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Heterogeneous Biocatalysts for the Final Stages of Deep Processing of Renewable Resources into Valuable Products

Galina Kovalenko and Larisa Perminova

Abstract

Heterogeneous biocatalysis is a part of biotechnology and it has commercial potential for industrial implementation, in particular the final stages of deep processing of renewable raw materials. The commercially attractive heterogeneous biocatalysts are prepared by immobilizing practically valuable enzymatic active substances onto solid inorganic supports. Heterogeneous biocatalytic processes of the target conversion of substrate into valuable market product are carried out in periodic or continuous modes using traditional batch and packed-bed reactors, as well as novel types of vortex reactors in accordance with the principles of green chemistry. Heterogeneous biocatalysts for the final stages of deep processing of vegetable raw materials such as starch and oils are described here. One of the biocatalysts is glucoamylase immobilized by adsorption on mesoporous carbon support Sibunit™ type. This glucoamylase-active biocatalyst is used at the stage of starch saccharification, i.e., hydrolysis of dextrin to treacle and glucose syrups used in food and confectionary industries. The second of the biocatalysts is recombinant *T. lanuginosus* lipase immobilized on mesoporous silica KSK™ type and macroporous carbon aerogel. These lipase-active biocatalysts can effectively compete with traditional organic synthesis catalysts, and they are used in low-temperature processes carried out in unconventional anhydrous media such as interesterification of vegetable oils' triglycerides with ethyl acetate for producing ethyl esters of fatty acids (biodiesel and vitamin F) and esterification of fatty acids with aliphatic alcohols for synthesis of various esters used as fragrances, flavorings, odors, emollients, and nonionic surfactants in perfume and cosmetics industries. The prepared heterogeneous biocatalysts due to their high enzymatic activity and operational stability are promising for practical implementation.

Keywords: glucoamylase immobilized on mesoporous carbon, recombinant lipase immobilized on mesoporous silica and macroporous carbon aerogel, heterogeneous biocatalysts, starch saccharification, esterification of fatty acids

1. Introduction

Biocatalysis is a part of biotechnology that is inherently interdisciplinary and comprehensive, and its achievements are determined by the state of art in the fields

of microbiology, molecular biology, biochemistry, chemical technology, and engineering sciences. Biocatalysis is a scientific field fully focused from the very beginning on practice, and the main task is to study and use only one selected enzymatic reaction for the purposeful target biotransformation of the initial reagent—substrate (S)—into the valuable product (P) demanded by the market [1]. Heterogeneous biocatalysis is a very important part of biocatalysis that is based on immobilized enzymatic active substances such as individual enzymes, whole microorganisms, and partially or completely disrupted microbial cells. Heterogeneous biocatalysis as an interdisciplinary sphere of professional activities, undoubtedly, has great scientific importance and commercial potential for industrial implementation, including the processing of renewable raw materials into valuable market products. In accordance with the 12 principles of green chemistry listed in [1, 2], biocatalytic processes satisfy all the requirements and provide environmentally friendly and energy-saving technologies that are a promising alternative to traditional chemical processes.

Despite unique catalytic properties of soluble enzymes, such as 100% selectivity and high reaction rates under very mild, usually ambient conditions, as well as chemo-, regio-, and stereo-specificity, their industrial applications are limited due to the main disadvantages, namely, homogeneous conditions of periodic processes and inability to reuse enzymes that fall into wastewater. Immobilization of enzymes on/in support may overcome this drawback. Immobilization is defined as the fixation of enzymatic active substances onto/inside water insoluble solid supports, accompanied by retaining their enzymatic activity at a high level and their significant stabilization (up to 10^3–10^5 times). Immobilization prevents the inactivation of enzymes and ensures their multifold reusability. The heterogeneous biocatalysts are prepared by immobilizing the enzymatic active substances. These biocatalysts are undoubtedly of great practical interest for widespread implementation in industrial periodic and continuous bioconversion processes using batch stirred-tank, packed-bed, and novel types of vortex reactors specially designed for the heterogeneous diffusion-controlled biocatalytic processes in order to overcome diffusion limitations and enhance biocatalysts' productivity.

It is generally recognized that heterogeneous biocatalytic processes are more commercially attractive for large-scale implementation than homogeneous technologies due to considerable simplification and reduction (in 1.2–1.4 times) of the total production cost. Of course, the cost of the final product decreases with a decrease in the cost of the enzymatic active component of the biocatalyst, as well as the support and the method of immobilization. In order to reduce all expenses, both not purified enzymes but partially or fully disrupted, or whole nongrowing microbial cells, as well as inorganic support such as silica and carbon, and adsorptive immobilization are preferable for preparing commercial biocatalysts.

For successful commercialization of the heterogeneous biocatalytic processes, for example, the process of starch dextrin hydrolysis (saccharification), a biocatalyst has to convert 45% of the substrate during 15–20 min with the inactivation half-time ($t_{1/2}$) of 30–120 days, which corresponds to 3–12 months operation of the reactor without reloading. A high value of $t_{1/2}$ (50–100 days) is essential to increase productivity up to the recommended value from 100 kg to 10 tons of final product per 1 kg of biocatalyst [3].

Heterogeneous biocatalysts for two important bioconversion processes are briefly described here. One of the biocatalysts is glucoamylase immobilized by adsorption on mesoporous carbon support Sibunit™ type. This glucoamylase-active biocatalyst is used at the stage of starch saccharification, i.e., hydrolysis of dextrin to treacle and glucose syrups. The second of the biocatalysts is recombinant *T. lanuginosus* lipase immobilized on mesoporous silica KSK™ type and macroporous

carbon aerogel. These lipase-active biocatalysts can effectively compete with traditional organic synthesis catalysts, and they are used in low-temperature processes carried out in unconventional anhydrous media such as interesterification of vegetable oils' triglycerides with ethyl acetate for producing ethyl esters of fatty acids (biodiesel and vitamin F) and esterification of fatty acids with aliphatic alcohols for synthesis of various esters used as fragrances, odors, emollients, and nonionic surfactants in food, perfume, and cosmetics industries.

2. Glucoamylase-active heterogeneous biocatalysts for starch dextrin hydrolysis

The glucoamylases (glucan 1,4-alpha-glucosidases, EC 3.2.1.3) hydrolyze the glycosidic bonds at the end of polymer (starch) or oligomer (dextrin) chains releasing glucose. The main areas of industrial application of these enzymes are as follows: (1) a large scale two-step hydrolysis of raw starch successively catalyzed by α-amylase then glucoamylase, for production of sweeteners such as treacle and glucose syrups used in food industry, and (2) large-scale processes of hydrolytic conversion of starch to fermentable sugars as feedstocks for the production of some commodity chemicals and the first-generation biofuel such as bioethanol. These industrial processes are conducted on an enormous scale. Although the enzymes involved are relatively inexpensive, they are used on a single-use, throw-away basis. As mentioned above, immobilization of enzymes ensures enzyme recycling that can provide significant saving in the cost of final products not less than 20%.

The glucoamylases are the main enzymes used in the key second stage of starch conversion—hydrolysis of dextrin (saccharification), following the stage of liquefication (dextrinization) of starch by amylases. It should be noted that the immobilization of glucoamylase (not amylase) is justified and appropriate because this enzyme converts relatively low molecular weight substrates such as dextrin (3–5 kDa), and diffusion limitations can be overcome, in particular via design of employed reactor. The development of heterogeneous biocatalysts with high glucoamylase activity and operational stability is of great importance since they can serve as the basis for modern technology for deep processing of renewable vegetable raw materials into demandable sweeteners.

