Multi-walled carbon nanotubes as lipase carriers for organic synthesis: current trends and recent update

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Abstract

Lipase-catalyzed organic reactions have been widely practiced in the past three decades. Especially interesting are insoluble/immobilized forms due to providing a possibility of facile use and recyclability, thus reducing process costs, and making the procedure more environmentally friendly. Carbon-based supports have been extensively exploited for this purpose, because of neutral and biodegradable nature and thermal and chemical stability. Their high specific surface area, characteristic surface morphology and lower mass transfer resistances play a vital role in the performance of the attached enzyme. This review paper presents an overview of the main aspects of lipase immobilized on multi-walled carbon nanotubes (MWCNTs). Moreover, different immobilization strategies to achieve a biocatalyst with improved performances are discussed. Furthermore, as lipases are considered to have high commercial worth for synthesis of valuable organic molecules, the second part of the paper is dedicated to the overview of the most important industrial sectors in which these nanobiocatalysts have been used. In specific, applications in biodiesel production, flavour ester synthesis and racemization are summarized.

Keywords: Enzyme immobilization; biocatalysis; biodiesel; flavour esters; racemization.

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1. INTRODUCTION

Organic syntheses strongly influence the quality of our lives because they have become an essential tool for preparing numerous molecules, which, due to their wide applicability, increase the modern living standard [1]. Considering that fundamental organic synthesis mostly harms the environment, attention is drawn to using enzymes as practical alternatives to fulfill the principles of green chemistry and sustainability [2-4]. The concept of enzymes as catalysts was introduced in the 1830s, while their importance in modern organic synthesis was recognized in the second half of the 20th century when the techniques for enzyme isolation and purification were developed [5]. Enzyme-based technology has been attracting substantial interest in many scientific fields as a substitute for conventional synthetic methods due to high specific activity and selectivity [6]. However, a couple of serious drawbacks at large-scale application, limited stability under working conditions in organic synthesis, and challenged recovery from reaction media restricted miscellaneous usage of enzymes.

Different cost-reduction techniques have been appraised to make biotechnological processes more favorable and to enhance enzyme stability and utilization [7]. The most elegant solution is enzyme immobilization and transformation into a heterogeneous solid catalyst which can be readily recovered from the reaction medium and reused [8]. Immobilization also decreases the mobility of the enzyme molecule by adjusting to the solid support surface, making the conformation more rigid and the preparation of biocatalysts more resistant to elevated temperatures and organic solvents. In short, immobilization can enable applications that would not be economically viable with the use of free enzyme, and it can facilitate the use of enzymes in cost-effective continuous flow technologies [9].

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The most widely used enzymes in organic synthesis are hydrolases [10]. Amongst them, especially important are lipases, triacylglycerol acylhydrolase, EC 3.1.1.3, which can catalyze the reactions of ester synthesis, hydrolysis, inter-esterification, and trans-esterification, [11-15] as well as some unconventional interesting reactions [16]. The valuable selective properties of lipases allow them to serve as highly adaptable catalysts in industrial biotechnology, particularly in the food, chemical, and pharmaceutical industries. The first scientific research on enzyme immobilization dates back to 1916 [17], while during the 1950s and 1960s, modern enzyme immobilization methods were developed [18]. One of the most critical tasks for developing insoluble/immobilized enzymes is selecting a suitable carrier [19,20], and a variety of supports for lipase immobilization have been synthesized [21,22]. Although conventional mesoporous resins are the most frequently used [23,24], typically micron-sized, nanostructured materials have received extensive attention in recent years. Among them especially significant are carbon nanotubes (CNTs). CNTs generally provide a large surface area, low mass transfer resistance, and high enzyme loading capacity, while proper functionalization of their surface with specific groups makes it even more suitable for further lipase attachment.

The present review focuses on lipase immobilization on multi-walled carbon nanotubes MWCNTs, starting from 2010. The aim is to provide a comprehensive guide on different immobilization strategies and further application of such prepared catalysts. Special attention will be paid to applying these biocatalytic systems in typical lipase catalyzed esterification/transesterification processes: biodiesel production, flavor esters synthesis, and racemization reaction.

2. NANOSCALED MATERIALS AS SUPPORTS FOR LIPASE IMMOBILIZATION

One of the most important issues for successfully applying immobilized systems in organic syntheses is selecting a suitable material to support enzyme attachment. Clear and precise classification of materials is a very complex and complicated task because materials science has been expanding for the last 20 years. The most general division is based on chemical composition, *i.e.* materials can be organic or inorganic [25]. Additionally, different organic and inorganic materials can be combined to create novel hybrid/composite materials [26]. According to the origin, materials can be natural, artificial (man-made), or a combination. Furthermore, all mentioned types of materials can be grouped based on the particle size: bulk (size above 100 nm) and nanomaterials (at least one dimension in the range 1 to 100 nm). Based on dimensionality, shape and number of nanoscale dimensions, nanomaterials can be divided into four classes (Fig. 1).

