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REVIEW PAPER

The Enzyme Immobilization: Carriers and Immobilization methods

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Abstract

Strategies based on the enzyme application are increasingly replacing the conventional chemical procedures because of their efficiency, quicker performance and environmental protection. However, natural enzymes can rarely be used in industry since their beneficial features cannot endure the industrial conditions. Additional drawbacks of natural enzymes are their inhibition by reaction products and difficulty to be removed from the reaction mixture. The most promising technique to substantially improve the enzyme properties, such as activity, pH, thermal and organic-solvent stability, reusability and storage stability, in non-natural environments is by the enzyme immobilization. In this review different techniques used to immobilize enzymes to inert carriers were summarized. Different materials of both the organic and inorganic origin were used as carriers for the enzyme immobilization. A class of new materials where the enzyme performance was enhanced by combining different classical materials and shaping in specific forms was also summarized.

Keywords: enzymes, immobilization, macroporous, carriers, hydrogels.

1. INTRODUCTION

Enzymes are biocatalysts that are applied in diverse fields owing to their great features such as activity, selectivity, specificity and simplicity of production. They allow for the preparation of a wide range of products under mild and environmentally friendly conditions (Faber et al. 2011; Kobayashi, Uyama, & Kimura 2001). The enzymes reduce the number of reaction steps and required quantities of hazardous solvents, and thus, make a process not only environmentally-safe but also more inexpensive (Cowan & Fernandez-Lafuente 2011). Therefore, these biomolecules have become of great interest in many practical applications ranging from food to pharmaceutical industry (Wohlgemuth 2010).

However, a wide industrial application of enzymes is hampered by their structural instability, high production and separation costs. The enzymes lose their beneficial characteristics when used in combination with organic solvents. Also, they are not able to withstand some industrial conditions such as high temperatures, mechanical shear etc. The enzyme activity is not sustainable. After a while, the enzymes lose their activity and therefore, have to be replaced with the fresh ones. The enzyme recovery from reaction solutions, separation from the final product and replacement is laborious. In order to overcome these issues and allow for the wide industrial application, the enzymes are immobilized on various carriers. The immobilized enzymes are defined as "Enzymes that are attached to specific solid supports and thus confined, and which can be used repeatedly and continuously while maintaining their catalytic activities" (Katchalski-Katzir 1993).

Unlike native enzymes, the immobilized enzymes are reusable over successive catalytic cycles (Zhang, Ge, & Liu 2015). The immobilized enzymes have increased activity in organic solvents (Miletić, Nastasović, & Loos 2012; R. M. Prodanović et al. 2006; Prokopijevic et al. 2014). Moreover, they are more stable under various reaction conditions such as increased temperatures. The immobilized enzymes can easily be separated from the product,

which results in products of higher purity. (Marzadori, Miletti, Gessa, & Ciurli 1998; Mateo, Grazú, et al. 2007). Also, they showed remarkable long-term stability and increased enantioselectivity. The reaction can be easily controlled by removing the immobilized enzyme from the reaction mixture. The use of immobilized enzyme allows for the development of a multi-enzyme reaction system. The aforementioned benefits of immobilization make an enzyme-catalyzed process reliable, efficient and economical.

This review considers the properties of immobilized enzymes, which are determined by the properties of both the enzyme and the carrier. The existing methods used for immobilization were summarized, as well as their recent developments. Materials used as carriers and a new class of advanced materials used in the enzyme immobilization were reviewed.

2. PROPERTIES OF IMMOBILIZED ENZYMES

Immobilized enzymes have specific chemical, biochemical, mechanical and kinetic properties. These properties are determined by the properties of both the enzyme and the carrier, as well as by the interaction between the enzyme and the carrier.

