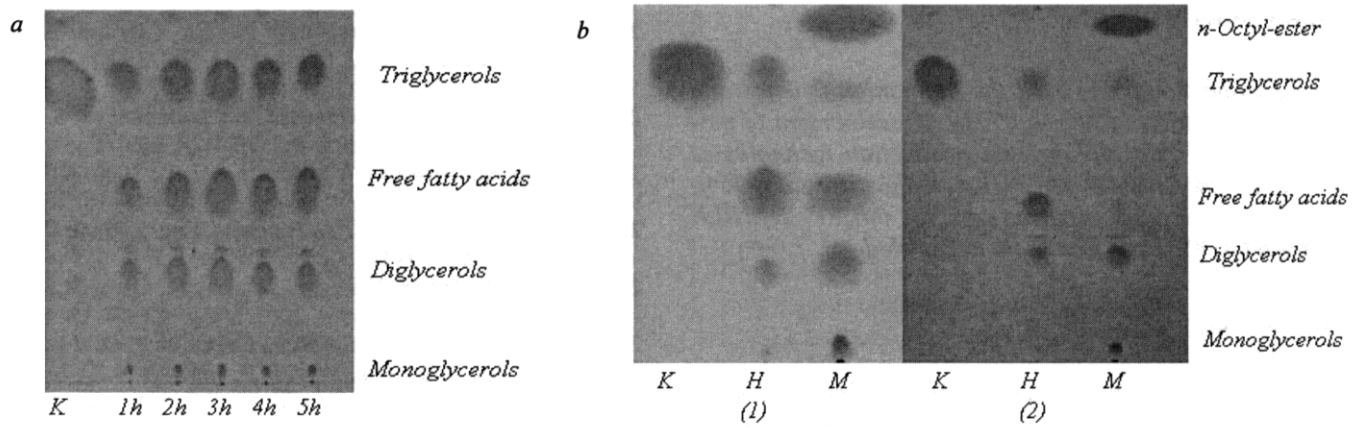

Fig. 6. Catalytic activity of *Enterobacter aerogenes E13* lipase immobilized on SHF lignin (E13-SHF). *a* – time course of rapeseed oil hydrolysis: *K* – control rapeseed oil, *1–5 h* – duration of hydrolysis, hours; *b* – transesterification of rapeseed oil with *n*-octanol: *K* – rapeseed oil, *H* –mixture of products obtained after hydrolysis, *M* – mixture of products obtained after transesterification; (*1*) – normal pressure (about 1000 mbar), (*2*) – further transesterification under pressure of 50 mbar

Transesterification of the hydrolysis products with *n*-octanol under normal pressure led to the appearance of *n*-octyl ester of rapeseed oil fatty acids after one hour of the reaction, which revealed itself as a new intensive spot on the TLC chromatogram (Fig. 6, b (1)). Simultaneously a sharp decrease in the content of triglycerols in the reaction mixture was observed. After one hour, transesterification was continued under vacuum and resulted in the further conversion of products due to a shift of reaction equilibrium [26] up to complete esterification of free fatty acids (Fig. 6, b (2)).

Transesterification of the non-hydrolyzed rapeseed oil with methanol is one of the conventional reactions of chemical catalysis for producing biodiesel [29]. Lipase immobilized on SHF lignin (E13-SHF) showed in this reaction a 58% efficiency as compared with the commercial product Lipozyme RM IM; the yields of the reaction product (methyl-ester) were 420 and 730 mg ml^{-1} respectively (by GC data).

Conclusions

Alternative supports for immobilizing lipase – wastes from lignocellulosic ethanol production – were proposed. Non-covalent–immobilization of lipase from

Enterobacter aerogenes E13 by a simple and inexpensive method of physical adsorption for biocatalyst design was considered.

It was shown that lignin obtained in the process of wood enzymatic hydrolysis for fuel ethanol production (SHF lignin) can be considered as a promising solid support for lipase because of a lowered content of hydrophilic carbohydrate wood components and the presence on its surface of enzymatic protein (previously sorbed by this lignin during hydrolysis), which increases the affinity of the immobilized lipase.

The usage of the column technique for lipase immobilization provided a much higher activity of the enzyme immobilized on SHF lignin in comparison with the samples obtained by the batch process.

Lipase immobilized on SHF lignin showed a high hydrolytic activity in an aqueous medium and esterification activity in non-aqueous / low-aqueous media. The catalytic activity of the novel biocatalyst is close to that of the known commercial product Lipozyme RM IM in the conversion of triacylglycerols by hydrolysis, transesterification of biodiesel and rapeseed oil by octyl alcohol as well as traditional transesterification of rapeseed oil by methyl alcohol. Immobilization of *Enterobacter aerogenes E13* lipase on lignin supports provides an enhanced operational and storage stability of the enzyme.

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ENTEROBACTER AEROGENES E13 LIPAZĖS IMOBILIZACIJA ANT MODIFIKUOTO LIGNINO NAUJAM KATALIZATORIUI SUKURTI

Santrauka

Pasiūlytas alternatyvus lipazių nešiklis – etanolio gamybos lignoceliuliozinės atliekos. Kuriant biokatalizatorių, buvo tirta lipazės iš *Enterobacter aerogenes E13* nekovalentinė imobilizacija nebrangiu paprastu fizikinės adsorbcijos būdu.

Parodyta, kad ligninas, gautas hidrolizuojant medieną etanolio gamybai (SHF ligninas), gali būti naudojamas kaip lipazės

kietas nešiklis, turintis mažai hidrofilinių sacharidų ir ant paviršiaus esančius baltymus (kurie pirmiausia sorbuojami lignino fermentinės hidrolizės metu), kurie padidina imobilizuotos lipazės afiniškumą.

SHF ligninas yra kietas lipazės nešiklis, suteikiantis imobilizuotai lipazei didesnę aktyvumo išeigą ir termostabilumą (iki 50 °C). *Enterobacter aerogenes E13* lipazės imobilizacija ant modifikuoto lignino padidina fermento regeneracijų skaičių ir stabilumą jį saugant: lipazė išlaiko per 50 % pradinio savo aktyvumo po 10-ies ciklų naudojimo; po trijų mėnesių laikymo imobilizuota lipazė išlaikė 80–97 % pradinio savo aktyvumo.

Lipazės, imobilizuotos ant SHF lignino, hidrolitinis aktyvumas vandeninėje terpėje ir esterifikacijos aktyvumas nevandeninėje / mažai vandens turinčioje terpėje buvo didelis. Šio preparato katalitinis aktyvumas yra artimas komerciniam produkto Lipozyme RM IM aktyvumui triacilglicerolių hidrolizėje, biodyzelino ir rapsų aliejaus transesterifikacijoje oktanoliu, taip pat tradicinėje rapsų aliejaus transesterifikacijoje metanoliu.