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Historical Perspective

Biochemical aspects of lipase immobilization at polysaccharides for biotechnology

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ABSTRACT

The design of immobilized enzyme preparations is an important and relevant area of modern sciences and technologies. Immobilization of enzymes from animal sources (component I) on natural carriers (component II) increases the system stability by protecting the active site of the enzyme from deactivation; facilitates the separation and accelerates the recovery of the enzyme. This makes reuse possible and provides a significant reduction in operating costs. Hydrolytic enzymes (such as lipases) and polysaccharides (such as chitosan) are the most promising of such pairs of components. The main attention here is devoted to the discussion on lipase immobilization on polysaccharide (mainly - chitin and chitosan). Based on the analysis of the available literature, the most adequate method is the immobilization of lipase from porcine pancreas (LPP) on polysaccharide particles (such as chitin or chitosan) pre-treated with ultrasound (to increase the particle surface area) and glutaraldehyde (for particle activation) that shows reasonably high LPP activity and stability. In order to increase further the activity of the lipase, some authors proposed to incorporate a spacer in the form of 1,3-diaminopropane (or 1,3-diaminobutane) prior to activation of the surface of the chitosan particles. In particular cases, the use of chitin (instead of chitosan) may be an alternative solution for biotechnological applications.

Recently the idea of constructing "supramolecular enzyme systems" realized in the so-called "coimmobilized multienzymatic systems" strategy. The most fascinating example is the combined assay of a mixture of native LPP, glycerol kinase (from *Cellulomonas*) and glycerol-3-phosphate oxidase (from *Aerococcus viridans*) linked by glutaraldehyde to chitosan (as shell for inorganic nanoparticle core). This material was placed on a Pt-electrode as biosensor and was successfully applied for amperometric determination of the triglyceride level in the serum of healthy and diseased person. Thus, the whole innovative research-production sequence is described by Aggarwal V. and Pundir C.S.: from simple components to advanced material and further biomedical application.

Thus, the following approach of lipase immobilization appears the most promising for future applications: a few types of lipases or the combination of LPP with some other enzymes immobilized simultaneously on multifunctional carriers (as nanohybrids of inorganic core and polysaccharide shell).

Contents

Abbreviations: AFM, atomic force microscopy; BAC, biologically active compounds; CALA, lipase A from *Candida Antarctica*; CALB, lipase B from *Candida antarctica*; CLSM, confocal laser scanning microscopy; CV, cyclic voltammetry; DM, degree of chitin deacetylation (important for chitosan evaluation); EIS, electrochemical impedance spectroscopy; FTIR, Fourier-transform infrared spectroscopy; LCC, lipase from *Candida cylindracea*; LHP, lipase from human pancreas; LPP, lipase from porcine pancreas; LWG, lipase from wheat germ; MCNCs, magnetic cellulose nanocrystals; PALF, pineapple leaf fibers; PALMF, pineapple leaf microfibers; PCL, lipase from *Pseudomonas cepacialipase*; SEM, scanning electron microscopy; SES, supramolecular enzyme systems; TAG, triacylglycerol; TEM, transmission electron microscopy; TGA, thermal gravimetric analysis; VSM, vibrating sample magnetometry; XRD, X-Ray diffraction..

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1. Introduction

The preparation and use of enzymes in their various forms in veterinary and biomedical, zootechnical and biotechnological, biochemical and food industries is an important and relevant field of modern science and technology [1–7]. Immobilization of enzymes (for example, lipases [4,6–8]) on natural and synthetic carriers increases their stability by protecting the active site of the enzyme from deactivation; facilitates the separation and accelerates the recovery of the enzyme; makes reuse possible; provides a significant reduction in operating costs. There is a large number of materials and methods for such enzyme immobilization [1–3,6–8]. It is important that their choice is carefully considered and justified taking into account the specifics of the pair of enzyme-carrier components and the catalytic process. To our opinion, hydrolytic enzymes (such as lipases, component I) and polysaccharides (such as chitosan, component II) are the most promising for immobilization procedure.

There are numerous reviews [8–18] on purification, structure-function relations, immobilization and application of various lipases. For example, production, three-dimensional structures and biocatalysts properties of major bacterial lipases were thoroughly discussed in reviews [9–11,16], whereas immobilization and biotechnological applications of bacterial lipases were a subject of all these and the following papers [19–23]. One of the reasons to initiate this review is that the lipase from porcine pancreas (LPP) was mentioned just slightly only in a few of these reviews, whereas the major protocols on LPP immobilization and its potential biotechnological applications in aqueous and organic media was discussed in detail only in 2012 by Mendes et al. [24].

In recent years, the idea of constructing supramolecular enzyme systems (SES) has become more and more attractive for researchers. The most representative example of such strategy is the so-called "coimmobilized multienzymatic systems" that is described in the recent review of Sizhu Ren et al. [25]. A broader view of the chemistry of different reactive groups (including amino, thiol, carboxylic, hydroxyl, and epoxy groups) for enzyme immobilization is presented in the recent review of Bilal et al. [1] The authors called it "smart" chemistry of biologically active compounds (BAC) immobilization on various supports (microspheres, hydrogels and polymeric membranes, nanoparticles, nanofibers, composite and hybrid or blended support materials). [1]. Numerous biopolymers such as alginate, chitosan, cellulose, agarose, guar gum/guaran, agar, carrageenan, gelatin, dextran, xanthan and pectins are thoroughly discussed in the more recent review of Bilal et al. [2]. This remarkable (in many aspects) review [2] includes only a few examples of the lipase immobilization. In particular, there is just one bacterial lipase mentioned in Table 2 entitled "list of various chitosan support materials for enzyme immobilization…" [2], but there are 8 examples of galactosidases and 6 examples of other proteins.

There is a very sophisticated research on lipase immobilization [26] which can serve as example of our suggestion concerning the component II. Multifunctional carriers (called "magnetic cellulose nanocrystals" or MCNCs) consisting of magnetic nanoparticles ($FeCl_2 4H_2O$ and $FeCl_3 6H_2O$) with chitosan and sodium tripolyphosphate (incorporated into microcrystalline cellulose) were prepared. These MCNCs were used for immobilization of lipase from *Pseudomonas cepacialipase* (PCL) by chemical attachment using glutaraldehyde as covalent cross-linking agent. All obtained PCL-MCNCs were characterized by combination of the modern methods: atomic force microscopy (AFM), confocal laser scanning microscopy (CLSM), X-Ray diffraction (XRD), Fourier-transform infrared spectroscopy (FTIR), vibrating sample magnetometry (VSM), scanning electron microscopy (SEM), transmission electron microscopy (TEM) and thermal gravimetric analysis (TGA) [26]. For example, the mean diameter and height of the "relatively compact" PCL-MCNCs were about 75.82 nm and 1.7 nm, respectively [26]. The best PCL-MCNC sample had PCL loading about 82.2 mg per 1 g of MCNCs and activity recovery of 95.9% by the hydrolysis of 4-nitrophenyl palmitate (after 5 min., 40 °C, measured at 405 nm) [26]. This sample (called by authors as "bionanocatalyst") was able to produce about 83.3% of the product by asymmetric hydrolysis of the (*R,S*)-"Ketoprofen Ethyl Ester" (final concentration 3 mM, reaction time of 2.5 h) [26]. The most impressive result is the possibility of the "bionanocatalyst" reuse with high efficiency: after 2, 4 and 6 technological cycles (2.5 h per used batch with further recovering by magnetic forces and washing) over 95%, 85% and 66% of the PCL-MCNC initial activity retained [26].

The more fascinating and successful example seems to be the combined assay of a mixture of native lipase (from porcine pancreas), glycerol kinase (from *Cellulomonas*) and glycerol-3-phosphate oxidase (from *Aerococcus viridans*) linked by glutaraldehyde to chitosan (shell) covered nanoparticle core consisting of $Zn(NO_3)_2 4H_2O$ that was published in the book series "Methods in Enzymology" 2016 [27]. This material was placed on a Pt-electrode as biosensor and characterized by electrochemical impedance spectroscopy (EIS), high resolution SEM, FTIR, and cyclic voltammetry (CV) [27]. This biosensor was successfully applied for amperometric determination of the triglyceride level in the serum of healthy and diseased person (in the range of 88–195 and 210–590 mg/dL, respectively) [27]. The last example [27] is especially impressive, because it shows the whole innovative research-production row: from simple components to advanced material and real biomedical application.

The purpose of this article is to review experimental studies on the methods of immobilization of some major lipases (mainly - lipase from porcine pancreas) on polysaccharides (mainly - on chitin and chitosan), including discussion on enzyme activity and their potential as the pattern of functional biomaterials.

Scientists currently active in this field may skip the next two parts of this chapter and procced directly to chapters 2 and 3. But for the readers from the other research fields (such as chemists, physics, engineers, etc.) or Ph.D.-students it is important to get a broad view on this topic. Therefore, we will discuss briefly the major properties of LPP and some polysaccharides before turning to the immobilization in chapters 2 and 3.

1.1. Lipases

Lipases (triacylglycerolacyl hydrolases EC 3.1.1.3) are widely distributed enzymes [3–6] and well-known by their hydrolytic activity - the hydrolysis of fats and oils in the aqueous media [5–15]. However, in the aqueous-organic media (at low water content) their hydrolytic activity can be transformed in synthetic: esterification, transesterification or interesterification activity [5,15,24,28]. By changing the reaction conditions, some lipases are capable to transfer the acyl groups of the complex esters on other nucleophiles, such as amines, oximes, and thiols [28].