Back in 1970s, Corning Glass Co. carried out the fist pilot plant tests of a packed-bed reactor filled with a heterogeneous biocatalyst prepared by covalent immobilization of glucoamylase on macroporous silica; the glucose productivity was 450 kg/day. The main requirement for the commercial glucoamylase-active biocatalysts was a sufficiently high operational stability at pasteurization temperature of 60°C or higher. Inactivation half-times ($t_{1/2}$) of the tested Corning Glass biocatalyst were 520, 150, and 75 h at 55, 60, and 70°C, respectively [4]. The best result described later in 2000 for a biocatalyst prepared by immobilization of glucoamylase on polystyrene was that $t_{1/2}$ = 300 h at 50°C [5]. The best result described in the recent papers during 2008–2019 is that the glucoamylase immobilized by formation of cross-linked enzyme aggregates (CLEA) has "excellent recyclability, retaining over 45% of the relative activity after 24 runs" over a broad range of temperature (55–75°C) [6]. According to the opinion of the specialists working on the R&D projects of heterogeneous stage of saccharification the low thermal stability of the immobilized glucoamylase at elevated temperature (above 50°C) was the main reason why this process has not been commercialized so far.

Reputedly, the inexpensive and available carbonaceous materials with appropriate texture parameters are promising supports for adsorptive immobilization of enzymes, in particular of glucoamylase, for the preparing of commercially

attractive heterogeneous biocatalysts. Sibunit™-type supports from a new class of carbonaceous materials are porous carbon-carbon composites that combine the advantages of both graphite such as chemical stability and electrical conductivity, and active carbons in particular high specific surface area and adsorption capacity. These supports are characterized by a high volume of mesopores and a narrow controllable pore size distribution; some types have a large proportion of pores with a size of 5–20 nm or a bidisperse meso- and macroporous structure suitable for enzyme immobilization. Indeed, the sizes of globular molecules of most enzymes in aqueous solutions are approximately 10 nm. And it can be argued that the specific accessible surface area can be calculated using pore size distribution diagrams on the assumption that pores with a diameter more than 10–15 nm are available for immobilization of enzymes.

Textural parameters of the Sibunit support are as follows: total specific surface area, $S_{sp\ BET}$ = 550 m^2/g; total pore volume, V_Σ = 0.86 mL/g; and average pore diameter, D_{pore} = 18 nm as follows from pore size distribution diagram (**Figure 1a**). Specific surface area accessible for enzyme immobilization was estimated to be 92 m^2/g, that is 16% of $S_{sp\ BET}$. Surface of Sibunit formed by round coke deposits of pyrolytic carbon looks rough and porous on the scanning electron microscopic (SEM) images (**Figure 1b**).

The properties of the best glucoamylase-active biocatalyst (designated as GlucoSib) prepared by physical adsorption of commercial enzyme preparation GlucoLux™ type (produced by Sibbiopharm, Novosibirsk, Russia) on mesoporous carbon support Sibunit™ are described in [7] and briefly here.

It was found that the porous texture of the carbon supports plays a key role in stabilization of glucoamylase [7]. Predominant mesopores (10–20 nm in diameter) of Sibunit and bulk catalytic filamentous carbon are appropriate in size to hydrated enzyme molecules, whereas micropores of activated carbon (4 nm in diameter) are too small. Exactly, mesopores provide multipoint binding enzyme molecules inside the support and, as a result, ensure great stabilization of the activity. The thermal stability of glucoamylase adsorbed on mesoporous carbon supports, measured in dextrin solutions (32–53 w/v%) was found to be $\sim$10^5 times higher than for soluble enzyme [7].

Macrokinetics of dextrin hydrolysis by immobilized glucoamylase was investigated. Internal diffusion of dextrin inside the porous space of the biocatalysts toward adsorbed glucoamylase was found to be a rate-limiting stage of the saccharification process. Indeed, the rate of dextrin hydrolysis significantly reduced if the granule size was larger than 1 mm; for example, from 400 to 180 U/g for 1.2- and 3-mm granules, respectively [7]. The GlucoSib-type biocatalysts were prepared using support granules with diameter less than 1 mm; for example, the activity of the
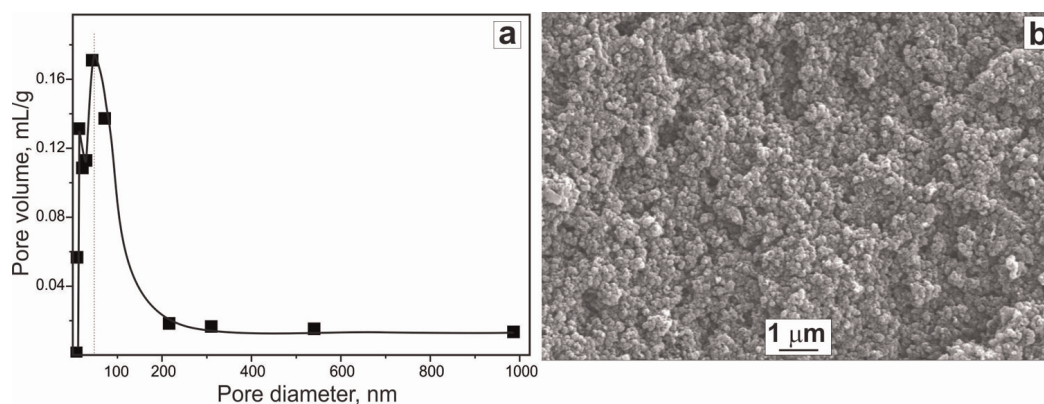

Figure 1.
(a) Pore size distribution diagram and (b) SEM image of Sibunit surface.

biocatalyst prepared by adsorption of glucoamylase on Sibunit granules of 0.2–0.7 mm in size was determined to be maximal, 530 U/g.

In order to overcome external diffusion limitations, a novel type of reactor such as immersed vortex reactor (IVR) was designed and tested in lab scale [7, 8]. The reactor body was filled with biocatalyst granules and then was immersed in a substrate solution and rotated. Substrate solution of dextrin was sucked through a bottom hole upon the body rotation and then moved with various high speeds through the biocatalyst bed toward side holes. Thus, a significant intensifying mass transfer of a substrate toward immobilized enzymes and, as a result, overcoming diffusion limitations was achieved by rotating the reactor body immersed in a substrate solution. Another very important advantage of the vortex type of reactor is an absence of stagnant zones and jet streams inside the bed of biocatalyst. To prevent formation of stagnant zones, the profiled reactor body was designed (**Figure 2a**). Since the profile of each half of the prefabricated reactor body was made in accordance with hyperbolic equation, the annular cross-section area for a substrate solution stream was equal to $2\pi R \cdot h$ and remained constant during circulation of substrate solution under operation. During this movement, the liquid was obviously affected by hydrodynamic, centrifugal, and inertial (Coriolis) forces, which resulted in a vortex flow of the substrate solution. The centrifugal forces were responsible for slight compression of the biocatalyst bed, narrowing channels for liquid flowing between granules, which results in the additional increase of mass transfer. Because of high mechanical strength of the Sibunit support, the granules were not distorted during the operation. Thus, the formation of stagnant zones was minimized.

A lab scale setup of the immersed vortex reactor filled by the glucoamylase-active biocatalysts GlucoSib was studied in the heterogeneous process of starch dextrin hydrolysis [7]. To elucidate optimal conditions for the IVR operation, the effect of rotation rate of the IVR body on the activity was studied. The maximal activity of GlucoSib, 700–750 U/g, was measured at body rotation of 300–900 rpm (**Figure 2b**). At 1000–1200 rpm, the reaction rate decreased perhaps due to the formation of a funnel in the rotating reaction medium, and special profiled device was designed to remove this defect.