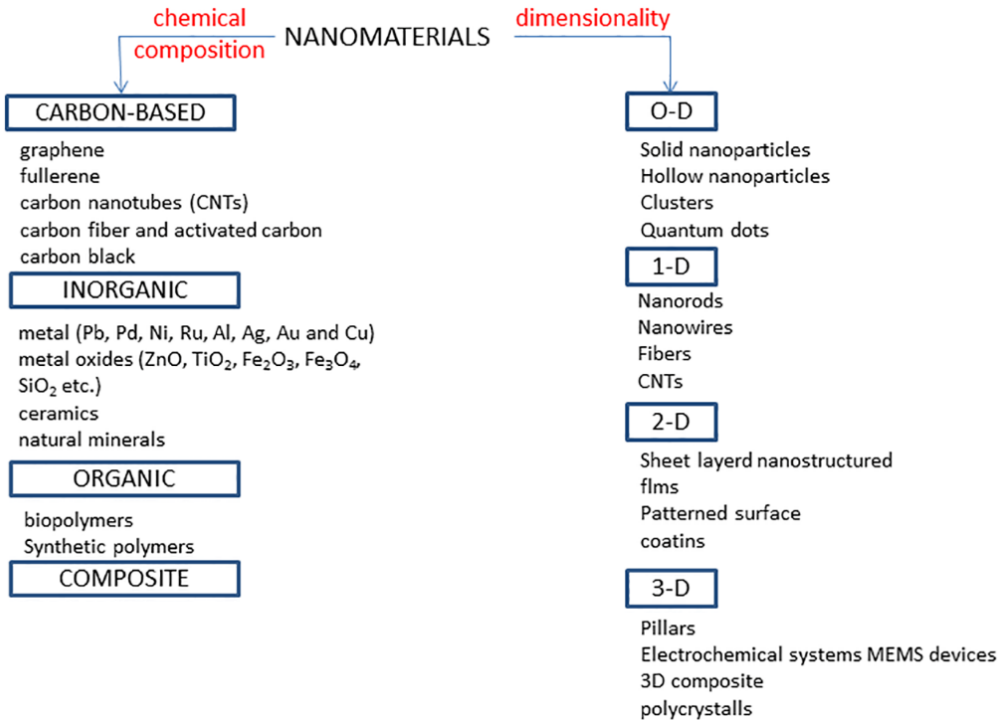

Figure 1. Classification of nanomaterials based on chemical composition and dimensionality (note that carbon materials are classified in a separate group)

Zero-dimensional (O-D) nanomaterials have all their dimensions within the nanoscale, one-dimensional (1-D) have one dimension outside the nanoscale, two-dimensional (2-D) have two dimensions outside the nanoscale and the three-dimensional (3-D) nanomaterials are composed of a multiple arrangement of nanosized crystals in different orientations [27]. Nanomaterials have proven to be ideal supports for enzyme immobilization, considering the possibility of balancing the key factors that determine the efficiency of biocatalysts. Many studies have reported that high specific surface area, characteristic surface morphology, and lower mass transfer resistances achieved by reducing the size of the enzyme immobilizing support play a vital role to achieve high enzyme loading, stabilization, and better performance of the attached enzyme [28,29]. Classification and examples of materials regarding composition and dimensionality are presented in Figure 1. It should be noted that carbon materials are classified in a separate group due to their variety of forms and distinctive properties.

2. 1. Carbon nanomaterials for lipase immobilization

Carbon is the most abundant element in the environment, and due to the possibility to exist in several allotropic modifications, carbon-based materials are considered a separate class of nanomaterials [30]. They include graphene, nanotubes (single- and multi-walled), fibers, activated carbon, and carbon black. Carbon-based supports have been extensively exploited because of neutral and biodegradable nature and thermal and chemical stability [31]. Due to the hydrophobic nature of the surface, these materials are particularly suitable for lipase immobilization. MWCNTs and graphene oxide (GO) are the most widely used, with the former being the focus of this review including modification routes for improving lipase performances.

Carbon nanotubes (CNTs), also called buckytubes, were discovered in 1991 [32]. Since then, they have been considered the most intensively studied nanostructured materials [33]. Structurally, CNTs consist of a two-dimensional hexagonal network of carbon atoms rolled up into a cylinder, with diameters typically measured in nanometers [34]. There are two types of CNTs, *i.e.* single-walled carbon nanotubes (SWCNTs) and multi-walled carbon nanotubes (MWCNTs). Due to lower costs and easier dispersibility immobilization research was almost exclusively performed on MWCNTs [35]. Structure of the MWCTs is presented in Figure 2.

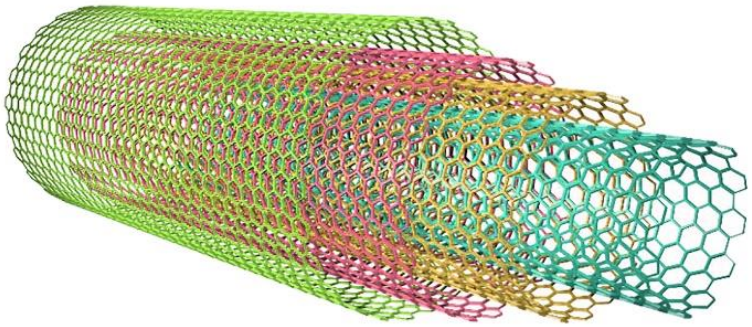

Figure 2. Schematic presentation of multi-walled carbon nanotubes (MWCNTs)

MWCNTs have one nanoscale dimension, providing a high specific area and possibility to load high amounts of the enzyme. Their length can be controlled, thus, providing the possibility for recovery by simple filtration [36]. Within various applications, due to their remarkable mechanical strength and specific surface chemistry, carbon nanotubes are recognized as unique and practical support for lipase immobilization. The success of lipase immobilization on a hydrophobic support greatly depends on the specific mobile structural domain called "lid", located over the active site. As a result, lipases show two different states in catalytic activity: closed - inactive and open - active form. Movement of the lid in the interaction with hydrophobic surfaces is a phenomenon known as "interfacial activation" and related to the increased lipase activity [37]. Hydrophobicity of the CNT surface can shift the structural balance of lipase to the open form, thus improving the catalytic activity [36]. MWCNTs became superior carbon nanomaterials for lipase immobilization due to greater hydrophobicity. At almost the same enzyme loading as on graphene oxide, MWCNT-composite showed higher activity by 1200 % [38].