In an organic medium, for example, lipases can catalyze the direct interaction of carboxylic acids and hydrogen peroxide with formation of peroxycarboxylic acids [29]. This ability of lipases is widely used for indirect epoxidation of various unsaturated organic compounds under mild conditions [30–33]. Recently, it has been demonstrated that these enzymes are able to catalyze a sufficiently wide range of completely different reactions - the formation of carbon-carbon, carbon-heteroatom, heteroatom-heteroatom bonds [34]. This diversity of catalytic behavior causes their wide application in various industrial processes [35].

Lipases are isolated from various sources: bacteria, fungi, seed germs, humans and a number of animals (pigs, cows, sheep, etc.) [6–24]. As a rule, lipases of animal origin for biotechnological use are isolated from the pancreas of adult individuals.

It is known that lipases of human and animal pancreas are very sensitive to colloidal nature of the environment and substrate (so-called "interfacial activation" effect [10,12,16–18,36]). The substrates (various triacylglycerols) must be emulsified in microdroplets with bile acids, phospholipids, etc. On the other side, the small protein-helper (called colipase) is the key hinge (hook) to bind the non-catalytic site of the enzyme and interact with the peptidic "lid" at the catalytic site. The joint action of the microdroplets and colipase opens the "lid" and loops to enable the substrate molecule to enter the hydrophobic channel of the enzyme active site (lipase "open" conformation) [10,12,16–18,36]. This scheme was modified in our research [6] on lipase activation by polyelectrolytes (instead of colipase) and emulsified substrate that convert the enzyme from "closed" to "open" conformation (Fig. 1).

Lipases are well-known enzymes for various applications that are permanently discussed in the literature [4–18]. Therefore, we summarize the major possible applications of lipases in the table below (Table 1).

In order to enhance the technical and economic advantages of lipase preparations, it is recommended to use them in an immobilized form to increase stability and reduce costs by reusing them in comparison to a soluble form. Some materials and methods of immobilization of lipases have been studied [9,74–85]. Immobilization of the enzyme changes the activity in relation to different substrates, shifts the pH-optimum, increases the working temperature and stability of the enzyme [74–85] that will be discussed in the chapter 2.

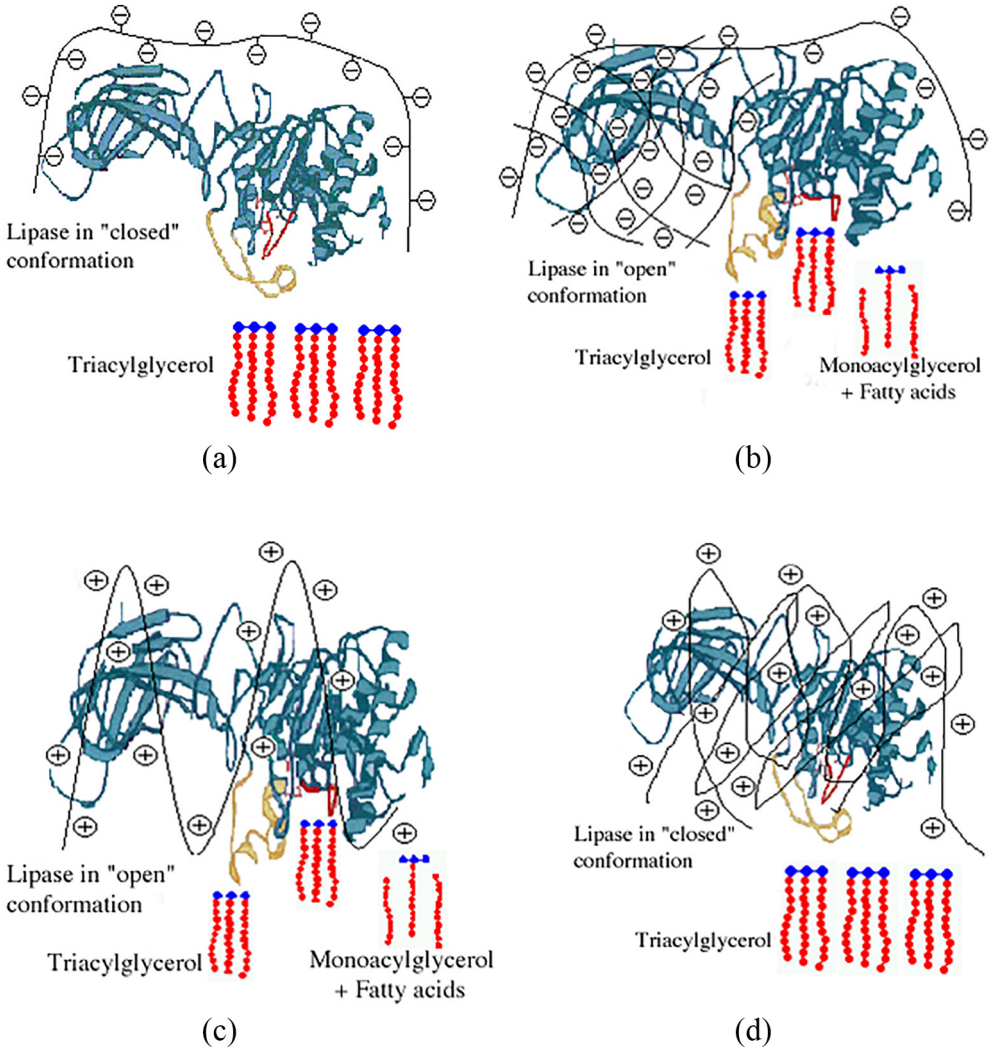

Fig. 1. Scheme of the LPP in the "closed" (A,D) and "open" (B,C) conformations and the triacylglycerol hydrolysis by activation with polyelectrolytes and emulsified fat (adapted from the book [6], inspired by R. Verger [36]).

Table 1
Biotechnological and industrial application of various lipases (adapted from [5,6]).

Process/product	Reaction	References
Aromatization of the diet products	Hydrolysis	[37,38]
High quality paper and related cellulose materials	Hydrolysis	[4]
Fur and leather quality improvements	Hydrolysis	[39]
Utilization of oil-containing rests from oil industry	Hydrolysis	[40]
	Alcoholysis	[41,42]
Utilization of fat-containing rests from husbandry and animal farming	Interesterification	[43,44]
Laundry detergents	Hydrolysis	[45,46]
Biodisel production	Alcoholysis	[40,47–50]
Food flavorings	Esterification	[51–54]
	Interesterification	[55,56]
	Alcoholysis	[57]
Food antioxidants	Esterification	[58,59]
Artificial cocoa milk	Interesterification	[60]
	Acidolysis	[44]
Margarine production	Acidolysis	[61,62]
Food and cosmetic detergents	esterification	[49,63,64]
	Alcoholysis	[65,66]
	Aminolysis	[67]
Fur cleaning	Esterification	[68]
Optically pure isomers	Stereospecific hydrolysis	[69,70]
	Stereospecific esterification	[71–73]

1.2. Chitin and chitosan

Chitin, the homopolymer of *N*-acetylgalactosamine, is the second most common natural biopolymer after cellulose [86–96]. Chitin is the main component of shells of crustaceans, such as crabs, shrimps and crawfish, and therefore it is a by-product of the fish industry. Chitosan is a rigid-chain biopolymer (Fig. 2) classified as aminopolysaccharide, derived from chitin by deacetylation [87–92].

Its most important parameters being the molecular weight and the degree of deacetylation. Over the past few decades, chitin polymers and, especially, chitosan have attracted increasing attention as promising renewable polymeric materials for a wide range of applications in the pharmaceutical [86], cosmetology and biomedical industries, for immobilization and purification of enzymes, in the chemical industry for wastewater treatment and in the food industry as a binder, a gelling agent, stabilizing and thickening agents [6,86–96]. The use of biopolymers as a matrix for the immobilization of lipase has a number of advantages, both from a technological and ecological point of view, in comparison with other materials - there are the absence of toxicity and chemical reactivity; easy fixation of the enzyme, biocompatibility, biodegradability, etc. [6,86–96]. The presence of free amino groups in chitosan provides binding sites for proteins. In addition, the low cost of chitosan/chitin polymers and their possibility for biodegradation should be noted [87–90].

Interesting aspects of biomedical or pharmaceutical applications of chitosan or chitin preparations were summarized in some recent review [86]. We do not discuss these aspects in detail here, but just mention the major application directions: cosmetics, artificial skin, functional food components for human and animal nutrition, ophthalmology, photography, water engineering (including the materials for metal capture from wastewater, color removal from textile mill effluents due to dye-binding properties of chitin and chitosan, etc.), paper finishing, solid-state batteries, drug-delivery systems, etc. [90–96].

2. Results on the immobilization of lipases on chitin and chitosan and their discussion

There are several methods of immobilization of enzymes, which can be divided into two categories: physical methods and chemical methods (Fig. 3). Physical methods include adsorption, encapsulation and inclusion (in gel); chemical methods include crosslinking and covalent binding. Recently, a combination of these methods has been used (Fig. 3) [7,97]).