The stability of the glucoamylase-active biocatalysts determined during continuous operation in saccharification heterogeneous stage was sufficiently high. Thus, the half-life time ($t_{1/2}$) exceeded 700 and 350 h at 50 and 60°C, respectively.
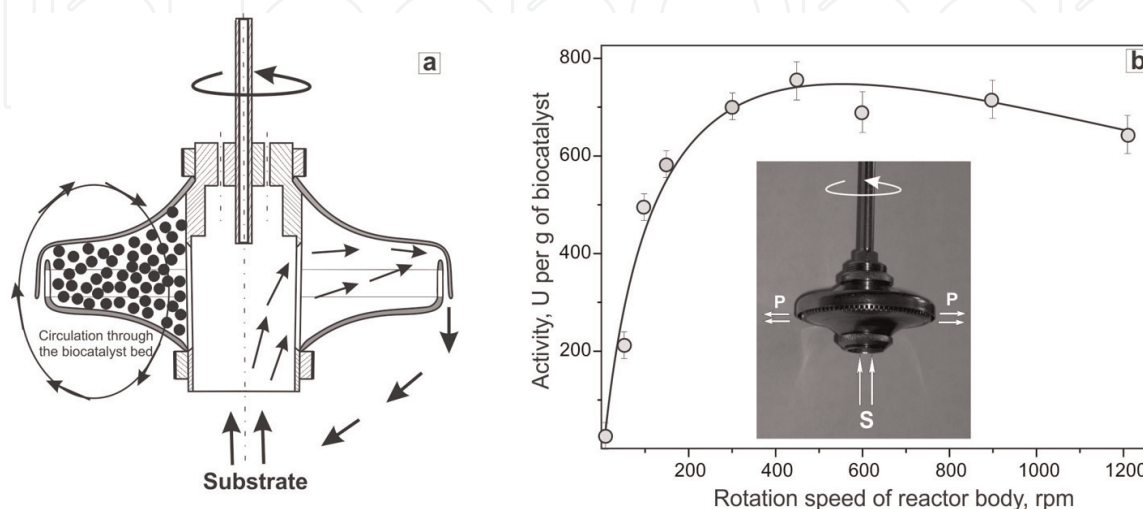

Figure 2.
Lengthwise cut of IVR body (a) and glucoamylase activity of the biocatalyst depending on rotation rate of reactor body (b). Photo of IVR body. Conditions of dextrin hydrolysis: 50°C, 0.05 M acetate buffer pH 4.6, 10 w/v% solution of potato dextrin as substrate.

Comparing these data with the Corning's results in [4], the $t_{1/2}$ of GlucoSib biocatalysts was estimated to be higher, 350 h vs. 150 h at 60°C, respectively. Long-term stability was sufficient also; the biocatalysts retained initial activity for 10 months upon storage at ambient temperature (18–22°C).

A technological scheme has been proposed and tested on a laboratory scale using the GlucoSib biocatalyst and immersed vortex reactor IVR for production of starch treacle and glucose syrups by heterogeneous dextrin hydrolysis. The advantages of this technological scheme are as follows: (1) significant acceleration of dextrin hydrolysis; (2) energy and resource saving in comparison with traditional starch processing; (3) a high quality of the final products due to the lack of protein impurities; and also, very importantly, (4) easily regulated carbohydrate composition of the treacle by simply stopping rotation of reactor body. It should be noted that when comparing the efficiency of the process of dextrin hydrolysis in vortex reactor with parameters of traditional packed-bed reactor the productivity of the IVR was higher by 1.2–1.5 times. The productivity in a novel proposed technology was calculated to be 5.3 tons of glucose per 1 kg of biocatalysts GlucoSib that is quite commercially attractive.

2.1 Conclusion for the part 2

The highly active and stable heterogeneous biocatalysts for dextrin hydrolysis were prepared by adsorption of glucoamylase on mesoporous carbon support Sibunit™. Under technological conditions (32 w/v% dextrin, 60°C, pH 5), the maximal activity was observed to be equal to 750 U/g, and inactivation half-life time ($t_{1/2}$) was 350 h. The immersed vortex reactor designed specially for the biocatalytic diffusion-controlled heterogeneous processes was used to carry out starch saccharification with enhanced productivity roughly estimated as 5 tons of glucose per 1 kg of biocatalysts.

3. Lipase-active heterogeneous biocatalysts for vegetable oil and fatty acid bioconversion

Heterogeneous lipase-active biocatalysts are of great importance due to remarkable properties of their enzymatic active component, namely lipase (triacylglycerol acyl hydrolase, EC 3.1.1.3.), that catalyzes a variety of reactions involving triglycerides—hydrolysis, esterification (synthesis of esters), inter-(or trans-)esterification (intra- and intermolecular exchange between fatty acids' residues), alcoholysis, and aminolysis [2, 9]. Nowadays, the main areas of industrial application of lipases and lipase-active biocatalysts are as follows: (1) hydrolysis of oily and fat stains on clothes by smart washing powders containing lipases as additives; (2) a large scale process of interesterification of the oil-fat blend to produce valuable ingredients, in particular for various spreads and margarine; (3) alcoholysis (methanolysis/ethanolysis) of triglycerides of vegetable rapeseed and soybean oils, as well as waste cooking and algal oils, into methyl/ethyl esters of fatty acids for production of biodiesel as an additive to fuel for engines; and (4) esterification of organic acids and synthesis of marketable valuable products including various esters of fatty acids for food and cosmetic industries, as well as enantiomers for pharmaceutical industry.

The most interesting and unique property of lipases is their ability to catalyze reaction in anhydrous media of organic solvents with water content less than 1%. Despite nonconventional conditions, these processes are characterized by high efficiency such as 100% selectivity, high conversion, and yield of final product.

One of the best results of oils' methanolysis by commercial biocatalyst Novozym® is that conversion of triglycerides into methyl esters of fatty acids (biodiesel) was ∼99% for 50 h at 45°C [10]. In 2007, the first biocatalytic process of the biodiesel production was implemented by methanolysis of edible oils' waste with the productivity of 10,000 tons; the biocatalyst was prepared by immobilization of lipase from *Candida* sp. [11].

NOVO (NOVOZYMES) Company is a leader in the production and sale of heterogeneous biocatalysts prepared by immobilizing the recombinant lipases on various supports. The Lipozyme® TL IM biocatalyst is prepared by immobilization of recombinant thermostable 1,3-specific *T. lanuginosus* lipase on silica. This biocatalyst is widely used in the industrial interesterification of fat-oil blends in order to produce valuable products, such as specialized fats and spreads without undesirable *trans*-isomers of fatty acids, as well as substitutes of cocoa butter and dairy fat. The Novozym®435 biocatalyst is prepared by immobilization of nonspecific *Candida antarctica* lipase on a macroporous polyacrylic polymer. The commercial Novozym® biocatalysts type are used in biodiesel production by methanolysis of vegetable oil (rapeseed and soya been) and waste oils of cooking. Nowadays, the NOVO biocatalysts are intensively studied for application in various processes, including organic synthesis.

It is well-known that methanol and ethanol inactivate enzymes rapidly. Therefore, in order to reduce the biocatalysts' inactivation, methanol was added stepwise in small portions during the reaction cycle of the biodiesel production. Another acylating reagent—methyl or ethyl acetate—was examined to be used. It was found that this reagent did not reduce the activity of the commercial biocatalyst Novozym® even at a molar ratio of oil to methyl acetate equal to 1:12; and under optimal operating conditions, the yield of methyl esters was equal to 96% and the biocatalyst's half-life time ($t_{1/2}$) increased 20-fold in comparison with $t_{1/2}$ in reaction with methanol [12]. Acyl derivatives of glycerol produced in interesterification of vegetable oils with methyl or ethyl acetate are valuable commercial products also. For example, mono- and triacyl glycerol are employed as fuel additives. Triacylglycerol (triacetin) is widely used in the food industry due to its good moisture-retaining properties. If the linseed oil is used in biocatalytic interesterification with ethyl acetate, the produced mixture of ethyl esters of ω3-, ω6-unsaturated fatty acids (vitamin F) is a valuable product for cosmetics industry and fodder additives production.