2. 2. Strategies for lipase immobilization on carbon nanotubes

Summary of various lipase immobilization methods onto MWCNTs, together with the tested catalytic properties and biotechnological applications, is given in Table 1, while the immobilization strategies are illustrated in Figure 3. Immobilization on MWCNTs can be generally classified as non-covalent and covalent; regardless of the interaction type, they have a great lipase loading capacity. Direct coupling or physical adsorption on unmodified (*i.e.* raw or pristine) nanotubes is driven by hydrophobic interaction and π-π stacking. Results of a dozen of papers published since 2010 [39], have demonstrated that MWCNTs could adsorb high amounts of lipase [40-44]. Interfacial activation of lipases has led scientists to make CNTs surfaces even more hydrophobic. A heterogeneous biocatalyst was developed by adsorbing type B *Candida antarctica* lipase (CALB) on MWCNTs modified with hydrophobic polytetrafluoroethylene (PTFE), which showed high activity and stability in the synthesis of levulinate esters [45,46]. Adsorption on raw-MWCNTs can be also enhanced by the addition of surfactants such as Tween 80 and Triton X-100 [47,48]. Even though the very hydrophobic surface of the MWCNTs has a positive effect on increasing lipase activity, the major disadvantage of adsorption on the unmodified surface is the agglomeration of both the particles of the carrier and the enzyme molecules. This problem can be easily overcome by increasing hydrophilicity of the surface by functionalization [49]. Further improvement of enzyme immobilization in the sense of stabilization and better orientation of the molecule and higher dispersibility in aqueous media is induced by functionalization of the CNTs surface providing positively and/or negatively charged moieties. Among various techniques, acid treatment has been the most widely used. A carrier modified in this way (o-MWCNT) has polar carboxylic groups on its surface (negatively charged under all immobilization conditions), which, in addition to the mentioned π-π stacking, enable hydrogen bonding and electrostatic interactions with polar moieties present on the enzyme surface [35,39,40,50,51]. In addition to carboxyl, amino groups can be also introduced to the surface directly to raw-MWCNTs by using amino plasma treatment [52,53], or indirectly starting from o-MWCNTs, using bifunctional reagents [38,54-58].

Table 1. Summary of various lipase immobilization methods onto MWCNTs, together with the tested catalytic properties and biotechnological applications

Support	Lipase source	Modification reagents / surface group	Immobilization method	Application	Ref.
MWCNT	CRL	-	Hydrophobic interactions	*p*-NPP hydrolysis	[41]
MWCNT N-MWCNT Fe-MWCNT	CALB	-	Hydrophobic interactions	Baeyer-Villager oxidation	[42]
MWCNT	CRL	-	Hydrophobic interactions	Olive oil hydrolysis	[75]
MWCNT	CALB	-	Hydrophobic interactions	Diester plasticizers synthesis	[43]
MWCNT	PFL	-	Hydrophobic interactions	Commercially important esters	[76]
m-MWCNT	RML	-	Hydrophobic interactions	(OPO)-Rich human milk fat	[44]
MWCNT	CALB	→PTFE	Hydrophobic interactions	Levulinate esters synthesis	[45,46]
MWCNT	CALB	→plasma treated/-COOH → amine/-NH_2	Adsorption	Biodiesel (rapeseed oil)	[56]
MWCNT	PCL	→Tween 80	Adsorption	*p*-NPP hydrolysis	[47]
MWCNT	CRL	→Triton X-100	Adsorption	Emulsifier synthesis (methyl oleate)	[48]
MWCNT	*Pseudomonas sp.*	→acid treatment/-COOH	Adsorption	Flavor ester synthesis (ethyl butyrate)	[40]
MWCNT	CRL	→acid treatment/ -COOH	Adsorption	Emulsifier synthesis (methyl oleate)	[50]
MWCNT	CRL	→acid treatment/-COOH	Adsorption	Flavor ester synthesis (geranyl propionate	[51]

Support	Lipase source	Modification reagents / surface group	Immobilization method	Application	Ref.
MWCNT	CRL	→acid treatment/-COOH	Adsorption Covalent (coupling with EDC+NHS)	*p*-NPP hydrolysis	[66]
MWCNT	CALB	→acid treatment/-COOH →acid treatment; HMDA/-NH_2 →acid treatment; OABr and HDBr/-COOR	Adsorption	Ester synthesis (butyl caprylate)	[39]
MWCNT	CRL	→acid treatment/-COOH	Adsorption Covalent (coupling with EDC+NHS)	Oily waste water treatment	[49]
MWCNT	CALB	→acid treatment/-COOH	AdsorptionCovalent (coupling with EDC+NHS)	Ester synthesis (geranyl acetate)	[61]
MWCNT	Alkaline PFL	→acid treatment/-COOH	Adsorption Covalent (coupling with EDC+NHS)	Solketal esters	[63]
MWCNT	PFL	→acid treatment/-COOH	Covalent (coupling with EDC+NHS)	Resolution of (*RS*)-1-phenylethanol	[62]
MWCNT	Amano AK from PFL	→acid treatment/-COOH	Covalent (coupling with EDC+NHS)	Biodiesel (Jatropha seed oil)	[64]
MWCNT	YLL	→acid treatment/-COOH	Covalent (coupling with EDC+NHS)	Resolution of (*RS*)-1-phenylethanol	[65]
MWCNT	Isolated lipase	→acid treatment/-COOH	Covalent (coupling with EDC+NHS)	Resolution of (*RS*)-1-phenylethanol	[67]
MWCNT	CALB	→acid treatment/-COOH →acid treatment; APTES; succinic acid/-COOH	Covalent (coupling with EDC+NHS)	Flavor ester synthesis (pentyl valerate)	[55]
MWCNT	Amano AK from PFL	→acid treatment/-COOH →$Fe_2SO_4{\cdot}6H_2O$; H_2O_2/-OH →AZDA/-NH_2	Covalent(coupling with CDI, DVS or GA)	Solketal esters	[38]
MWCNT	Amano AK from PFL	urea/-NH_2	Covalent (crosslinking with GA)	Biodiesel (sunflower oil)	[77]
m-MWCNT	RML	→acid treatment; APTES; PAMAM/-NH_2	Covalent (crosslinking with GA)	Biodiesel (vegetable oil)	[73]
m-MWCNT	BCL	→acid treatment; $SOCl_2$; EDA; methyl acrylate; PAMAM/-NH_2	Covalent (crosslinking with GA)	Biodiesel	[74]
MWCNT	CRL	→acid treatment; PDA/-NH_2	Covalent (crosslinking with GA)	Flavor ester synthesis (ethyl butyrate and isoamyl acetate)	[54]
MWCNT	CALB	-NH_2	Covalent (crosslinking with GA)	Biodiesel (Jatropha seed oil)	[70]
MWCNT	TLL	→acid treatment; EDA/-NH_2	Covalent (crosslinking with GA)	Hydrolysis long chain ester	[71]
MWCNT	CRL, CALA, CALB	→acid treatment; HMDA/-NH_2	Covalent (crosslinking with GA)	Ester synthesis (butyl caprylate)	[72]
MWCNT	CALB	→D-glucose based IL treatment	Adsorption Covalent	Ester synthesis (n-butyl acrylate)	[69]
MWCNT	CALB, CRL, AOL	→ IL treatment	Adsorption Covalent	Baeyer-Villiger oxidation of ketones	[68]