2.1. Physical adsorption of the enzyme

Due to the rather large number of studies on immobilization of various lipases on chitin and chitosan (using different approaches and methods), only a brief review of the literature (mainly concerning the lipase from porcine pancreas) is considered here. The most important data from these publications are summarized in Table 2.

It is supposed that physical adsorption of enzyme on carrier occurs primarily due to electrostatic interactions between positively charged chitosan (CHI) and negatively charged lipase. Thus, in the investigation of Bassani et al. [75], the molecular mechanism of interaction between LPP and CHI from crab shells was investigated in details (the degree of deacetylation of CHI was at least 75%). The evidence of complex formation of LPP-CHI due to electrostatic interactions is that charge neutralization leads to precipitation in aqueous media at a ratio of 0.011 mg CHI per 1 mg LPP. The complex dissolves as the ionic strength of the system increases. Turbidimetry showed that even at high ionic strength (1 M NaCl aqueous solution) there was no complete dissolution of the

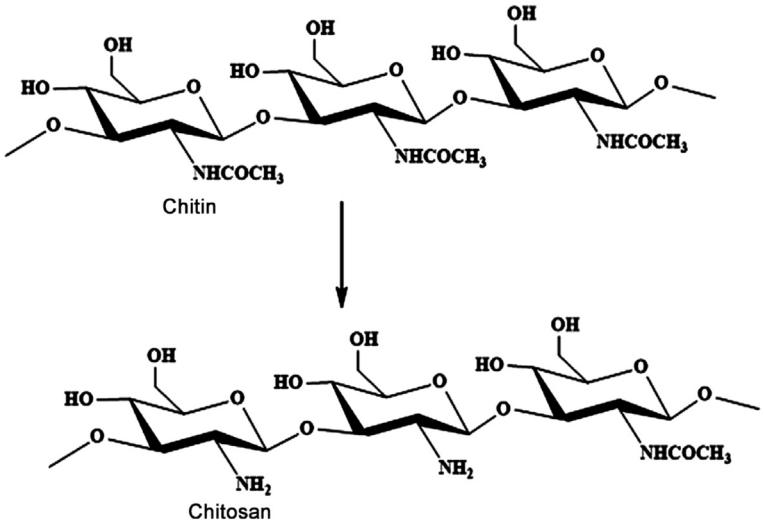

Fig. 2. Scheme of the chitin deacetylation into chitosan (adapted from the [83]).

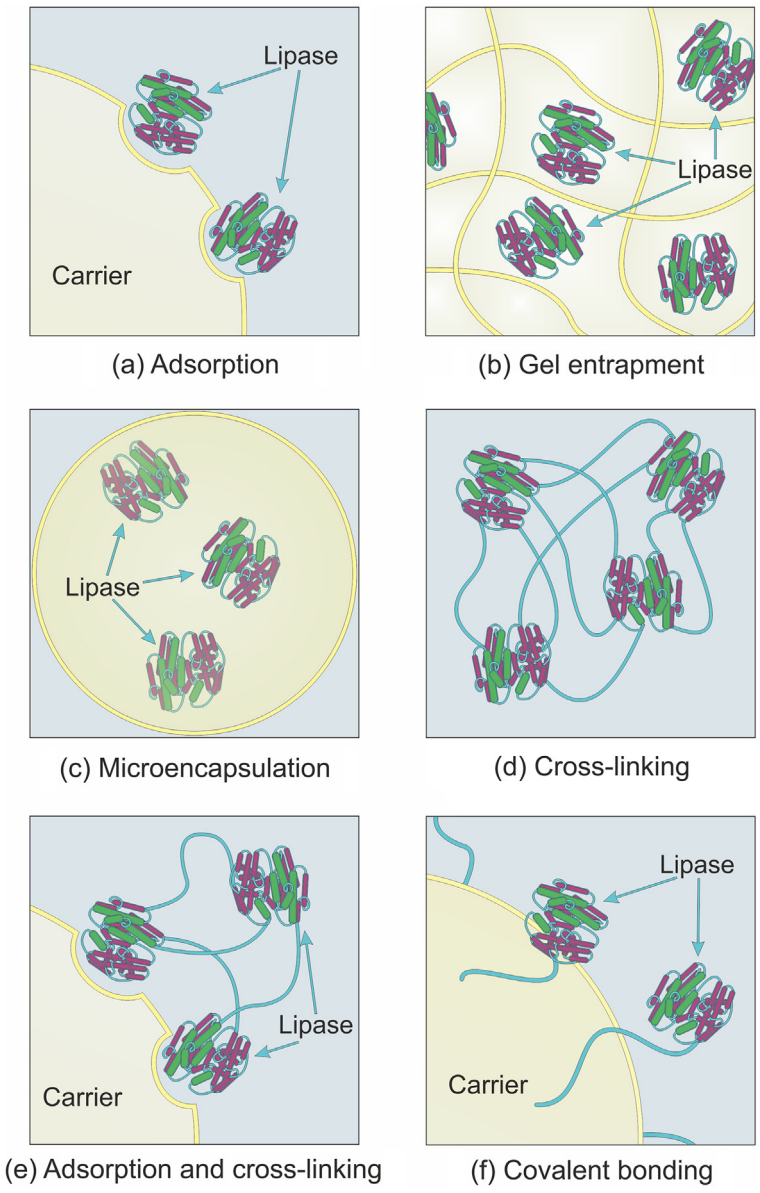

Fig. 3. Methods of enzyme immobilization (modified from [7]).

precipitate, which allowed the authors of [75] to suggest the existence of the "superposition" of all forces for complex formation, including hydrophobic interactions.

Pereira B. et al. [80] investigated these interactions in comparison with the hydrolytic and synthetic activity of LPP immobilized on chitosan flakes (a) and porous chitosan (b) by physical adsorption (olive oil emulsion as substrate). The degree of binding for (a)-samples was ~54%, whereas for (b)-samples this was ~72%; the hydrolytic activity was 8.6 and 42.6 l/min•mg, respectively (Table 2). In contrast, the synthetic (esterification) activity was almost the same for both preparations - 33.3 and 31.9 μmol/min•mg, respectively [80]. Thus, for (b)-samples (LPP on porous chitosan) an almost five-fold increase in the hydrolytic activity occurred, while the esterification activity decreased insignificantly (Table 2) as compared to the samples of the first type (a).

In a number of studies (only major papers cited here [98,99]) Russian scientists from the Voronezh State University prepared and studied biocatalyst based on two enzymes (LPP and trypsin adsorbed on chitosan). It is strange that the chitosan (used in the work) has not been characterized by the authors. The results [98,99] show that the order of components sorption does not affect the proteolytic activity of the complex (93%). However, the greatest retention of lipolytic activity (65%) appears with preliminary immobilization of lipase on the carrier and subsequent attachment of trypsin [99]. This effect can be associated with the stabilizing properties of the polyelectrolyte matrix with respect to the protein globule of lipase (but the degree of deacetylation has not been characterized). In comparison, lipase adsorbed on chitosan in the absence of trypsin retains up to 90% of its activity [99]. The reasonable concentrations of trypsin and lipase in solutions were about 10^{-4} and $5{\cdot}10^{-5}$ mol/L [99]. The authors [98] did not explain that the lipase immobilized on chitosan is not very sensitive to changes in the pH of the medium, the percentage of "conservation of activity" (with a decrease in pH from 7.0 to 5.8) is about 75% of the initial. It is important that the catalytic activity of co-immobilized enzymes (7.8 U/g or 7.2 U/g) was highest in the case of phosphate or phosphate-citrate buffer. In contrast, the use of acetate and succinate buffer systems leads to a sharp decrease in the catalytic activity of both enzymes [98,99] that was not explained in details. In any case, these studies [98,99] can be considered as the best examples of the applied SES (discussed in the Introduction) based on chitosan.

Table 2
Major data on immobilization of lipases on chitin and chitosan (using different approaches and methods), hydrolytic and synthetic activity of the obtained samples.