Nowadays, enzymatic esterification is considered as a competitive alternative to the chemical organic synthesis of various esters that are valuable commercial products commonly used in manufacturing flavors, fragrances, emollients, lubricants, antimicrobial agents, and nontoxic surfactants. The requirement of consumers for such natural products is constantly increasing. Compared with organic synthesis using strong liquid and solid acids as catalysts and temperature above 100°C (usually, 120–150°C), the enzymatic esterification is currently of great commercial interest since this method of esters' production proceeds efficiently at a low temperature (usually, at 20–40°C) without the formation of any by-products and with high specificity toward substrates. The heterogeneous lipase-active biocatalysts for the low-temperature esters' synthesis are prepared, as mentioned above, by immobilizing lipases on solid supports by various chemical origins and texture. These heterogeneous biocatalytic processes realized in periodic or continuous modes in anhydrous reaction media fully satisfy the requirements of "green" chemistry and they are promising for implementation into the organic synthesis industry [2].

The authors and their collaborators have developed and researched systematically the heterogeneous biocatalysts in which the enzymatic active component was a

recombinant *T. lanuginosus* lipase. These lipase-active biocatalysts were prepared both by entrapment of fully disrupted cells (lysates) of recombinant strain-producer r*E.coli*/lip inside silica xerogel and its nanocarbon-containing composites [13, 14, 17] and by adsorption of *T. lanuginosus* lipase produced by recombinant lipase on silica [15–18] and carbon aerogel [19]. These biocatalysts were studied in the reactions of tributyrin hydrolysis [13, 19], interesterification of oil-fat blends and vegetable oil triglycerides with ethyl acetate [13, 14], and esterification of fatty acids [15–19]. The two biocatalysts prepared by adsorptive immobilization of recombinant *T. lanuginosus* lipase are described briefly below.

3.1 Biocatalysts prepared by adsorption of recombinant lipase onto mesoporous silica

The enzymatic active component of the developed heterogeneous lipase-active biocatalysts was recombinant *T. lanuginosus* lipase (designated as r*Pichia*/lip) that was produced extracellular by the methylotrophic yeast *Pichia pastoris* X-33 strain specially constructed by the following genetic engineering manipulations: (1) chemical synthesis of the gene of a mature *T. lanuginosus* lipase taking into account the nucleotide sequence found in the Protein Data Bank (PDB-database); (2) cloning of the synthesized gene into a plasmid vector and production of the constructed recombinant plasmid in *E. coli* cells; (3) transformation of competent *P. pastoris* cells with the obtained plasmid and selection of recombinant yeast clones; and (4) analysis of the selected clones for the ability to produce extracellular and secrete recombinant *T. lanuginosus* lipase into the nutrient culture medium. Finally, conditions for the cultivation and intensive growth of the r*Pichia*/lip strain-producer were optimized in order to increase the concentration of the secreted target enzyme. The lab scale production of recombinant lipase was carried out in a 10-liter BIOK gas-vortex bioreactor (ZAO Sayany, Russia); the lipase concentration in nutrient media reached 2 g/L. Partial purification of recombinant lipase was carried out by precipitation of the secreted r*Pichia*/lip with ammonium sulfate (up to 75% saturation) at 4°C for 16 h. The precipitates were dissolved in distilled water, and further dialysis against a 25 mM acetate buffer pH 4.0 was carried out. The samples of dialyzed and lyophilized r*Pichia*/lip were used for the preparing lipase-active biocatalysts.

Mesoporous silica (SiO_2) of KSK™ type was used as a support for immobilization of recombinant *T. lanuginosus* lipase; its textural parameters are as follows: the specific surface area 157 m^2/g; total pore volumes (V_Σ) 0.76 mL/g; average pore diameters 19 nm; and porosity 58%. The lipase-active biocatalysts were prepared by both "spontaneous" and "forcible" adsorption using r*Pichia*/lip solutions at the same concentration. Spontaneous adsorption was carried out while contacting support with lipase solution at a ratio of support weight to solution volume of 1:(3–10) for 24–48 h. For such a long contact, adsorptive immobilization of lipase occurred due to electrostatic and acid-base interactions of enzyme molecules with silica surface. Forcible adsorption of lipase was carried out via moisture capacity impregnation of mesoporous silica by lipase solution, followed by drying granules. When biocatalyst was dried, enzyme molecules lost their hydrated shells and stuck to the surface. In both cases, the amounts of adsorbed enzyme were close in magnitude and equal to 4.2 and 5.4 mg/g for spontaneous and forcible adsorption, respectively. It was found that under exactly the same reaction conditions of esterification and synthesis of *n*-butyl heptanoate, the stationary activity of the spontaneously adsorbed lipase was two-fold less than the activity of forcibly adsorbed enzyme, for example, 2.4 vs. 5.0 U/g, respectively (recall that 1 U = 1 μmol/min). The method of forcible adsorption was characterized not only by comparatively high enzymatic

activity but also by simplicity of its implementation and economical enzyme consumption. For example, a minimal volume of lipase solution used for spontaneous adsorption on 1 g of silica was 3.0 mL, whereas for forcible adsorption, it was 0.8 mL equal to total pore volume (V_Σ) of SiO_2. All results described here referred to the lipase-active heterogeneous biocatalysts (designated as LipoSil) prepared by forcible adsorption on silica of recombinant lipase, which were used predominantly in esterification processes [15, 16, 18].

Biocatalytic processes of enzymatic esterification were performed at ambient conditions (20 ± 2°C, 1 bar) in unconventional anhydrous media of organic solvents such as hexane and diethyl ether. The saturated fatty acids differing in the number of carbon atoms (C2–C10, C18), as well as aliphatic alcohols differing in the structure of the molecules, namely, the number of carbon atoms (C2–12, C16), the isomerism of the carbon skeleton (*n-* and *iso-*) and OH-group position (*prim-*, *sec-*, and *tert-*) were studied as substrates in esterification by LipoSil. There were some peculiarities of operation of the preliminary dried lipase-active biocatalysts in nonaqueous organic solvents. A considerable increase of the activity was observed during the 1st–3rd reaction cycles. This phenomena, named preconditioning stage, was due to the ongoing accumulation of formed product—water—in the vicinity of the adsorbed lipase inside the silica-based biocatalyst, and this stage proceeded faster and the higher than the activity of the biocatalyst. For example, if the biocatalytic activities were about 5 and 500 U/g, then the activation of the biocatalysts (preconditioning) proceeded within 24 and 0.5 h, respectively. Calculation showed that under studied conditions upon full conversion of fatty acid, maximal 0.1 mL of water was formed inside for one reaction cycle. Since the total pore volume of silica (0.8 mL/g) was multifold greater than the volume of the water formed, this amount of H_2O was firmly held inside KSK™ silica commonly applied as a dehumidifier for industrial gases. Therefore, during esterification, the favorable aqua microenvironment was created for adsorbed lipase, and the activities of the dried biocatalysts increased by 2–4 times. After preconditioning stage, the biocatalytic activity, named stationary, was measured in batch reactor during several tens of reaction cycles. Each reaction cycle was completed to the full conversion of acid, close to 85–90%. Then, the reaction medium was removed by decantation and the biocatalysts were washed by solvent for 20 h. The next reaction cycle was started by adding fresh reaction medium containing substrates of lipase—acid S_1 and double molar excess of alcohol S_2. Stationary activity of the biocatalysts was fluctuated in magnitude during the consecutive reaction cycles of the periodic esterification process, perhaps due to the presence of ester (product) residues inside the operating biocatalyst. As it can be seen in **Figure 3**, the operational stability of the prepared biocatalyst was sufficiently high; its stationary activity was retained completely after 38 cycles (∼900 h) of esterification of various fatty acid. Also, the biocatalysts possessed a high long-term stability; the activity was determined to be ∼80% of initial one after storage for 9 months in the solvent (hexane and diethyl ether) at ambient temperature and in dried state in refrigerator.

Obviously, a very important property of heterogeneous biocatalysts is the high operational stability, since the productivity calculated by multiplying average activity by $2 \cdot t_{1/2}$ increases significantly. The esterifying activity of the prepared lipase-active biocatalysts did not practically change during 500–1000 h of operation. Under studded conditions, the productivity was evaluated as ∼2 tons of product per 1 kg of LipoSil biocatalyst.