CRL - *Candida rugosa* lipase; CALB - *Candida antarctica* lipase B; PFL - *Psudomonas fluorescens* lipase; m-MWCNT - magnetic MWCNT; RML -*Rhizomucor miehei* lipase; PCL - *Pseudomonas cepacia* lipase; BCL - *Burkholderia cepacia* lipase; YLL -*Yarrowia lipolitica* lipase; TLL - *Thermomyces lanuginosus* lipase; CALA - *Candida antarctica* lipase A;AOL - *Aspergilus oryzae* lipase PTFE - Polytetrafluoroethylene; OABr - tetra-n-Octylammonium bromide; HDBr - Hexadecyl bromide EDC - 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide; NHS - N-Hydroxysuccinimide; APTES - (3-Aminopropyl)triethoxysilane; AZDA - 10-Azidodecan-1-amine CDI - 1,1'-Carbonyldiimidazole; DVS - Divinyl sulfone; GA - Glutaraldehyde; PAMAM - Poly(amidoamine); PDA - Polydopamine; EDA - Ethylenediamine; HMDA - Hexamethylenediamine; *p*-NPP - *p*-Nitrophenyl palmitate, IL - ionic liquids

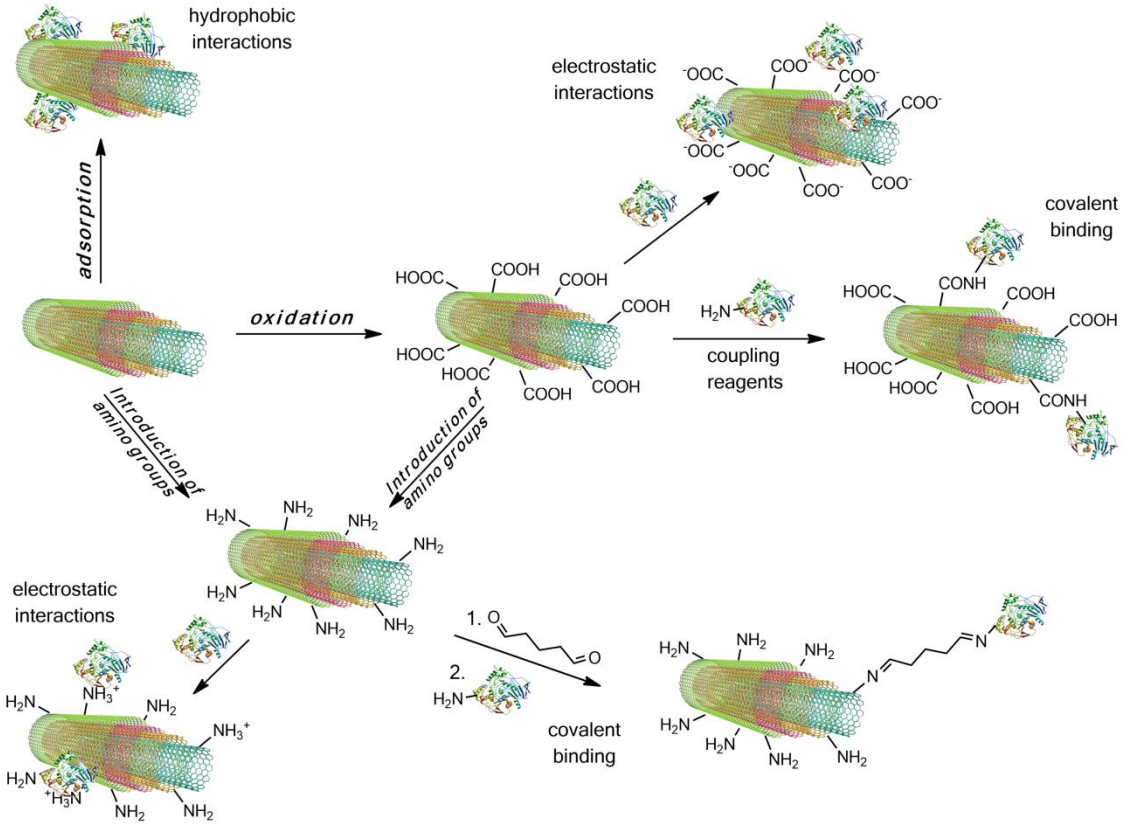

Figure 3. Lipase immobilization strategies onto MWCNTs

Modification of o-MWCNTs first implies formation of chloride (with the aid of thionyl chloride), and reaction with various diamines afterwards, or direct reaction with diamines using coupling reagents. In both ways, one amino group of diamine forms an amide bond with the carbonyl group at the MWCNTs surface, and the other remains free. Such a more hydrophilic surface with positively charged amino groups enables electrostatic interactions with oppositely charged groups from the lipase molecule surface. By a comparative study of lipase adsorption on MWCNTs functionalized with carboxyl-, amine-, and ester-terminal groups, it has been shown that a more hydrophilic surface can load a higher lipase amount, while a more hydrophobic surface provides biocatalysts with greater activity [39]. Covalent conjugation between the enzyme and support is recognized to provide durable attachment, enhanced stability, selectivity, and accessibility, and prevents enzyme leaching [59,60]. The most commonly used covalent method for immobilizing lipase is amide bond formation between carboxylic groups on MWCNTs and amino groups on the enzyme *via* carbodiimide chemistry [38,49,55,61-66]. Numerous examples can be mentioned to illustrate the advantages of this method. Lipolytic activity of MWCNT immobilized *Candida rugosa* lipase (CRL) was shown to be almost 3-fold higher under covalent promoting conditions compared to only adsorption [67]. At the same time, it was reported that covalently attached CRL retained 98 % activity of the native lipase, which is significantly higher than other usually used supports [49]. Raghavendra *et al*. used (3-aminopropyl)triethoxysilane (APTES) as a spacer arm to position lipase molecules away from the MWCNTs surface, but the biocatalyst activity was not improved [55]. Excellent results were obtained by covalently coupled Amano lipase AK on oxidized MWCNTs resulting in a biocatalyst with 100 % retained native activity [64].

As stability of the immobilized enzyme appears to be the most significant factor for industrial application, an interesting approach for lipase stabilization using ionic liquids (IL) has been developed [68,69]. The obtained biocatalysts showed exceptional properties, and the synthesis process fully corresponds to the sustainable development.