Ref. number	Sample	Immobilization, % or mg (protein)/ g(carrier)	Hydrolytic activity		Synthetic activity	
				Units		Units
[76]	Control		3.6	μmole/mg·min	–	
	Beads		3.2		–	
	ChiS*		2.3		–	
	ChiG*		3.0		–	
[77]	Control	–	100%		–	
	Beads +1 mg LPP	0.519 mg/g	31.2%		–	
	Beads +2 mg LPP	1.31 mg/g	31.30%			
	Beads +3 mg LPP	1.51 mg/g	31.20%			
	Beads +4 mg LPP	2.08 mg/g	39.6%			
	Beads +6 mg LPP	2.13 mg/g	39.0%			
[78]	Control	–	107.42	Unit#/mg (enz.)	$1.23 \cdot 10^{-3}$	μmole/min
	Chitin	23.0%	14.6		$5.23 \cdot 10^{-3}$	
	Chitosan	90.6%	16.1		$2.53 \cdot 10^{-3}$	
[79]	Covalent bonding	32.2 mg/g	–		325 unit/mg	μmole/mg (chitosan)
	Without	1.91 mg/g	–		1750	
	1.3 DAP**	1.51 mg/g	–		2050	
	1.4 DAB**	1.36 mg/g	–		1950	
	1.5 DAP**	0.67 mg/g	–		1925	
	1.6 DAH**	0.57 mg/g	–		1550	
[80]	Flakes	53.7%	8.6	μmole/mg·min	33.3	μmole/mg·min
	Beads	72.3%	42.6		31.9	
[81]	a***	33%	33.74	Unit/g (chitosan)	–	
	b***	74%	33.40		–	
	c***	63%	31.11		–	
	d***	74%	32.20		–	
	e***	86%	35.18		–	

*ChiS - lipase is adsorbed to particles obtained from chitosan beads, after sonication;
* ChiG - lipase is covalently attached to particles obtained from chitosan beads, after sonication, followed by activation of the surface with glutaraldehyde;
** 1,3 DAP-lipase is covalently attached to chitosan, by insertion: glutaraldehyde-1,3-diaminopropane-glutaraldehyde;
** 1,4 DAB - lipase is covalently attached to chitosan, by means of an insert: glutaraldehyde - 1,4-diaminobutane - glutaraldehyde;
** 1,5 DAP - lipase is covalently attached to chitosan, by means of an insert: glutaraldehyde - 1,5-diaminopentane - glutaraldehyde;
** 1,6 DAH - lipase is covalently attached to chitosan, by means of an insert: glutaraldehyde - 1,6-diaminohexane - glutaraldehyde;
*** a - lipase is adsorbed to chitosan beads;
*** b - lipase is covalently attached to chitosan beads;
*** c - lipase is adsorbed to chitosan beads, followed by binding with glutaraldehyde;
*** d - lipase is covalently attached to chitosan beads, washed and then further bound by glutaraldehyde;
*** e - lipase is covalently attached to chitosan beads, followed by binding with glutaraldehyde (including free lipase).
#. – definition of the unit .

2.2. Chemical stitching/binding of the enzyme

In the work of de Mello M.D. and co-authors [76], ultrasonic fragmentation of chitosan was successfully used to increase the surface area and make it more accessible for interaction with the lipase. Chitosan of low viscosity and lipase from porcine pancreas, as well as from plant origin, together with 4-methylumbelliferyl butyrate (MUB) as a substrate were used [76]. Immobilization was carried out with glutaraldehyde, the most universal chemical agent used to immobilize enzymes, which performed simultaneously two functions: it cross-links the polymer chains and activates the surface. According to microscopy, chitosan particles are balls with a diameter of about 2 μm, but after sonication their size is becoming significantly smaller (about 50–100 nm). Chitosan, after treatment with glutaraldehyde, had a particle diameter of 100–200 nm. The average particle size increased upon LPP immobilization up to 1000–2000 nm. Consequently, glutaraldehyde cross-links the polymer chains to each other, which results in a multi-layered packing of chitosan particles. The effectiveness of immobilization of lipase on various chitosan samples (1 - balls, 2 - balls sonicated, 3 - balls sonicated and treated with glutaraldehyde) was different. For example, in the first case the binding efficiency was about 61%, in the second - 64%, in the third – 84%, that authors [76] explained by the activation of the surface of chitosan particles with glutaraldehyde. In addition, the treatment with glutaraldehyde increased the hydrophobicity of the surface of chitosan particles (due to the conversion of free amino groups into amide derivatives), which, in the authors' opinion [60], leads to an increase in the lipase adsorption. It is known, that the presence of surfactants stabilizes the "active" (open) form of the lipase catalytic site [36]. Therefore, the authors [76] immobilized lipase in the presence of Triton X-100. For these samples, interesting data on specific activity and kinetic parameters were obtained in comparison with data for an unbound enzyme. For free lipase from porcine pancreas, the activity was 3.6 nM/(min•mg), $K_m = 0.5$ mmol, $V_{max} = 2.9$ nmol/min; for the first sample (balls) 2.2 nM/(min•mg); for the second (sonicated balls) – 2.3 nM/(min•mg), $K_m = 1.2$ mmol, $V_{max} = 1.7$ nmol/min; for the third (sonicated balls with glutaraldehyde) – 3.0 nM/(min•mg), $K_m = 1.1$ mmol, $V_{max} = 4.7$ nmol/min [76]. These results may be explained by immobilization of the lipase molecules in their "active" form in the presence of Triton X-100. Experiments on the thermal stability of the samples showed that the free lipase is most active at 35 °C, while immobilized LPP - at 45 °C. The relative lipase activity increased 1.5–1.6 times as compared with activity at 25 °C (for samples 1 and 2) and even - 1.8 times in the case of sample 3. The highest activity of lipase was observed at pH 6.5–7.0. Another important parameter of the samples (for their commercial use) is the possibility of enzyme re-use. The sample 2 (on ultrasonic treated chitosan beads) showed a sharp decrease in activity to 30% with repeated use, further 8 cycles led to a smooth fall in activity to 10%. This is due to the fact that in this sample the lipase is adsorbed on the surface of chitosan, rather than sewn to it covalently. Therefore, the sample 3 (lipase on chitosan beads treated with ultrasound and glutaric aldehyde) showed the best results - almost 100% activity on repeated use and about 85% after five cycles of use. Similar results were obtained

for samples of lipases from plant raw materials [76] and can be useful for further application.

Desai P.D. and co-authors [77] immobilized LPP on chitosan by covalent binding with glutaraldehyde and studied its hydrolytic activity. The activity of free and immobilized lipase was determined spectrophotometrically using olive oil as a substrate. Activity was maintained at 50% after five cycles. The optimum pH of the medium for both free and immobilized enzymes was about 9.0. The kinetic parameters were the following: $K_m = 4.0 \cdot 10^{-7}$ mmol and $V_{max} = 0.32$ nmol/min for unbound lipase, $K_m = 3.32 \cdot 10^{-7}$ mmol and $V_{max} = 0.32$ nmol/min for the immobilized LPP. This indicates a lack of significant conformational changes during immobilization, however, the immobilized enzyme showed improved heat resistance and storage stability [77]. These results [77] are less impressive as compared to the data of de Melo et al. [76] discussed above in details with respect to their further application.

2.3. Immobilization of the enzyme in a gel or sewing using a spacer between the substrate and the enzyme

In the study of Ozyilmaz [79], LPP (329 units/mg) was bound to chitosan by a covalent bond through a diaminoalkane and glutaraldehyde. An inserted spacer between the carrier and the enzyme was used, because an increasing distance between the enzyme and the carrier often leads to an increase in enzyme activity [79]. Optimization of diaminoalkane length showed that the sample obtained with 1,3-diaminopropane has the highest activity. In this work [79], LPP was included in the beads of a mixed gel of alginate and glutaraldehyde crosslinked chitosan. An independent optimization was carried out for the concentrations of sodium alginate, chitosan and glutaraldehyde to produce a lipase-containing gel at constant lipase concentrations of 3 mg/ml and calcium chloride equal to 2 mol/l [79]. Optimal concentrations of sodium alginate, chitosan and glutaraldehyde were 1.5%, 1.5% and 0.15%, respectively [79]. The activity of the lipase was determined by the reaction of isoamyl acetate formation from isoamyl alcohol and acetic acid. The determination of the required moisture level of the gel was carried out and the following effects were found: 1) when the humidity in the adjacent gaseous phase was reduced from 80% to 40%, the lipase activity in the samples gradually increased; 2) a jump in activity was observed for samples at 27% humidity; 3) with a decrease in humidity to 17%, lipase activity decreased [79]. Optimization of the solvent used (hexane, heptane, chloroform, toluene and xylene) demonstrated that the greatest yield of ester was observed when heptane was used. It is extremely important that in the obtained samples leaching of lipase did not occur even when they were kept in heptane for 24 h. Temperature optimization showed that the greatest activity of lipase was at 40 °C for samples based on mixed gel and at 45 °C for chitosan-based samples. The optimum concentrations of isoamyl alcohol and acetic acid, 50 mM, were also determined for both reagents. This reaction was carried out in a volume and on a column in order to study the dynamics of product formation. For the column method, the maximal amount of the reaction product was reached after 5 h (a further decrease in the yield of the ester appears to be due to excess water molecules resulting from the reaction and leading to hydrolysis of the product) [79]. The outputs of the reaction product in the volume of the system were 2 orders of magnitude greater than on the surface of the carrier (for the column method). This appears to be due to the low reaction rate on the carrier surface, as well as the slow diffusion of substrates across the column. The yields for samples based on mixed gel turned out to be an order of magnitude lower for chitosan-based samples, which is explained by the slow diffusion of substrates into the gel. At 40 °C, after 5–7 h, an activity increase of 40% was observed for a mixture of samples based on chitosan and 80% for samples based on a mixed gel, respectively. After 24 h, the activity for both samples remained at a level of 90% (of the original one). When samples were stored at 4 °C for 60 days, a gradual decrease in activity of up to 25% was observed for chitosan-based samples and up to 30% for samples based on mixed gel, respectively. Similar results were obtained for lipase from *Candida rugosa* [79] and proposed for further application.