The study of the functional properties of enzymes after their immobilization, such as activity, stability, and, importantly, specificity, is of great interest. Of particular scientific and practical interest is the research of the possibility of modulating these properties, by engineering heterogeneous biocatalysts, in particular, by
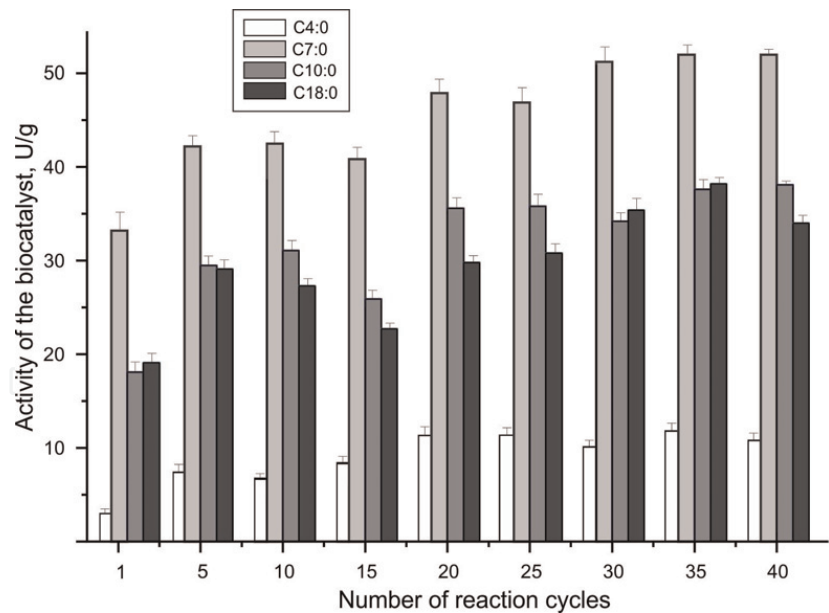
Figure 3.
Operational stability of the lipase-active biocatalyst in periodic esterification of various fatty acids such as butyric (C4:0), enanthic (C7:0), capric (C10:0), and stearic (C18:0) with n-butanol as a function of enzymatic activities depending on a number of reaction cycle. Conditions of esterification: 20 ± 2°C, 0.25 M acid, 0.50 M alcohol, hexane:diethyl ether = 1:1, content of biocatalyst in reaction medium 20.8 wt.%. The amount of adsorbed lipase is 14.0 mg/g.

Acids ↓	Alcohols →								
	C2	C3	C4	C5	C8	C10	C11	C12	C16
C2	0.00	–	–	0.03	0.02	–	–	–	0.02
C4	0.08	0.21	0.21	0.19	0.15	0.19	0.08	0.06	0.04
C5	0.09	0.23	0.28	0.15	0.17	0.28	0.10	0.08	0.07
C6	0.08	0.26	0.31	0.23	0.19	0.47	0.09	0.07	0.07
C7	0.54	0.98	1.00	0.88	0.85	0.81	0.72	0.53	0.47
C9	0.27	0.64	0.54	0.52	0.56	0.59	0.28	0.24	0.19
C10	0.30	0.57	0.64	0.63	0.58	0.54	0.32	0.26	0.27
C18	0.39	0.57	0.78	0.61	0.57	–	0.39	0.34	0.39

Table 1.
Matrix of relative activities of the immobilized recombinant T. lanuginosus lipase in reaction of esterification of various pairs of substrates—saturated fatty acids and aliphatic primary n-alcohols.

selecting the chemical nature of the supports [20, 21]. In our research, the specificity of heterogeneous enzymatic esterification was determined by comparing the reaction rates for various pairs of substrates—saturated fatty acids and aliphatic primary *n*-alcohols—and the matrix of relative units of activities for the LipoSil biocatalyst was composed (**Table 1**) [18].

The following conclusions can be drawn from the data presented in **Table 1**: (1) the rate of esterification of acetic (C2) acid is very low; thus, it is practically impossible to obtain acetate esters; (2) the rate of synthesis of ethyl ester (reaction with ethanol C2) is 1.3–2 times lower than that in reactions with C3–C16 alcohols; (3) the esterification of fatty acids with a number of carbon atoms more than six (C6) occurs 2–5 times faster than that of low molecular weight fatty acids C4–C6; and (4) the maximal observed rate is determined for esterification of enanthic (C7) acid with C3–C8 alcohols. Also, one can see that the immobilized on silica recombinant lipase is more sensitive to the molecular structure of saturated fatty acids than
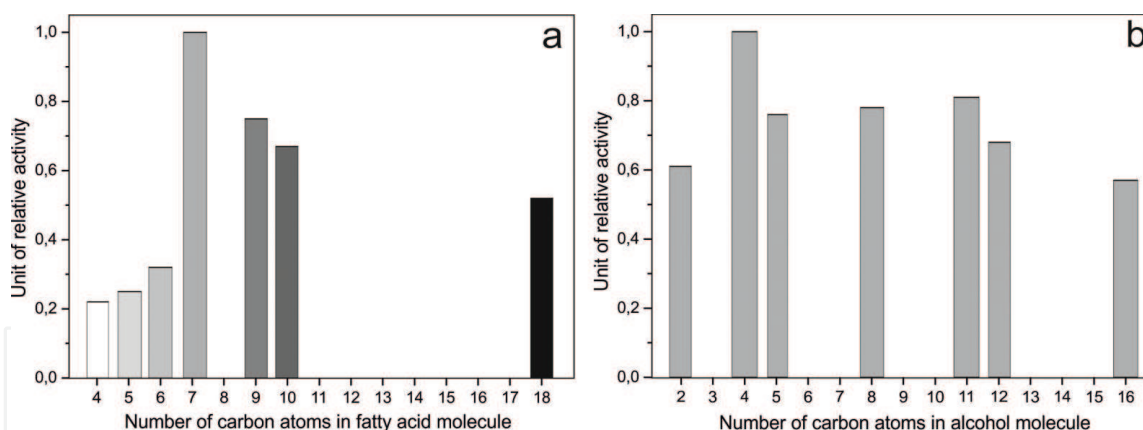
Figure 4.
Relative activities of the immobilized recombinant T. lanuginosus lipase in reaction of esterification of (a) different saturated fatty acids with n-butanol, and (b) enanthic acid with different alcohols. Conditions of esterification: 20 ± 2°C, 0.25 M acid, 0.50 M alcohol, hexane:diethyl ether = 1:1, content of biocatalyst in reaction medium 20.8 wt.% the amount of adsorbed lipase 14.0 mg/g.

that of aliphatic alcohols because the maximal differences in rates were ∼6 times for various acids (**Figure 4a**) and only ∼2 times for alcohols (**Figure 4b**).

It was found that immobilized on silica r*Pichia*/lip was not sensitive to isomerism of alcohols' molecule, and the rates of esterification of fatty acids with primary *n*-C4,C5 alcohols were almost 10 ± 4% lower than those with *is0*-C4,C5 alcohols [18]. The most pronounced specificity was observed toward the position of OH-group in alcohol molecules. The rates of esterification of fatty acids with secondary alcohol (*sec*-propanol and *sec*-butanol) was two order of magnitude lower than those with primary alcohols, for example, 0.36 U/g vs. 56.1 U/g for *sec*- and *prim*-propanol, respectively [18]. The esterification of fatty acid with ternary alcohols (*tert*-butanol) did not occur, and reaction rate was zero. Also, it was found that the substrates (both acid and alcohol) with aromatic and cyclic restudies reacted with very low rates [15, 19].