Amino functionalized surface of MWCNTs can be further activated by using glutaraldehyde (GA). To avoid cross-linking of the enzyme molecule, the nanomaterial is activated first. After that, aldehyde groups of GA can form stable covalent, imine, bond with amino groups of the enzyme. Various lipases have been attached using this approach [54,70-72]. Although biocatalysts obtained by forming a covalent bond typically express lower catalytic activity than the free lipase, their higher

stability and reusability justify the immobilization process. For example, *Thermomyces lanuginosus* lipase (TLL) immobilized by GA cross-linking exhibited significantly improved thermal stability and could be reused in up to 10 cycles for hydrolysis of long-chain esters [71]. The improved stability of lipase–MWCNTs conjugates over the free lipase is considered a consequence of a more rigid protein, resistant towards rapid unraveling for longer periods of time. Such property is useful, especially when dealing with extensive esterification process under harsh conditions.

Another interesting immobilization approach feasible for amino-functionalized MWCNTs (not depicted in Fig. 3), which significantly increases surface, represents dendrimer formation using a nanoscaled hyperbranched polymer as poly(amidoamine) (PAMAM). *Rhizomucor miehei* (RML) and *Burkholderia cepacia* lipases (BCL) immobilized on magnetic-MWCNT-PAMAM nanocomposites exhibited good stability and recycling performance [73,74].

Influence of different functionalized MWCNTs, namely alkylamino-, hydroxyl- and carboxyl- MWCNTs, was explored regarding the biocatalytic performance [38]. Nanobiocatalysts obtained by covalent immobilization of alkaline lipase from *P. fluoerscens* on MWCNT-NH_2 have been the most active (12 times higher than the native lipase), while MWCNT-COOH based bioconjugates emerged as the most enantioselective. Such results corroborate the finding that different properties of biocatalysts can be achieved by controlling the immobilization conditions, especially surface groups involved in the immobilization process.

3. APPLICATIONS

Lipases primarily catalyze hydrolysis of triacylglycerols to free fatty acids, di- and monoacylglycerols and glycerol. Still, their versatile nature enables them to catalyze hydrolysis of various other esters, as well as the formation of ester bonds between acids and alcohols (Fig. 4). Lipases occupy third place in the total world enzyme production, just behind proteases and enzymes that catalyze the hydrolysis of carbohydrates, while they are the most widely exploited enzymes in organic synthesis [78].

Transesterification *via* lipase use is referred to as a ping-pong bi-bi mechanism, where the lipase enzyme reacts with two substrates, triglyceride and the enzyme-substrate intermediate resulting in the formation of two products. The lipase active site has two functional groups vital for the catalytic activity: the hydroxyl group of serine that performs a nucleophilic addition forming an enzyme-substrate complex, and the second is a nitrogen atom on histidine acting as a proton acceptor [79].