Turkish scientists, Kilinc A. and co-authors [78], immobilized LPP on chitin and chitosan by adsorption followed by addition of glutaraldehyde, which was added before ("conjugation") or after ("cross-linking") the washing of unbound protein. Immobilization was carried out in phosphate buffer (pH 7.0). After adsorption, the carrier and enzyme were cross-linked with glutaraldehyde. The "conjugation" was determined to be more appropriate and effective than the "cross-linking" procedure [78]. The effect of pH on lipase activity was determined in phosphate buffer at pH 7.5–10.5. The pH optimum shifted from 8.5 to 9.0 for both types of immobilization. The activity vs. pH profile of the immobilized enzymes was significantly broader than that of the free enzyme; this is probably due to the formation of layers of the fatty acid produced during the reaction, which causes external diffusion limitations on the surface of the enzyme. The immobilization efficiency was low for chitin (23%) and high for chitosan (91%), but all immobilized enzymes showed good activity upon repeated use and high stability. The temperature dependence of the lipolytic activity was studied in the range from 25 °C to 55 °C. The optimum temperature shifted from 30 °C to 40 °C for chitin-enzyme and to 45 °C for chitosan-enzyme samples. The reaction rate for the free enzyme rises from 25 °C to 30 °C, and then rapidly decreases at higher temperatures. For immobilized enzymes, the temperature dependences were very different (the activity of free lipase at 30 °C was 60% of that at the optimum, lipase immobilized on chitin - 70%, and lipase immobilized on chitosan - 85%) [78]. However, the decrease in reaction rate at temperatures above the optimum was much slower than for free lipase. In summary, the immobilization improved the stability of lipase at increasing temperature [78]. The data is important for futher application of these preparations.

The hydrolytic activity of the lipase samples was determined by pH titration at 37 °C by measuring the rate of tributyrin hydrolysis. The immobilized lipase retained 88% and 67% of its original activity for chitin and chitosan enzyme, respectively, after repeated use at least 10 times. Chitin-enzyme retained 86.5% and chitosan-enzyme 67% of its original activity for about 45 days, whereas the free enzyme retained only 20% of its activity over the same period of time. When enzymes are used in a continuous production cycle, the stability of the enzyme is particularly important. The chitin-enzyme conjugate lost about 66% of its activity, whereas the chitosan-enzyme conjugate lost about 76% after 30 h [78]. Immobilized lipases were also suitable for catalyzing the esterification reaction of fatty acids and fatty alcohols with a middle long chain (e.g. C12). According to these results, the esterification activity of immobilized lipases was two to four times higher for the chitosan enzyme and chitin enzyme than for the free enzyme, respectively [78].

On the other hand, a fatty acid with an increased chain length can create spatial obstacles to the formation of an acyl-enzyme complex [78]. Apparently, therefore, the maximum activity of the enzyme was observed for a fatty acid with a middle length of the carbon chain. The results showed no activity in the esterification of butanol with fatty acids for all types of samples (C8-C16) that was not explained by the authors [78].

There are also several publications [100–103] concerning the immobilization of the lipases from various sources on chitosan in combination with other polysaccharides or/and inorganic materials like nanoparticles of some metal oxides that will be discussed here only briefly. For example, the studies [100–102] presented the immobilization of LPP and lipase from cattle pancreas in 0.5% gels of chitosan and sodium alginate or in microcapsules formed from chitosan-calcium-alginate shell. The kinetic and thermodynamic characteristics of various immobilized forms of pancreatic lipases were obtained. The authors [100–102] suggested that microencapsulation is the most effective way to immobilize pancreatic lipases. An interesting "stepwise" approach of the covalent binding of pancreatic lipase onto the dithiocarbamate/chitosan-based magnetite particles (abbreviation as Fe3O4@CS/NHCS-Lip) was proposed in the work [103]. The key steps in this approach are the

modification of the magnetic chitosan nanocomposite in order to produce dithiocarbamate moieties on the surface through amine functions. This approach is considered as a more promising in comparison to covalent binding by glutaraldehyde. The only problem is the 2–4 times lower hydrolytic activity of Fe3O4@CS/NHCS-Lip as compared to the free enzyme that was not solved by the authors [103].

Changes in the molecular weight of water-soluble chitosan (also using its fat−/cholesterol-binding capacities) directly influence the activity of the pancreatic lipase [104]. Another possibility to prevent the chitosan inhibition of the enzymatic action of human pancreatic lipase is the additional use of Langmuir monolayers of 1, 2-didecanoyl-glycerol [105]. Multipoint covalent immobilization of lipase on chitosan hydrogels influence the polyelectrolyte complex type and chemical modification of the catalytic properties of the biocatalysts [106]. As an example of the practical application of the lipase (immobilized at hydrophobic dodecyl-chitosan), the selective hydrolysis of some fish oil substrate by these complexes was demonstrated [20]. A significant impact of the chitosan functionalization on lipase activity, selectivity and stability was shown [20].

Thus, the following approach of lipase immobilization appears the most promising for future applications: a combination of LPP with some other enzymes immobilized simultaneously on multifunctional carriers (as nanohybrids of inorganic core and chitosan shell).

3. Comparison of the lipase immobilization on chitin/chitosan with that on some other carriers/matrices

There are numerous papers on lipase immobilization on various carriers/matrices (other than chitin or chitosan). In this chapter we will discuss only a limited number of examples of the major polysaccharide carriers and immobilization possibilities for lipases.

3.1. Cellulose and its derivatives

Cellulose or its derivatives are widely used for immobilization of various biologically active compounds (BAC) [107], but only a limited number of reports can be found for lipase immobilization (mainly concerning bacterial lipases [108–112]). One of the first and rather extensive work on immobilization of lipase from *Geotrichum candidum* on broad variety of polysaccharides and synthetic polymers, as well as on some inorganic materials, was done in the DDR [108]. There were seven polysaccharides (such as carboxymethylcellulose, p-aminobenzylcellulose, carboxymethyl-Sephadex, carboxyl-Agarose, Sepharose 4B, CH-Sepharose 4B, AN-Sepharose 4B) studied in combination with 6 methods for lipase immobilization [108]. Only a few of such combinations showed relatively high final lipase activity of 66% or 58% (carboxymethylcellulose and azid-method or p-aminobenzylcellulose and azo-method, respectively) [108]. The lipase activity for all other (>50) combinations was below 28%, with the exception of combination based on "Wofatit MC50" (modified by carbodiimide-method) [108]. The pH- and temperature optima were almost the same for lipase in the abovementioned combinations of matrices and individual lipase (8.50–8.75 and 35 °C, respectively) [108]. A small decrease was found only in the case of K_m values for the abovementioned combinations ($4.8–5.0*10^{-4}$ M/L) in comparison with individual lipase ($6.2*10^{-4}$ M/L) [108]. All these data were obtained with poorly characterized substrates such as sunflower oil, rapeseed oil and, even, rarely used linseed oil.

In contrast, recent research approaches are concentrated on native enzymes immobilized onto very special cellulose derivatives. In addition, the lipase (both, immobilized and pure) activity measurements were usually performed using classical well-characterized substrates such as I) triacetin or tributyrin (to measure the hydrolytic activity by pH-stat scheme) or II) phenylethanol interaction with vinylacetate (to measure the esterification activity by optical spectroscopy or gel chromatography). For example, pineapple leaf fibers (PALF) or microfibers (PALMF, with hydrophilic surface and diameter of about 3 μm) were used by researches from Thailand [109] to immobilize bacterial lipase from *Candida rugosa* with the advantages of easy separation and reusability. Two samples (PALMF-AY-1 and PALMF-AY-2) which differed only by the amount of the physically adsorbed lipase (of about 0.77 and 1.37 mg/g) showed high immobilization efficiency of 72.46% and 70.50%, as well as the highest relative activity of 82.62% and 72.52%. Whereas, 5 other samples showed a relative activity between 48.67% and 29.45% [109]. All samples were characterized by scanning electron microscopy and FT-IR. The hydrolytic activity of the first two samples remained at about 65%, 45% and 40% after the 2nd, 3rd and 4th recycles [109] using tributyrin substrate and pH-stat titration scheme.

These results [109] can be considered as achievements in comparison to the observed fast decrease in the lipase (from *Geotrichum candidum*) activity after the few substrate exchange cycles in the case of the abovementioned combinations (carboxymethylcellulose and p-aminobenzylcellulose) [108]: after second - only 46–48%, but after fifth - only 7–12% of the initial activity remained [108]. The authors [108] reported the complete disappearance of the lipase activity after sixth substrate exchange cycles that is critically negative, to our opinion, for biotechnological application. It is necessary to highlight the work of scientists from Thailand [109] because they used very simple immobilization steps (soaking, decantation and washing) for pineapple leaf fibers or microfibers obtained from agricultural residues that need to be recycled.

A very important point concerning efficiency and activity of the immobilization of the crude and purified lipase from *Candida rugosa* on microcrystalline cellulose was clarified in the work [110]. In both cases the lipase samples were physically absorbed onto cellulose with relatively high amount of 0.2 mg/m^2 at a pH near the isoelectric point (pH = 4.2) and 0.1 mg/m^2 at pH = 3.6 [110]. The authors explained the difference in the initial slope of the adsorption isotherms ("for purified lipase is higher than that for crude lipase" [110]) by presence of other proteins which compete in between for the cellulose surface. The lipase "specific" activity values were also the same (8–10 U/mg) for both preparations at the plateau range on adsorption isotherms that indicated the "preferential adsorption of the lipase from the crude preparation" [110]. The significant difference at low adsorbed amount (about 40 U/mg for purified lipase at 0.02 mg/m^2) the authors explained by the fact that "other components adsorb when there is not sufficient lipase available" and "with low degrees of occupancy of a sorbent, the specific activity of the enzyme is higher" [110]. The authors concluded that the obtained data established the correlations between the adsorption and "purification process" [110]. If this is really the case than it will make purification of the crude lipase "obsolete" for further immobilized enzyme applications.