The kinetic parameters such as Michaelis constant (K_M) for acid and maximal reaction rate (V_{max}) under studied conditions were determined in esterification of enanthic (heptanoic, C7:0) acid with a double excess of *n*-butanol. The kinetic curve as a function of the initial reaction rate from the initial fatty acid concentration lower than 1.0 M was satisfactory approximated by the classic hyperbolic Michaelis equation, $V = \frac{V_{max} \cdot C_0}{K_M + C_0}$, where V and V_{max} are the initial observed and maximum reaction rates, respectively, μmol/L·s^{-1}; C_0 is the initial substrate concentration, mol/L; and K_M is the Michaelis constant, mol/L. At a concentration of enanthic acid above 1.0 M, the reaction rate decreased perhaps due to inactivation of enzyme by high concentration of *n*-butanol as described previously for esterification of capric acid with *iso*-pentanol [17]. The values of K_M and V_{max} determined using Lineweaver-Burk linear approximation and the regression of hyperbolic equation by soft Origin' programs were 0.22 ± 0.05 mol/L and 66.7 ± 4.0 μmol/L s^{-1}, respectively. A comparison with data published earlier in [16, 17] showed that the Michaelis constant for enanthic (C7) acid was ∼3 times less than K_M for capric (C10) acid during esterification with C4,C5 aliphatic alcohols. This means, that the affinity of adsorbed recombinant lipase toward enanthic acid is higher; thus, the maximum rate V_{max} will be achieved at lower initial fatty acid concentrations, and it is important for practice.

3.2 Conclusion for the part 3.1

The catalytic properties of the lipase-active heterogeneous biocatalysts such as enzymatic activity, stability, and substrates specificity were investigated in the

esterification of various saturated fatty acids with aliphatic alcohols. These biocatalysts were prepared by immobilizing recombinant *T. lanuginosus* lipase onto mesoporous silica by impregnation method. Particular attention was paid to the study of the substrate specificity of immobilized lipase. It was found that forcibly adsorbed on silica *T. lanuginosus* lipase demonstrated broad substrate specificity. Saturated fatty acids with a number of carbon atoms 6 or more (till 18) and alcohols with a number of carbon atoms 3 or more (till 16) reacted with comparatively high reaction rates. The reaction rates depended slightly on isomerism (*n-* and *iso-*) of carbon skeleton of C4-C5 alcohols, whereas the rates depended strongly on the position of OH-group, and secondary and ternary alcohols did not react with fatty acids. Comparing the rates of esterification of various pairs of substrates using primary alcohols, a matrix of relative biocatalytic activities was composed. According to this matrix, the rates of synthesis of C4-C18 esters were sufficiently high, while the rate of synthesis of acetate ester was very low. Under the same reaction conditions, the maximal rate was observed in esterification of enanthic (C7:0) acid with butanol. The classical Michaelis-Menten kinetics was inherent for biocatalytic esterification of this acid with double molar excess of alcohol, and the main kinetic parameter, Michaelis constant for acid, was determined to be 0.22.mol/L.

It is well known that, from a practical point of view, the stability of heterogeneous biocatalysts is a very important characteristic that determines the biocatalyst productivity. The prepared lipase-active biocatalysts possessed considerably high operational stability in a periodic batch process of low-temperature esters' synthesis carried out in unconventional anhydrous media of organic solvents (hexane and diethyl ether). The enzymatic activity of the biocatalysts was completely retained for several tens of reaction cycles.

It was concluded that due to the remarkable catalytic properties combined with simplicity of immobilization method, the prepared lipase-active biocatalysts are promising for practical use in organic synthesis including the production of valuable esters.

3.3 Biocatalysts prepared by adsorption of lipase on carbon aerogel

Immobilization of recombinant *T. lanuginosus* lipase (SIGMA Co.) was carried out by spontaneous adsorption onto macroporous carbon aerogel [19]. It should be noted that carbon aerogels are a unique class of porous materials with a very low density (less than 0.1 g/mL) and a porosity of up to 90–99%. These materials are novel promising adsorbents for enzyme immobilization with great potential for practical implementation. Macroporous carbon aerogel (MCA) for our research has been produced by *in situ* synthesis of multi-walled carbon nanotubes (CNTs) via catalytic high-temperature decomposition of ethylene over the supported Fe:Co catalyst. The carbon aerogel was obtained in the form of ball-shaped granules of 1–10 mm in diameter (**Figure 5a**).

The three-dimensional framework and rigid macrostructure of MCA were formed by chaotic interlacing carbon nanotubes (**Figure 6a**). The number of walls and the diameter of CNTs determined by high resolution transmission electron microscopy (HRTEM) were equal to 12–14 and 15–25 nm respectively (**Figure 6b**). The density of MCA was 0.06 g/mL. The texture parameters of carbon aerogel were as follows: the specific surface area (S_{BET}) was 80–110 m^2/g, total pore volume (V_Σ) was 10-14 mL/g; macropores of 0.5–1 μm in diameter were predominant in texture; and the volumes of meso- and micropores did not exceed 2% of V_Σ.

When studying adsorption of recombinant lipase on MCA, it was found that adsorption graph contained two "plateaus" corresponding to adsorption of ~100 mg/g, or ~0.8 mg/m^2 of carbon aerogel for the 1st plateau, and 200 mg/g for
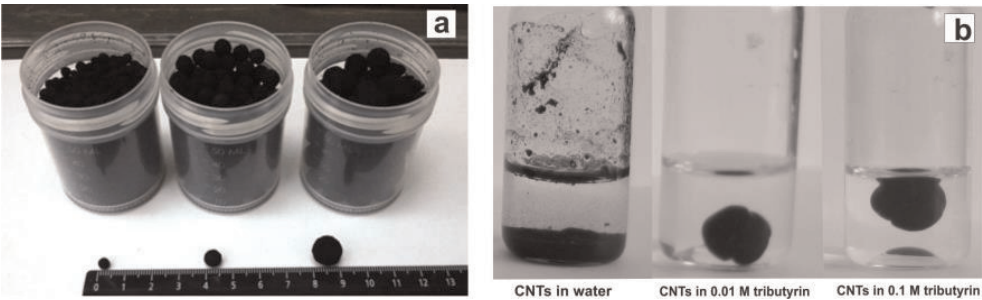
Figure 5.
(a) Photo of granules of macroporous carbon aerogel and (b) photo of agglomeration of fine dispersed carbon nanotubes (CNTs) under the influence of increasing concentration of tributyrin.
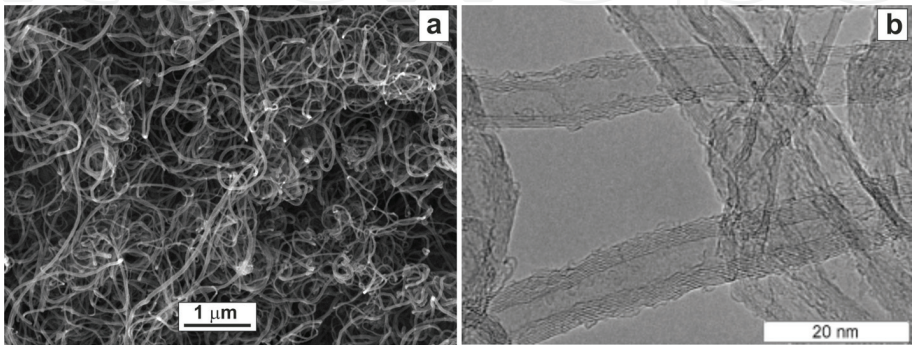

Figure 6.
(a) SEM image of the inside of the granules of macroporous carbon aerogel; (b) HRTEM image of carbon nanotubes forming MCA.
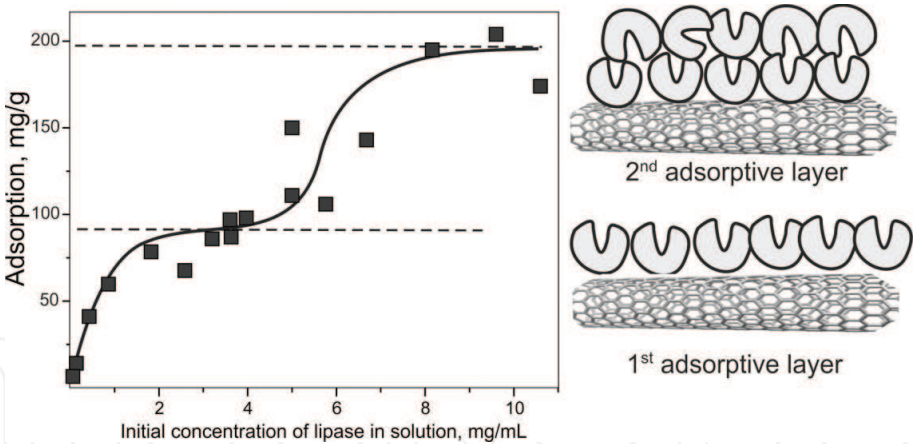