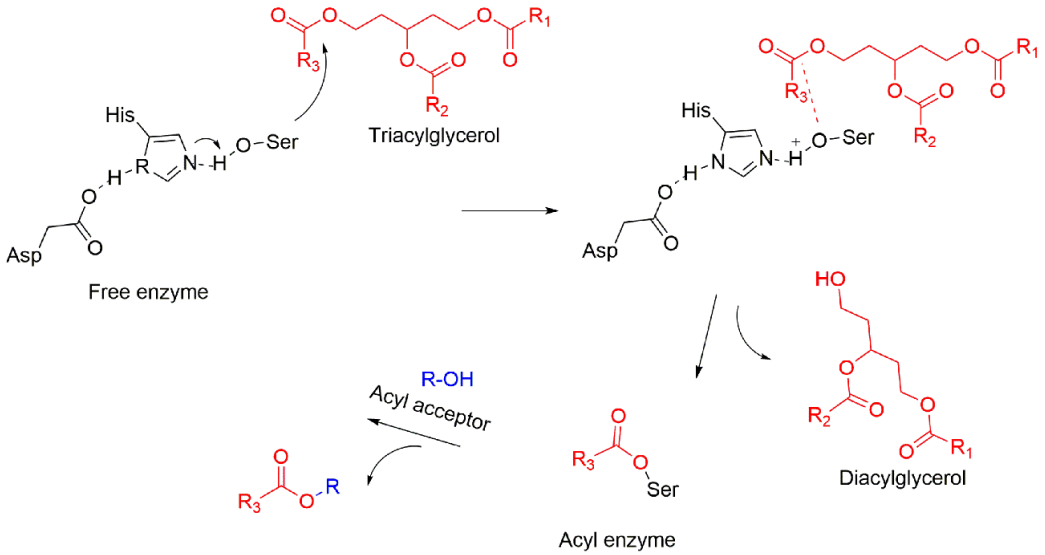

Figure 4. Schematic Illustration of lipase catalyzed transesterification reaction

An amphiphilic polypeptide loop covers the active center (in most lipases), the lid. In contact with hydrophobic substrates such as oil droplets, the lid acquires the open conformation for the active center and creates a large hydrophobic pocket [80]. In aqueous media, lipases in the open form can create dimeric and multimeric aggregates, which can lead to the lower lipase activity [81]. This can be overcome by enzyme immobilization [82] providing also

lower costs by possibilities of reuse [83] as the high cost of lipases has slowed down its commercialization for the production of bulk chemicals. Synthesis of biodiesel and other bulk chemicals is generally carried out in a non-aqueous or organic environment; hence, many researchers focus on obtaining novel lipases with enhanced activity and stability in organic solvents. High activity and stability of CALB in the presence of alcohols have drawn attention to this enzyme as a biocatalyst for biodiesel production [84]. However, some challenges need to be resolved. The immobilized lipase on a hydrophilic carrier may show low activity in biodiesel production because of the low accessibility of oil as the hydrophobic substrate. Also, the hydrophilic matrix has a higher affinity for methanol than oil, and methanol may easily deactivate the immobilized lipase. On the other hand, immobilization of lipase on the hydrophobic surface induce conformational changes of protein which result in increased stability [85].It is required to improve the existing methods and to explore new techniques to make immobilized lipase useful for industrial applications. Many papers deal with advanced techniques of enzyme immobilization and their application to obtain commercially valuable compounds [86].

3. 1. Lipase immobilized on MWCNTs for biodiesel production

Biodiesel (fatty acid alkyl esters, FAAE) is one of the best alternatives to fossil fuels. It has similar physical and chemical properties to petroleum diesel but it is renewable and sustainable with lower carbon dioxide, sulfur, and particulate emissions [82]. Biodiesel can be derived *via* transesterification of vegetable oil, animal fats, or microbial oil with alcohol by either chemical or enzymatic catalysis [87].

Most of the scientific studies and patents filed on biodiesel production have focused on improving the catalyst technology for efficient conversion. Other biodiesel research areas predominantly deal with feedstock, reactor configuration, byproduct recovery, and combustion performance [89]. Generally, biodiesel is produced *via* the transesterification/esterification reaction of acylglycerols and free fatty acids into fatty acid alkyl esters using short-chain alcohols (methanol) (Fig. 4). The acyl migration with short-chain alcohols is a time-consuming process, and, therefore, catalysts or pre-conditions (super/subcritical) are required for quicker alkyl ester formation. The formed FAAE (biodiesel) has a lower viscosity and pour point than its precursor oil/fat [89].

Chemical catalysts, consisting mainly of acids (H_2SO_4, BF_3, H_3PO_4) and bases (KOH, NaOH), are industrially used in biodiesel production. High temperatures are required, and wastewater is generated in large volumes. Using chemical catalysts also produces metal ions and salts that present a disposal challenge [88]. To reduce energy consumption and the amount of wastewater generated and to avoid producing inefficient end products, classes of enzymes, especially lipases, are being successfully explored as substitutes for chemical catalysts [89]. Compared with chemical catalysis, biodiesel production by lipase displays numerous advantages, such as easy product separation, minimal wastewater treatment requirements, easy glycerol recovery, and the absence of side reactions [90]. Recently, acidic, alkaline, and immobilized lipase catalysts were compared regarding efficiency in converting waste cooking oil to biodiesel. Alkaline and lipase catalysts showed >85 % biodiesel conversion in <120 min. Unlike the alkaline catalyst, the immobilized TLL used for the study was recycled three times [91].

As described previously, enzymes can be immobilized on the surface of MWCNTs by adsorption or covalent binding, resulting in improved catalytic performance and stability and showing great potential in biodiesel synthesis. A biocatalytic Pickering emulsion using MWCNT-immobilized CALB (CALB@PE) was developed to produce biodiesel, with *Jatrophacurcas L*. seed oil and methanol as substrates. A yield of 95.2 %, consistent with the predicted 95.5 %, was obtained. CALB@PE could be reused up to 10 times without substantial activity loss; it also exhibited better reusability than the commercial Novozym 435 in biodiesel production [70].

A nanoscale carrier was created as a support for CALB immobilization, and the catalytic performance was evaluated in methanolysis of rapeseed oil using *terc*-butanol as the reaction solvent. The carrier was plasma-treated MWCNTs (MWCNT-COOH), while further functionalization of the obtained oxidized MWCNTs was performed by using butylamine (BA) and octadecylamine (OA). The yield of biodiesel for the two of these three supports, namely, MWCNT-COOH and MWCNT-BA was similar at about 92 %, while 86 % was the yield for the reaction catalyzed by the lipase immobilized on MWCNT-OA [56].