Four different methods for lipase (from *Candida rugosa*) immobilization on the bacterial cellulose (BC) membranes with the use of glutaraldehyde were proposed by Wu et al. [111]. The immobilized lipase exhibited 82.6%, 87.0%, 45.7% and 93.5% lipolytic activity (by methods 1, 2, 3 and 4, respectively) in the case of olive oil emulsion as substrate [111]. The most important advantage of the preparations obtained by 4th immobilization method (BC membranes were dried at 60 °C for 20 min. Two times, before and after addition of glutaraldehyde solution, with further lipase solution application for 6 h) was their high reuse ability (76.7% and 60.0% after 6 and 15 repeated usages) [111].

In order to complete the section concerning the cellulose preparations it is necessary to consider just one example [112] of immobilized lipase with high esterification activity. This activity (of 97% at 60 °C) of the lipase (from *Candida antarctica*) was observed after its covalent binding by glutaraldehyde to cellulose functionalized with amino (APTES) groups [112] as compared to commercial "Novozyme 435" sample (of 80% at 60 °C). Interesting results of the "recyclability" of the immobilized lipase with the following conversion efficiency were obtained: 85%, 60%, 54%, 51% and 43% after 1st-5th technological cycles (as compared to 84%, 71%, 56%, 26% and 20% in the case of "Novozyme

435" sample) [112]. These results (in particular those with the commercial "Novozyme 435" sample [112]) looked strange, but the authors thoroughly characterized all samples by various physical chemical methods (HPLC, thermographic and IR analysis, X-Ray photoelectrone scpectroscopy, scanning electron and laser confocal microscopy) to prove the abovementioned data.

There is a limited number of the results available on the porcine pancreatic lipase (PPL) immobilization on cellulose or its derivatives. The study from the year 1990 [113] on the activity and thermostability of immobilized LPP can be considered here, but with definite criticism. It is interesting that the authors [113] developed and compared three immobilization techniques such as: immobilization via formation of azo-bonds between the *p*-aminophenylsulfonyl-derivatized (0.08 mmol of reactive groups per gram of wet carrier) cellulose beads and PLL (Preparation I); immobilization using cyanuric chloride-activated bead cellulose (Preparation 2); immobilization by adsorption on trityl cellulose (Preparation 3). The values of the activity of the immobilized PPL were about 82%, 38% and 17% for the Preparations 1, 2 and 3, respectively; as well as 3.28, 1.51 and 0.69 mg of protein per g of carrier were obtained. The abovementioned results were obtained carefully and reliable, but, to our opinion, not correlated with the data on specific or relative activities presented on Figs. 1 and 2 [113]. There were no digital data with experimental errors (no tables with activity data available, but just two figs. [113]). The authors consider that the samples with covalently immobilized PPL (Preparations 1 and 2) were less active (comparing the lipolysis of the 5% olive oil emulsion with 0.5% of the ethylene oxide additive "Slovasol EL" as surfactant in the sodium carbonate buffer at pH 9.0) than that adsorbed onto trityl cellulose (Preparation 3) [113]. Preparation 1 showed only about 0.5–1.0% of the relative activity (our estimates from Fig. 2 [113]), i.e. was almost inactive in the 5% olive oil emulsion (at all condition studied). To our opinion, the reported activity differences of 3–4 times between the Preparations 1 and 2 (or 3) are not realistic, especially as compared to the cited amount of the LPP on carriers (opposite dependence discussed above [113]). The authors wrote "the covalent linkage decreased the relative activity of immobilized lipase" [113]. However, why is the specific activity of the Preparation 2 roughly the same as that of Preparation 3 (Fig. 2 [113]). The authors suggested that carriers with "higher hydrophobicity" enhanced lipase activity (using olive oil as a substrate) as compared to those with "lower hydrophobicity" [113]. This correlated with the significant differences in lipase activity values presented in Fig. 3 [113] and could be considered as reliable (even no experimental errors were presented).

Our criticism is supported by other facts: the Preparations 1 and 2 were unreally thermostable with the activity about 109% and 103% after 80 min. [113], whereas the Preparation 3 or free lipase remain only about 36% or 2% of the initial 100% activity after 80 min. (our estimations from Fig. 3 [113]). The authors wrote "Lipase reaction was performed in well-shaken flasks at 28°C. After an appropriate time interval, free fatty acids were determined in 1 cm^3 samples by titration…." [113]. The activity and thermostability data obtained in the abovementioned conditions and presented in such a manner can not be considered as reliable.

In contrast, recent approaches are combining a few polysaccharides in order to obtain an advanced matrix, such as bacterial cellulose (BC) covered by sodium alginate (SA) as proposed in the study [114]. This shows a certain "advanced bridge" between the cellulose and alginates as matrices for lipase immobilization and will be discussed below.

3.2. Alginates and related polysaccharides

Two aspects are important in enzyme engineering: 1) understanding the biochemical mechanisms of enzyme structure-function relationship and 2) preparing biomaterials that will be not only highly effective, but also friendly to the natural environment. Very promising in this sense is the study [114] with bacterial cellulose prepared by cultivating *Gluconacetobacter xylinus*. A volume of 1 ml of the obtained suspension (about 80 mg dry cell weight) was mixed with 4 ml of sodium alginate (SA, 3% *w*/w). This mixture was added drop-wise into 1 L of 0.2 $BaCl_2$ at a rate of 50 μL/min. and beads with average diameters from 2.5 ± 0.1 to 3.5 ± 0.1 mm depending on the cultivation time and other conditions were obtained [114]. Lipase from *C. rugosa* was physically adsorbed onto the hydrogel beads (SA/BC), alginate-free BC beads (CEL) or alginate beads (ALG1 and ALG2) at different SA fraction without BC. For example, the maximum values of specific activity of lipase on SA/BC beads were 187%, 245% and 381% higher than that adsorbed on ALG1, ALG2, and CEL beads, respectively [114]. When the adsorption process reached equilibrium, the specific activity values of lipase on SA/BC beads were 159%, 192% and 245% higher than that adsorbed on ALG1, ALG2, and CEL beads, respectively [114]. Thus, the SA/BC surface was more favorable than that of ALG1, ALG2, and CEL beads for lipase immobilization.

The "hit" result of this work was the high biodegradability of SA/BC nanocomposite beads found: "about 85% of cellulose in these beads was hydrolyzed into glucose by cellulase from *Trichoderma reesei*" [114]. Thus, the cellulose of SA/BC beads "can be degraded by cellulase-producing bacteria, when the beads are exposed to the environment" which is very important for the "applications in environmental fields" [114].

The effect of immobilization conditions (such as alginate concentration, $CaCl_2$ concentration, ratio by weight of enzyme to alginate and bead size) on "loading efficiency" of the lipase from *Candida rugosa* (CRL) (i.e. "percent of total enzyme entrapped") and "immobilization yield" ("specific activity ratio of entrapped lipase to free lipase") for various sodium alginate (SA) beads were investigated [115]. For example, the beads (SA-CRL) were "coated with chitosan and silicate" (SA-CS-CRL) in order to prevent the enzyme leakage. The last multifunctional combination SA-CS-CRL showed the highest catalytic activity and reusability, illustrating the effectiveness of the such coating method as compared to SA-CRL beads [115].

Candida antarctica lipase B (CALB) was immobilized in sodium alginate beads (SAB-CALB) and freeze-dried SAB (fdSAB-CALB) [116]. The chiral reaction of α-phenyl ethanol (substrate) with vinyl acetate in organic phase was much more effective in the case of SAB-CALB. The conversion of the substrate ($e.e_s$) increased strongly with increasing CALB concentration (from 400 to 800 μL/mL) and became almost constant (at about 100%) when the enzyme concentration was >800 μL/mL [116]. The authors suggested that an excessive amount of CALB led to "enzyme protein unfolding" [116]. The influence of other reaction parameters, such as the substrate concentration, temperature and molar ratio of the substrate to the solvent, was investigated and discussed. Reuse experiments for the substrate (1000 μL/mL) chiral resolution ($e.e_s$) demonstrated that the activity of fdSAB-CALB was maintained at high levels (98%, 94%, 90% and 96%) in 2nd, 3rd, 4th and 5th cycles [116].

A completely different approach was presented in few papers [117,118] concerning the action of the water-soluble pancreatic lipase (PPL) on SA-oil beads [118]. So, the LPP was not immobilized, but the oil (triacylglycerols) droplets were incapsulated in: **system 1**) 2.0% (*v*/v) dispersion of microbeads (MicB); **system 2**) 2.0% (*v*/v) dispersion of macrobeads (MacB); **system 3**) an equivalent amount of non-encapsulated emulsion and MicB+MacB ("as made for the production of the alginate beads, but before addition of the alginate") or remained as initial oil emulsion (**system 4** for reference as 100% fatty acids released) [117]. The free fatty acids (FFA) released from oil incapsulated in MicB or MacB after addition of LPP were measured by pH-stat titration (as "*in vitro* digestion model") [117,118]. In the case of MacB (d = 2.4 mm) the FFA values reduced to about 12% after 120 min. (as compared to 100% fatty acids released in the **system 4**) [117,118]. The rate and extent of triacylglycerol "digestion" increased with decreasing bead size (down to 0.8 mm for MicB), decreasing calcium or alginate concentrations (i.e., "lower degree of cross-linking"), and decreasing triacylglycerol molecular weight (i.e., "tributyrin > trioleoylglycerol ≈ corn oil") [117,118]. The authors concluded that "these results have important implications for the design of delivery systems to protect and

release lipophilic bioactive components within the human body, as well as to modulate satiety/satiation by controlling the rate of lipid digestibility" [117,118].