Figure 7.
Adsorption of recombinant lipase on macroporous carbon aerogel depending on initial concentration of soluble lipase. Conditions of adsorption: 20 ± 2°C, 0.02 M Na-phosphate buffer pH 7.0, 24 h.

the 2nd plateau (**Figure 7**). Considering the molecular weight of the lipase, ~30,000 Da, we calculated the surface concentration of the adsorbed lipase, 2×10^{15} molecules per 1 m^2, and then the area occupied by one molecule of the adsorbed lipase, ~500 nm^2. The diameter of one adsorbed molecule of lipase was estimated to be ~20 nm as confirmed by HRTEM images [19]. It is known from literature [22] and PDB-database, that crystallographic size of the lipase molecule is $3.5 \times 4.5 \times 5.0$ nm; in aqueous media, the diameters of hydrated molecules increase twice. The *T. lanuginosus* lipase has the ability to be associated into dimers due to hydrophobic interaction between hydrophobic lids and pockets of active site [23]. So, the first plateau corresponded to the formation of the 1st adsorptive layer that was dense and uniform in distribution of the adsorbed lipase on the surface of MCA. The second plateau can be attributed to the 2nd also dense adsorptive layer formed

probably by further dimerization of lipase molecules. As it turned out, under studied conditions, the lipase adsorbed molecules formed two dense adsorptive layers; the amount of lipase in each adsorptive layer was equal to ~100 mg per 1 g of MCA (**Figure 7**).

It was found that 85–90% of the amount of adsorbed lipase was very firmly attached to the surface of carbon aerogel and did not desorb [19]. Desorption was investigated using various selective reagents in order to clarify mechanism of lipase binding on carbon surface. For example, the amount of lipase desorbed using 1 M sodium chloride did not exceed 2%; hence, electrostatic interactions were negligible. The most effective desorption, $10 \pm 2\%$, was observed using distilled water and C2-C4 alcohols' solutions; hence, weak Van der Waals interactions were predominant. The lipase was also efficiently desorbed by emulsifier (gum arabic) perhaps due to breaking hydrophobic interactions. Thus, study on selective desorption showed that the strong adsorption of *T. lanuginosus* lipase on carbon nanotubes forming aerogel occurred exclusively due to hydrophobic-hydrophobic interactions.

Lipase-active biocatalysts prepared by adsorptive immobilization of recombinant *T. lanuginosus* lipase on macroporous carbon aerogel were studied in the periodic processes of bioconversion of triglycerides and fatty acids such as tributyrin hydrolysis, interesterification of vegetable oil with ethyl acetate, and esterification of saturated fatty acids (butyric C4:0, capric C10:0, and stearic C18:0) with *iso*-pentanol.

The activity of the lipase-active biocatalysts and specific activity of adsorbed on MCA lipase were measured in hydrolysis of emulsified tributyrin under conditions when there were no diffusion limitations for mass transfer of the substrate from reaction media toward the adsorbed lipase [19]. Note that specific activity of adsorbed enzyme (in U/mg) is calculated by dividing the experimentally observed activity of the biocatalyst (in U/g) by the amount of adsorbed enzyme (in mg/g). The specific activities of the lipase adsorbed both on MCA granules and nonporous fine powders of carbon nanotubes were compared with each other; the values were found to be 160 and 12 U/mg, respectively. This significant difference was due to the relative rigidity of ball-shaped aerogel granules that prevented agglomeration of carbon nanotubes in "oil-in-water" triglyceride emulsion as presented in **Figure 5b**. Since water-immiscible hydrophobic triglyceride molecules were adsorbed efficiently on hydrophobic carbon nanotubes, with increasing tributyrin concentration, agglomerates of round shape containing this substrate were formed from CNTs (**Figure 5b**). As a result, the required hydrolysis water is displaced from the vicinity of adsorbed lipase and its specific activity fell down as mentioned above.

The maximal hydrolytic specific activity of the adsorbed *T. lanuginosus* lipase was measured to be 700 U/mg (vs. 14,000 U/mg for soluble lipase), i.e., activity significantly decreased upon adsorption of lipase on MCA. Another reason discussed below may be probably incorrect orientation of adsorbed lipase on a highly hydrophobic carbon surface of CNTs.

The results of study on dependence of the specific activity of adsorbed lipase on the adsorption value are presented in **Figure 8a**. Initially, when adsorption of the lipase was small, the observed activities were extremely low. The specific activity increased dramatically and reached maximal values, >700 U/mg, in a region close to the formation of the 1st protein dense layer at adsorption of 100–110 mg/g (**Figure 8a**). Then, the specific activity fell down as adsorption increased (**Figure 8a**). As a result, maximal hydrolytic activity of the biocatalysts, 75,000 U/g, was observed at the adsorption corresponding to the formation of the 1st protein layer. The activity of the biocatalyst with the double adsorptive layer was found to be 1.6-fold lower, 47,000 U/g. In order to explain maximum on the curve in **Figure 8a**, we proposed that the adsorbed lipase can be oriented differently on the
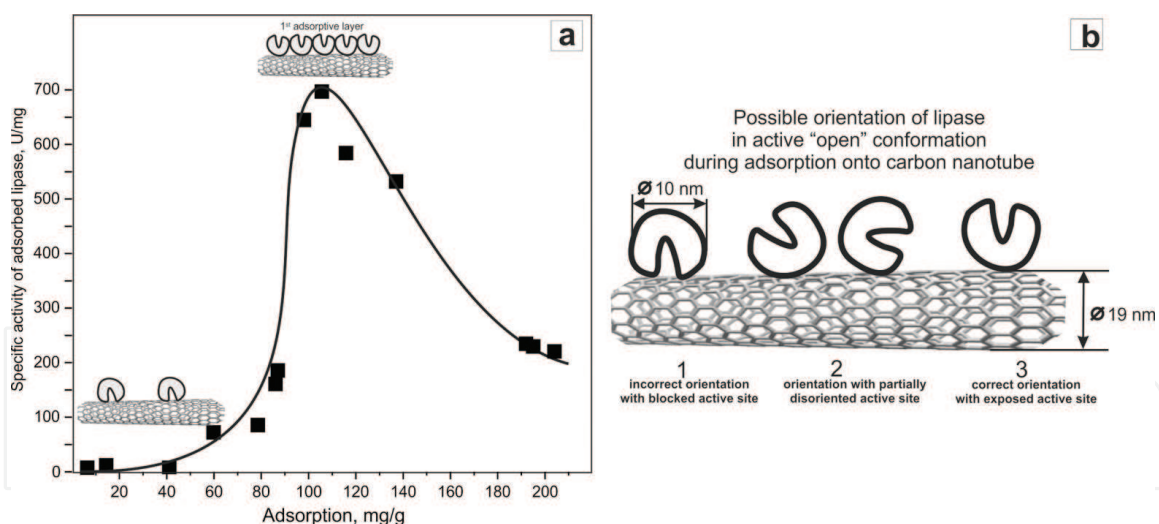
Figure 8.
Specific hydrolytic activity of lipase adsorbed on carbon aerogel depending on adsorption. Conditions of hydrolysis: 20 ± 2°C, 0.02 M Na-phosphate buffer pH 7.0, 0.02 M tributyrin, 1.0 M glycerol, 0.6% gum arabic, content of biocatalyst in reaction medium 3.3 wt.%.

surface of carbon nanotubes, namely correctly and incorrectly (**Figure 8b**). The incorrect orientation (No. 1 in **Figure 8b**) with blocking the active site by the surface was realized more likely in the incompact 1st adsorptive layer; in this case, the observed lipase activity had a minimum value. The correct orientation (No. 3 in **Figure 8b**) may be realized in the dense 1st adsorptive layer, so the formation of active enzyme-substrate complex occurred; and in this case, the observed lipase activity had a maximum value. A further decrease in specific activities after the formation of the 1st adsorptive layer was due to covering the active lipase by the molecules in subsequent 2nd adsorptive layer (scheme in **Figure 7**) and possible partially correct orientation (No. 2 in **Figure 8b**). The obtained data suggest that the main reasons for a loss of enzymatic activity upon adsorption of the lipase on hydrophobic carbon aerogel are as follows: (1) incorrect orientation of enzyme molecule on the carbon surface and blockage of the active site, as well as possible deformation of enzyme molecule; and (2) dehydration of the biocatalysts due to removal of essential water from the vicinity of adsorbed lipase via efficient adsorption of hydrophobic triglycerides.