The small size of carbon nanotubes, one of its best properties, is also one of its disadvantages because the recovery after the end of the reaction is challenging. A solution for this problem could be combination of carbon nanotubes with magnetic nanoparticles, or functionalization with other molecules [92]. Magnetic multiwalled carbon nanotubes (m-MWCNT) were used to immobilize RML. The obtained esterification activity was 27-fold higher than that of the free lipase. The immobilized lipase was employed as a biocatalyst in biodiesel production from waste cooking oil in a *tert*-butanol solvent system yielding high biodiesel conversion of 94 % under optimal conditions. Additionally, the immobilized lipase could be recovered easily without showing a significant decrease in conversion rates after 10 cycles of reuse [73]. Same authors made superparamagnetic MWCNTs by filling the nanotubes with iron oxide and further modified them by linking polyamidoamine dendrimers (m-MWCNTs-PAMAM) on the surface. BCL was successfully immobilized on the obtained carrier *via* a covalent method. The maximal activity of the immobilized lipase was 17-fold higher than that of the free enzyme. The immobilized lipase displayed significantly enhanced thermostability and pH-resistance and could efficiently catalyze transesterification to produce biodiesel at a conversion rate of ~93 % [74].

Deep *et al*. demonstrated preparation of a lipase-conjugated MWCNT catalyst *via* a chemical reaction. They examined the catalyst efficiency for biodiesel production *via* methanolysis of crude Jatropha oil and compared it with that of free lipase. The results revealed that the production of fatty acid methyl esters (FAME) (1 h reaction time) increased linearly with increasing the fraction of the catalyst support. The maximum yield of the desired product was achieved in the presence of 15 % of the immobilized catalyst (calculated as the mass of catalyst in the total reaction mixture). Moreover, it was also reported that the developed carbon-based nanocatalysts could be reused up to 10 times without any loss of enzyme activity. A maximum 90 % FAME yield was recorded for lipase-conjugated MWCNT catalyst, compared to only 10-15 % obtained with the free lipase [64].

3. 2. Flavor esters

Although several flavors and fragrances are obtained either by chemical synthesis or by extraction from plants, applying biocatalysts for the sake of a safe and productive pathway by more sustainable chemical processes is the main alternative. Optimization of variables that influence this lipase-catalyzed synthesis, such as enzyme formulations, solvent-free media, and acetylating agents, is crucial to achieving higher conversions[93].

Lipase from *C. rugosa* was immobilized by surface modified MWCNTs and used to synthesize ethyl butyrate and isoamyl acetate, which have characteristic pineapple and banana flavors. Magnetic MWCNTs were produced by incorporating cobalt and functionalized with aminated polydopamine. The immobilized CRL retained around 84 % of its initial hydrolytic activity and showed high-yields in enzymatic synthesis of ethyl butyrate and isoamyl acetate, amounting to 78 and 75 % ester yield, respectively [54].

Compared with geraniol, geranyl esters are preferred for their lower toxicity and higher bioactivity in food, pharmaceutical, cosmetic, and other fields. Enzymatic synthesis of geranyl esters is the most effective, especially with lipase as a catalyst [94]. Geranyl propionate was enzymatically synthesized from geraniol and propionic acid using CRL immobilized on acid-functionalized MWCNTs [51]. Bourkaib*et al*. combined advantages of MWCNTs as a support and supercritical CO_2 as a solvent for the synthesis of geranyl acetate catalyzed by CALB [61].

CALB-MWCNT conjugates were shown to be highly efficient in synthesis of pentyl valerate under non-aqueous conditions as reflected by the esterification yields, storage stabilities and storage stabilities and reusability studies [55].

Alkyl esters of levulinic acid are important flavors and fragrances in pharmaceutical, cosmetic and food industries. Enzymatically, they are usually prepared in the conventional esterification reaction as demonstrated using CALB immobilized on commercially available MWCNTs and polytetrafluoroethylene (PTFE) - CALB/MWCNT-PTFE as a biocatalyst. n-Butyl levulinate has been synthesized in a high yield (99 %) and with high selectivity (>99 %). The catalyst used retained its activity and stability after sixth reaction cycles, with 69 % yield of ester[45]. However, as lipase can catalyze ester hydrolysis at the same time, to overcome limitations regarding equilibrium, lactones can be used as alternative substrates. Szelwicka et al. used the same CALB/MWCNT-PTFE conjugate as a biocatalyst and α-angelica lactone as a substrate and obtained quantitative yields of n-butyl levulinate. The developed biocatalyst was stable in the six consecutive reaction cycles [46].

3. 3. Racemization

One of the most complex and challenging problems in the field of synthesis of valuable chemicals is production of optically active compounds [95]. Due to the possibility of functional conformation lipases are capable for the resolution of racemic mixtures of optically active compounds. As the enantiomers of 1-phenylethanol represent valuable intermediates in several industries, the lipase catalyzed resolution of (*R*,*S*)-1-phenylethanol is a relevant research topic.

Pseudomonas fluorescens lipase (PFL) was covalently immobilized on oxidized MWCNTs and applied in the kinetic resolution of racemic 1-phenyl ethanol with good enantioselectivity and recyclability (8 cycles) [62].

To produce effective and recyclable catalysts for enantioselective transesterification of three vinyl esters (acyl donors) by racemic Solketal esters, PFL was non-covalently immobilized on raw and oxidized MWCNTs (MWCNTs and o-MWCNTs, respectively). Depending on the structure of the acyl donor, MWCNTs were found to be 2.2- to 4-fold more active than their oxidized counterparts (and up to 9 times more activating than the native enzyme), whereas enantioselectivity was higher for the composites based on o-MWCNTs [63]. The same research group chemically synthesized different functionalized MWCNTs for the same purpose. The studies revealed that different chemical functionalization of morphologically identical nanotube supports led to various enzyme loadings, catalytic activities, and enantioselectivities [38].