3.3. Agarose and its derivatives

There are some recent studies [119–125] concerning the modification of agarose with octyl-glyoxyl groups and further immobilization of various lipases. To our opinion, this can be the most promising approach and will be discussed first. The technical procedures of agarose modification by octyl- and glyoxyl-groups, of lipase immobilization via interfacial activation on octyl-glyoxyl-agarose, covalent immobilization of the adsorbed lipases by reduction of the imine bounds to secondary amino ones were thoroughly discussed in the paper [119].

This group [120] recently presented two-step immobilization of lipase B from *Candida antarctica* (CALB): 1) on octyl-agarose (C8A); 2) C8A modified with polyethyleneimine (PEI). The enzyme activity was almost the same after each step and close to pure CALB, whereas the thermal stability was significantly improved in the last case [120]. It is interesting that enzyme release from the support by incubation in the non-ionic detergent Triton X-100 was drastically reduced after the PEI-coating. This may be due to "intermolecular physical crosslinking" [120]. The stability of the C8A-CALB-PEI was significantly higher than that of C8A-CALB during inactivation in mixtures of aqueous buffer and organic co-solvents (as evidenced by SDS-PAGE analysis). Thus, the authors concluded that "the modified CALB not only has a strong anion exchange nature, while maintaining the activity, but it also shows improved stability under diverse reaction conditions without affecting the reversibility of the immobilization" [120]. The same lipase (CALB) was immobilized on C8A in different ways: 3) rapidly, in 5 mM sodium phosphate (85% immobilization yield after 30 min), or 4) slowly, in the presence of 30% (v/v) ethanol (40% immobilization yield after 30 min) [121]. Both biocatalysts were treated with glutaraldehyde and showed very high stability both by thermal inactivation (at following pH: 5, 7 and 9) or by addition of 50% (v/v) dimethylsulfoxide [121]. The authors described such simple and controlled treatment of C8A-CALB with glutaraldehyde as "effective way to obtain a biocatalyst with improved activity and stability under different conditions" [121].

The same group [122] reported immobilization of lipase A from *Candida antarctica* (Novocor ADL) on octyl-agarose (C8A) beads in comparison with previously obtained lipase B from *Candida antarctica* [120,123] and "Eversa" as a promising commercial lipase from "Novozymes" (Alcobendas, Spain) [122]. All these preparations exhibited relatively high activity and stability at different reaction conditions. C8A-CALA was less stable than C8A-CALB at pH 9, but more stable at pH 5 [122]. In contrast, C8A-Eversa was much more stable than C8A-CALB at pH 9, but it was less stable at pH 5 [123].

Other steps in this direction are connected with immobilization of lipases from *Pseudomonas fluorescens* (PFL) [124–126] and *Rhizomucor miehei* (RML) [126] separately and in some combinations [126]. Layer-by-layer technique was used as novel modification in the carrier construction of the C8A-$(PFL)_x$-$(PEI)_y$ with "x,y" values in the range of 1–3 in various combinations [125]. A strong dependence between the loading amount and lipase activity was observed in all cases [125]. The main problem in this approach appeared that $(PFL)_2$-$(PEI)_2$-(PFL) carrier construction exhibited significantly lower PFL activity as compared to the (PFL)-(PEI)-(PFL) composition using any of the two substrates: pNPB or thriacetin [125]. The first problem occurred with comparing the absolute values of the lipase activity (better may be relative data given in %). A second problem (even more serious, to our opinion) appeared with discussing the obtained activity values, because these substrates (pNPB and thriacetin) were used at different conditions (25 °C in phosphate buffer and 4 °C in the presence of 30% acetonitrile, respectively) [125]. The authors suggested that these differences in methods will be helpful to overcome the "substrate diffusion limitations" [125], but the measurement procedure at 4 °C in the presence of organic solution, to our opinion, is not reasonable for the study of lipase activity.

The most promising approach of "coimmobilization" of the following three lipases (Lecitase Ultra, PFL and RML) on the same C8A matrix (beads) was presented in the fascinating work of the same group, headed by Fernandez-Lafuente [126]. In the cases of LU or RML, separate immobilization onto octyl-agarose increased the activity by a factor of 5, while in the case of PFL the increase was only around 10%. The enzymatic activity was measured by lipolysis of 50 μL of *p*-nitrophenyl butyrate (p-NPB) substrate (50 mM in acetonitrile) in mixture with 2.5 mL of 25 mM sodium phosphate at pH 7 (the product, p-nitrophenol, released during the hydrolysis of p-NPB, was measured at 348 nm). The "coimmobilization" of RML, LU, and PFL led to the higher stability at various conditions (pH, temperature, solvent, etc.), if the PFL was the first enzyme that covalently attached to the support because "RML and LU could be 'coimmobilized' with it just via interfacial activation" [126]. The authors suggested that "PFL activity after desorption of the inactivated LU or RML was maintained for 5 different inactivation, desorption, and new enzyme coimmobilization cycles, where the combi-biocatalysts are incubated at different pH values and in the presence of different organic solvents" [126]. Thus, the proposed approach ("coimmobilization" of different enzymes) allowed to prepare multifunctional materials working under a broad range of conditions [126]. The applicability of the proposed approach was additionally verified in the case of "coimmobilization" of the following enzymes: CALB, RML and Lecitase Ultra [127].

"Aminated" lipases from *Candida antarctica* (CAL), *Thermomyces lunuginosus* (TLL), and the recombinant *Geobacillus thermocatenulatus* (BTL2) were used for immobilization onto glyoxyl–agarose support (Gx) at neutral pH with anthranilic acid (AA) or DTT as additives [128]. The immobilization yields were > 70% and activities from 37.5% to 76.7% for these systems. The spectroscopic (FTIR) studies evidenced the effective adsorption of these lipases at the carriers. The authors [128] reported: "subsequent basification drastically increases the stability of BTL2–glyoxyl derivatives under harsh conditions ($t_{1/2}$, from 2.1–54.5 h at 70°C; from 10.2 h–140 h in 80% dioxane)". Further: "The novel BTL2-derivatives were active and selective in fish oil hydrolysis (1.0–1.8 μmol of polyunsaturated fatty acids (PUFAs) $min^{-1} \cdot g^{-1}$) whereas the selected TLL-derivative was as active and stable in biodiesel production (fatty ethyl esters, EE) as the commercial Novozyme®-435 after ten reaction cycles (~70% EE)" [128]. It is difficult to compare the last data (presented as absolute values [128]) with those from the previous publications of the R. Fernandez-Lafuente group (given in % [122–127]).

It is interesting that the "open" lipase structures obtained by addition of detergent (Triton X-100) was "stabilized" by the glutaraldehyde treatment [129]. The authors explained: "in the presence of detergent … a form of the lipase with the serine residue could be more exposed to the medium and much more active" [129] that is in contrast to the classical approaches of "concurrent inhibition". This strategy was proven with the use of the lipases from *Candida rugosa* (CRL) and lipase isoforms A and B from *Candida antarctica* (CALA and CALB) immobilized on monoaminoethyl-N-aminoethyl (MANAE)-agarose beads [129]. The specific values of these lipase activities were dependent on the type of the substrates used for enzymatic hydrolysis. In any case the lipase immobilized by addition of "Triton X-100″ showed an increase of the activity of 1.3 to 2.3 times (for CRL), of 1.5 to 6.1 times (for CALA) and of 2.8 to 6.4 times (for CALB) [129]. This approach is useful to improve the lipase activity "to be used in macroaqueous media" [129].

Lipase from *Candida rugosa* (CRL) was immobilized by dropwise addition of sodium alginate (SA) forming spheres of about 2 mm in diameter [130]. The authors showed [130] that spheres prepared from 1.5% SA solution with CRL had the following properties: a) the CRL release in the solution was maximal after 30 min. and stayed the same for at least 24 h; b) the pH-optimum shifted from 7.6 to 8.3; c) the temperature optimum shifted from 35 °C to 45 °C; d) activation energy of

nitrophenyl propionate (NPP) hydrolysis decreased from 40 to 21 kJ/mol, i.e. "resulting in a higher catalytic efficiency" [130]. The most important fact for practical use is that immobilized CRL was very stable after numerous cycles (during long time): after 2 (30 min.), 4 (60 min.), 6 (24 h) and 7 (7 days) cycles the remained activity was about 95%, 93%, 83% and 64%, respectively [130].

3.4. Dextran and related polysaccharides

There are no recent papers on lipase adsorption on pure dextran, probably, because it can be considered as "low active" polysaccharide and can be used as carrier mainly after derivatization with amphiphilic groups [131–134].