It has been found that activity and stability of the prepared lipase-active heterogeneous biocatalysts depended strongly on the type of enzymatic reaction performed either in aqueous reaction media (hydrolysis) or in nonaqueous media (interesterification and esterification). The stability of the biocatalysts was quite low when triglycerides participated in reactions as lipase substrates. For example, the biocatalysts lost ~90% of initial activity during six reaction cycles of tributyrin hydrolysis [19]. Another example, the stability of adsorbed on MCA r*Pichia*/lip was determined during interesterification of linseed oil with ethyl acetate in order to produce the valuable product, vitamin F—ethyl esters of ω-3 fatty acids. The conversion of triglycerides was 87 and 66% in the 1st and 3–5th reaction cycles, respectively, and then biocatalysts inactivated due to rapid dehydration.

As mentioned above, esterification of acid with alcohols was accompanied by the formation of water and the corresponding ester, and, as a result, accumulation of essential water molecules inside biocatalysts in the vicinity of the adsorbed lipase occurred. So, the stability of the prepared biocatalysts was much higher in esterification than in hydrolysis and interesterification. And the biocatalysts operated during esterification without a loss of activity for more than several hundred hours in the nonaqueous media of anhydrous organic solvents. As seen in **Figure 9**, the
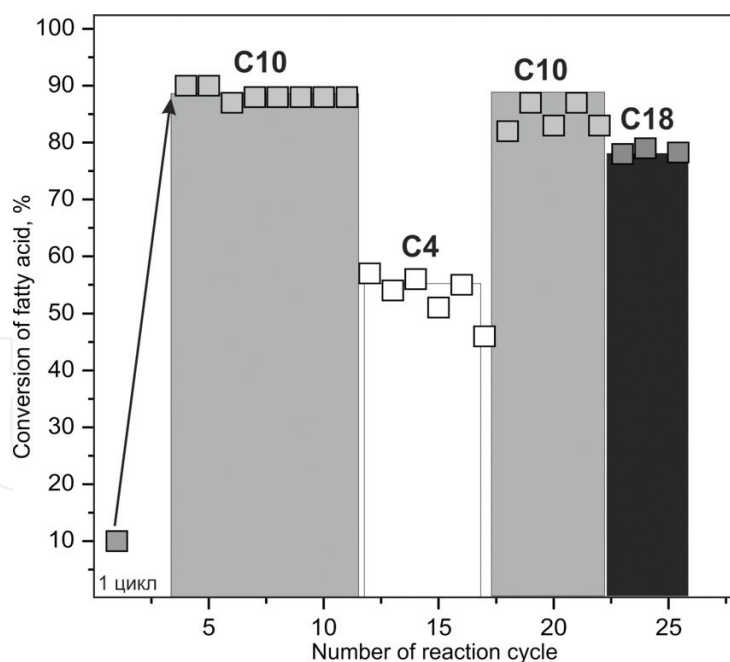
Figure 9.
Operational stability of the biocatalyst in periodic esterification of various fatty acids such as butyric (C4), capric (C10), and stearic (C18) with iso-pentanol as a function of the acid conversion depending on a number of reaction cycle. Conditions of esterification: 40°C, 0.10 M acid, 0.40 M alcohol, hexane:diethyl ether = 1:2, agitation 70 rpm, content of biocatalyst in reaction medium 3.3 wt.%. The amount of adsorbed lipase is 206.0 $mg\,g^{-1}$.

esterification activity of dried biocatalysts sharply increased during the 1st reaction cycle; a pre-conditioning stage was observed as described above. The esterified activity depended on the structure of fatty acid, namely of a number of carbon atoms in molecule. Similar to LipoSil biocatalysts described above, the rate of esterification of butyric C4 acid was the lowest compared to capric (C10:0) and stearic (C18:0) acids (**Figure 9**). In the process of the synthesis of *iso*-pentyl caprinate in binary (hexane and diethyl ether) solvent at 40°C, the prepared biocatalysts demonstrated high operational stability and operated without losing activity for a few hundred hours [19].

3.4 Conclusion for the part 3.2

Macroporous carbon aerogel (MCA) obtained by *in situ* catalytic synthesis of the carbon nanotubes was studied as the efficient promising support for adsorptive immobilization of enzymes, in particular recombinant *T. lanuginosus* lipase, due to the rigid 3D-framework of granules and high adsorption ability of MCA. Heterogeneous lipase-active biocatalysts were prepared by adsorption of the enzyme on carbon nanotube (CNT)-forming aerogel, and the processes of bioconversion of substrates into valuable products were organized in periodic regimes under mild ambient conditions.

Recombinant *T. lanuginosus* lipase was adsorbed on macroporous carbon aerogel very firmly exclusively due to hydrophobic interactions between enzyme and carbon nanotubes. Two dense adsorptive layers were formed by lipase molecules; the amount of the lipase in each layer was nearly 100 mg/g.

Activity and stability of the prepared biocatalysts strongly depended on the type of reaction media, namely aqueous or nonaqueous. In the hydrolysis of triglycerides (aqueous reaction medium), the specific activity of the *T. lanuginosus* lipase significantly (in 20 times) decreased upon adsorption on MCA, because orientation of adsorbed lipase on the carbon surface was probably incorrect. The maximal activity

of the biocatalyst, 75,000 U g^{-1}, was measured at the adsorption corresponding to the formation of the 1st dense adsorptive layer. The activity and stability of the prepared biocatalysts decreased during the periodic process of tributyrin hydrolysis because a displacement of essential water via efficient adsorption of triglyceride on CNTs occurred, which was followed by progressive dehydration of biocatalysts.

In esterification of fatty acids with alcohol (nonaqueous reaction media), hydrophobic MCA did not prevent the accumulation of the produced essential water in the vicinity of adsorbed lipase. The lipase-active biocatalysts possessed high stability in the synthesis of esters of fatty acids with *iso*-pentanol and operated for a few hundred hours under mild condition of the synthesis of *iso*-pentyl caprinate in binary (hexane and diethyl ether) solvent at 40°C.

As a general conclusion based on all results of adsorption/desorption and activity/stability, we can convincingly note that texture and chemical nature of supports such as mesoporous silica and macroporous carbon aerogel greatly affect the biocatalytic properties of the adsorbed recombinant *T. lanuginosus* lipase. This influence was due to not only hydrophilic-hydrophobic properties of the adsorbents but also specific molecular features of lipase and peculiarities of biocatalysis carried out in aqueous and nonaqueous reaction media. The main reason of inactivation of the prepared biocatalysts during bioconversion of hydrophobic triglycerides, such as hydrolysis and interesterification, was their dehydration. The accumulation of essential water inside the lipase-active biocatalysts during esterification prevented their inactivation. Analyzing our own and literature data, it was concluded that due to the prominent catalytic properties, such as enzymatic activity and operational stability, in combination with simplicity of the immobilization method, the biocatalysts prepared by adsorption of recombinant lipase onto mesoporous silica and macroporous carbon aerogel are promising for practical implementation for reactions of organic synthesis, in particular for production of fatty acids' esters.

Acknowledgements

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