3. 4. Miscellaneous applications

Progressively demanding regulations of industrial processes concerning safety and waste disposal have compelled modification of several chemical processes. A new method for the chemo-enzymatic Baeyer–Villiger oxidation of cyclic ketones to lactones in the presence of a new heterogeneous nanobiocatalyst consisting of CALB immobilized on MWCNTs has been developed. Activities of the obtained biocatalysts were compared with the commercially available Novozyme-435. Recycling studies demonstrated the possibility of utilizing the most active MWCNTs-lipase biocatalyst five times without any significant activity loss [42]. The same nanobiocatalyst was used to synthesize diester plasticizers based on aliphatic diesters (dicarboxylates). The superior activity of the CALB immobilized on MWCNTs was obtained resulting in a significant reduction in reaction times compared to those reported in the literature [43].

Wastewater generated from various industries (food, paper, textile, oleochemical, pharmaceutical) contain high concentrations of oil and grease difficult to treat by using inadequate and costly conventional chemical and/or biological methods. Therefore, considering the primary function of lipase to catalyze hydrolysis of fat and oil it is not surprising that they represent an appropriate "green" choice for lipid-rich wastewater treatment. Lipase can degrade complex oily materials through hydrolysis to free fatty acids that are easier to extract. There are a number of studies about treatment of oily wastewater using lipases[49,96-101]. However, most of them are directed towards exploiting native enzyme and synthetically prepared/model wastewater. Only few studies used the immobilized lipase and/or real industrial water, among which application of CRL immobilized on MWCNTs was reported [49]. High thermal and operational stability of lipase immobilized on oxidized MWCNTs in the presence of EDC and NHS as cross-linkers demonstrated the potential for improved resistance to severe conditions in industrial applications [49]. In another study it was shown that immobilization of CRL on MWCNTs significantly improved its activity in olive oil hydrolysis reactions. MWCNTs affected diffusion and distribution of the substrate and products in the lipase reaction environment [75].

Production of methyl oleate, an important ester used in detergents, emulsifiers, wetting agents, and intermediate stabilizers was optimized by using the adsorbed CRL onto raw-MWCNT by dint of Triton X-100 and o-MWCNT. The obtained biocatalysts produced around 80 % of methyl oleate. o-MWCNT-CRL could be recycled up to 5 times, retaining 50 % of its activity [48,50].

Lipases have the capability to catalyze unconventional reactions as aldol condensation, Hantzsch, Canizzaro, Morita-Baylis-Hillman, Knoevenagel, UGI and Mannich reaction, Michael addition, perhydrolysis, oxidation and some cascade reactions [16,78]. It was shown that PPL immobilized on magnetic-MWCNT can catalyze a three-component reaction to obtain 2H-chromenes at high yields (95 %) [102], while Szelwicka et al. used biocatalyst designed in the presence of supported ionic liquid-like phase for chemo-enzymatic Baeyer-Villiger oxidation of cyclic ketones. The obtained

biocatalysts showed high conversion of substrate (92 %) in the model oxidation of 2-adamantanone, under favorable conditions [68].

4. CONCLUSION

This review aims to summarize and point out the most important advances in the lipase-catalyzed synthesis of biotechnologically important compounds, with a particular focus on MWCNTs-immobilized biocatalysts. According to the data presented in this review, it is clear that the development of processes catalyzed by immobilized lipase represents a focus for many researchers due to the high use of these reactions in important industries. Esterification is a well-established reaction type in organic synthesis used for manufacturing various valuable esters used prevalently as food ingredients, in skincare products, pharmaceuticals, and fuels. The fact that enzymatically synthesized products gain label of natural products makes this technology even more market appealing. Additionally, a lipase-immobilized process offers significant advantages, including higher chemical and thermal stabilities than the free form of lipase as well as easy recovery and reuse. However, many challenges need to be overcome. In the future, developing additional viable and cost-effective methods is required. This can be achievable by enhancing capabilities for biocatalyst recycling, minimizing lipase production costs using molecular biology and genetic engineering techniques, or by designing supports from inexpensive and readily available waste biomass (for example, MWCNT from cotton or corncobs).

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Višeslojne ugljenične nanocevi kao nosač lipaza za organsku sintezu: pregled najnovijih trendova

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Izvod

Lipaze su poslednjih decenija široko rasprostanjeni katalizatoriu raznovrsnim organskim reakcijama. Posebno su interesantne u imobilisanom/nerastvornom obliku jer je na ovaj način olakšana njihova upotreba uz mogućnost recikliranja i ponovne upotrebe čime se smanjuju troškovi samog procesa i postupak je ekološki prihvatljiviji. Kao nosači za vezivanje nanomaterijali na bazi ugljenika, posebno ugljenične nanocevi, su našli primenu zbog svojih izuzetnih fizičkih, mehaničkih i hemijskih svojstava. Njihova velika specifična površina, karakteristična površinska morfologija i smanjen otpor prenosu mase igraju vitalnu ulogu u performansama vezanog enzima. Ovaj pregledni rad predstavlja prikaz glavnih aspekata lipaze imobilisane na višeslojne ugljenične nanocevi i različitih strategija imobilizacije za dobijanje biokatalizatora sa poboljšanim svojstvima. Takođe, kako su lipaze enzimi od velikog komercijalnog značaja za organsku sintezu i primenu u biotehnologiji, drugi deo rada posvećen je pregledu najvažnijih industrijskih sektora u kojima su ovi nanobiokatalizatori našli primenu. Shodno tome, dat je pregled proizvodnje biodizela, mirisnih estara i racemizacije.

Ključne reči: Imobilizacija enzima; biokataliza; biodizel; mirisni estri; racemizacija