For example, the "potential" of O-pentynyl dextran (PyD) as carrier for immobilization of lipase from *Rhizopus arrhizus* (by adsorption method) was compared thorouhgly with ion exchange resins (Lewatit VP OC 1600, Amberlite XAD 761 and Duolite A568) in the work of Nazir et al. [131]. In the case of PyD, with a degree of substitution (DS) of 0.43, a higher lipase adsorption and a significantly higher esterification (of octanoic acid with geraniol in n-hexane) activity (249 $l^*mol^*min^{-1}*g^{-1}$) than those of the other supports (67–83 $l^*mol^*min^{-1}*g^{-1}$) were obtained [131]. Moreover, a lipase-PyD system retained almost full esterification activity for 14 weeks and about 90% yield (after eight working cycles), whereas other systems based on various resins became completely inactive within 8 weeks (storing at −10 °C) and the yield decreased rapidly after a few cycles [131].

The lipase from *Burkholderia cepacia* was immobilized on "cross-linked enzyme aggregates" (CLEAs) based on a specially prepared dextran-aldehyde derivative (100–200 KDa) [132]. For such system the hydrolysis activity (between 3186 ± 21 and 4800 ± 30 U/g-of-CLEA) and particle sizes (between 52.6 ± 18.7 and 126.2 ± 53.5 μm) were higher than those for CLEAs prepared with glutaraldehyde (activities between 894 ± 16 and 2874 ± 20 U/g-of-CLEA) and particle sizes between (21.2 ± 5.1 and 83.4 ± 24.9 μm) [132]. In contrast, the thermal stability dependences for these two systems had opposite directions. Therefore, the authors finally, nevertheless, proposed to use glutaraldehyde as cross-linking reagent (at pH 9.5, concentration of 3.5 g/l, enzyme/albumin ratio of 15) for CLEAs preparation in order to achieve more stable biocatalysts [132].

An unusual or so-called "complex" approach has been proposed [133]: 1) to make the "chemical amination" of the enzymes (the parts which are not contacting with the hydrophobic octyl-Sepharose support) and 2) to "cross-link" the system (with immobilized enzyme molecules) using aldehyde-dextran polymers (in order to increase the stability). Using such "complex" approach the lipases from *Thermomyces lanuginosus* (TLL), *Rhizomucor miehiei* (RML) and *Candida antarctica* B (CALB) were immobilized, retaining a relatively high lipase activity ("higher than 70% with regard to unmodified systems" [133]) by hydrolysis of pNPB as substrate. The thermal stability of the obtained system (fully aminated and cross-linked using aldehyde-dextran) "with immobilized CALB increased by 4.2-fold with regard to the unmodified system" [133]. The authors [133] mentioned that "the thermal stabilities of the obtained preparations (fully aminated and cross-linked using aldehyde-dextran) with immobilized RML and TLL increased by 250- and 40-fold, respectively" [133]. It is difficult to believe in such values, especially because the authors [133] did not describe the experimental details for the last two cases and did not provide the complete characterization of these systems after the treatment at 60 °C and pH 7.0 by combination of the modern methods (only SDS-PAGE analysis is insufficient to support such incredible results).

The authors of [134] described the "synthesis of new dextran—lipase conjugates for use in immobilizing low molecular weight haptens onto glass planar waveguides for immunosensor development". Here, the major attention was devoted to immunochemistry, but not to lipase activity or "engineering" [134]. Lipase was just one element for this construction, because the hydrophobic "pocket" of the bacterial lipase (*Geobacillus thermocatenulatus* lipase 2, BTL2) can strongly interact with the hydrophobized (with 1,1,1,3,3,3-hexamethyldisilazane) glass interface to obtain highly ordered and homogeneous molecular architectures as confirmed by AFM [134]. This lipase was immobilized onto agarose "macroporous" beads, followed by covalent coupling to dextran networks of variable MM from 1500 to 40,000 [134]. Later the microcystin LR (MCLR) was covalently bound to the dextran—BTL2 conjugates for determination of MCLR (in amount of a few picograms per liter) by fluorescence measurements of attached "DyLight649-labeled secondary antibody" [134]. The lower limit of detection was about 0.007 ± 0.001 ng L^{-1} and lower IC_{50} value was 4.4 ± 0.7 ng L^{-1} [134]. The authors [134] suggested that the proposed approach could be very effective "for the development of high- performance biosensing platforms".

There is a limited number of results concerning the immobilization of genetically modified enzymes (lipase or another protein) in combination with native lipase on some multifunctional carriers which we will discuss at the final part of this chapter. For example, in order to bind lipase B (from *Candida antarctica*) by "translational protein fusion" [135], functionalized polyhydroxyalkanoate (PHA) beads, recombinantly produced in *Escherichia coli*, were used. These beads were characterized with respect to protein content, functionality, long term storage capacity and re-usability. The direct fusion of the PHA synthase (PhaC) to lipase B yielded the active PHA-lipase beads, which were capable of hydrolyzing glycerol tributyrate. The authors concluded that PHA-lipase beads showed stable activity "over several weeks and re-usability without loss of function" [135].

A highly stable cellulosic nanogel (CNG) was prepared by hydrolysis of microcrystalline cellulose, onto which the native lipase and the "fusion protein of this lipase" were immobilized by covalent binding [136]. The CNG-bound lipase showed optimum activity at 55 °C and pH about 10 [136]. Furthermore, the immobilized lipases showed "two-fold increase of relative activity" in the presence of some organic solvents such as DMSO, isopropanol, isoamyl alcohol and n-butanol. The immobilized lipases retained ~50% activity after eight repetitive hydrolytic cycles, and showed a 1.24 fold increase in V_{max} and 5.25 fold increase in k_{cat} towards p-NPP hydrolysis [136]. On the other hand, the CNG-bound lipase was successfully tested to synthesize a biodiesel ester (ethyl oleate) in DMSO by varied oleic acid and ethanol concentrations. The maximum concentration of ethyl oleate (about 111.2 ± 1.24 mM) was obtained under optimized reaction conditions in organic medium [136]. These results suggest that this CNG-bound lipase can be used as an efficient tool for the "biotransformation reactions on an industrial scale" [136].

4. Conclusions and perspectives

Immobilization of particular lipases is a general tool for their application as materials for biotechnology and biomedicine due to the following properties: high stability, easy separation, reusability, shift in pH-optima and, in few cases, even higher activity than the individual enzyme. For example, samples of LPP-(porous)chitosan showed an almost five-fold increase in the hydrolytic (lipolytic) activity, while the esterification activity decreased insignificantly as compared to the reference samples [80]. The most simple and reliable method is the immobilization of LPP on chitosan particles pre-treated with ultrasound (to increase the particle surface area) and glutaraldehyde (for particle activation) [79]. The repeated addition of glutaraldehyde at a certain time after immobilization of the enzyme leads to an increase in the activity and stability of the preparations due to the binding of a larger number of lipase molecules on the surface [79].

Recent strategies of constructing supramolecular enzyme systems (SES) [6] or so-called "coimmobilized multienzymatic systems" [25] has become more and more attractive for researchers working with immobilized lipases. These strategies can be combined with the broader possibilities of the chemistry of matrices with different reactive groups

(including amino, thiol, carboxylic, hydroxyl, and epoxy groups) for enzyme immobilization on various supports (microspheres, hydrogels and polymeric membranes, nanoparticles, nanofibers, composite and hybrid or blended support materials) [1,2].

The perspective of this field can be the design of SES consisting of a few types of enzymes immobilized on multifunctional carriers. The most promising and consolidating approach, to our opinion, can be the immobilization of crude lipase preparations, containing additionally some esterases (assigned as component I) onto the fluorescent, radiolabeled or magnetic nanoparticles covered with (modified by) chitin/chitosan (assigned as component II). The idea concerning component I is supported by the publications cited above [6,76–81,108–110]. There is a very sophisticated research from China [26] which can serve as example of our suggestion concerning the components I and II, respectively. A combined assay of a mixture of native LPP, glycerol kinase (from *Cellulomonas*) and glycerol-3-phosphate oxidase (from *Aerococcus viridans*) linked by glutaraldehyde to chitosan (shell) covered nanoparticle core consisting of $Zn(NO_3)_2 4H_2O$ published recently [27] and presented the modern innovative research-production row: from simple components to advanced material and real biomedical application.

A fruitful further development in this field is protein engineering which mainly consists of the following approaches: directed evolution and rational design [45]. Despite recent advances, engineering of lipases (as for many other proteins) remains more an "art" than a technique, because many aspects are still not entirely understood [45]. To overcome this problem, some hybrid approaches can be used for lipase "improvement", in particular: "circular permutation, iterative saturation mutagenesis (ISM), combinatorial active-site saturation testing (CASTing), cassette mutagenesis, restricted libraries and structure-guided consensus" [45]. However, a crude lipase preparation is still the cheapest and reliable way for large scale production cycles. Advanced engineering of lipases is going on for the application in bionanotechnology.

Thus, the following approach of lipase immobilization appears the most promising for future applications: a few types of lipases or the combination of LPP with some other enzymes immobilized simultaneously on multifunctional carriers (as nanohybrids of inorganic core and polysaccharide shell).

Declaration of Competing Interest

The authors declare no conflict of interest regarding the publication of this paper.

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