The molecular weight of the enzyme, its functional groups and purity are of great importance for the immobilization process and properties of the enzyme-carrier system. Due to the steric effect, the enzymes with high molecular weights and large molecules attach to the carriers to a lesser extent. The functional groups on the enzyme surface determine the type of interactions between the enzyme and the carrier. Impurities, present in the enzyme, can interrupt the enzyme attachment to the carrier surface.

The properties of the immobilized-enzyme systems are also determined by the characteristics of the carrier. Its chemical structure provides information about attachment of the enzyme to the carrier surface. When highly porous materials are used as carriers, pore size and pore size distribution are of great importance for the properties of immobilized enzymes. The presence of small pores in the carrier can cause diffusion limitations. As a consequence, the structure of the enzyme is rearranged and the enzyme loses its activity. On the other hand, too large pores cause clustering of enzyme molecules and thus, their inactivity. Size of the enzyme carrier determines the enzyme distribution on the carrier and therefore, properties of the immobilized enzyme. B. Chen, Miller, Miller, Maikner, and Gross (2007) immobilized CALB on methyl methacrylate resins with the same pore diameter and surface area, but different particle size (35 to 560–710 μm). They discovered that large carrier particles (560–710 and 120 μm) had a non-uniform distribution of enzyme with the most of enzyme on the outer region of particles, whereas lowering in the particle size resulted in increasing uniformity of the enzyme distribution through the resin (B. Chen et al. 2007).

Depending on the application, carriers for the enzyme immobilization should have certain mechanical properties. When applied in a stirred tank, the carrier must be resistant to abrasion. The immobilized enzymes used in columns require carriers resistant to flow.

3. IMMOBILIZATION TECHNIQUES

Several methods were used to immobilize an enzyme on a solid surface. They can be divided into four different groups: 1. Adsorption, 2. Covalent bonding, 3. Entrapment, 4. Cross-linking.

3.1. Adsorption

Immobilization by adsorption is commonly divided into 3 subcategories: 1. Physical adsorption, 2. Electrostatic binding, 3. Hydrophobic adsorption.

3.1.1. *Physical adsorption*

Physical adsorption is a simple, low-cost and straightforward technique for enzyme immobilization. An enzyme is bound to a carrier by Van der Waals forces, hydrogen bonding and hydrophobic interactions. The immobilization procedure is quite simple. A carrier is immersed into an enzyme solution and stirred for a certain period of time. The unattached enzyme is removed by rinsing the carrier with a buffer. This process does not require functionalization of the carrier surface nor use of destructive chemicals. Therefore, the activity of enzymes is maintained. Physical adsorption is reversible which allows for reuse of the carrier. Inactive enzymes can easily be removed by changing temperature, pH or ionic strength and replaced with fresh one. However, immobilization by physical adsorption is not suitable for industrial application since Van der Waals forces, hydrogen bonding and hydrophobic interactions are relatively week and can be broken by industrial conditions (a high reactant or product concentration, high ionic strength) or changes in pH, temperature or solvent polarity (Miletić et al. 2012; Mohamad, Marzuki, Buang, Huyop, & Wahab 2015). Therefore, the leakage of enzyme can occur, not all enzyme can be reused and the product can be contaminated with enzyme which would require purification.

Physical adsorption was used in the immobilization of lipases. When *Yarrowia lipolytica* lipase was adsorbed on octyl-agarose and octadecyl-sepabeads, higher

yields and stability greater than that of native lipase were obtained (Cunha et al. 2008). A high activity of the lipases immobilized on octadecyl-sepabeads was caused by the hyper-activation of the enzyme due to interfacial adsorption to hydrophobic surfaces (Mateo, Grazú, et al. 2007; Mateo, Palomo, Fernandez-Lorente, Guisan, & Fernandez-Lafuente 2007). *Candida rugosa* lipase immobilized by physical adsorption on poly(3-hydroxybutyrate-co-hydroxyvalerate) had 94% of maximum activity after 4 h at 50∘C and reusability till 12 cycles (Cabrera-Padilla et al. 2012). Temperature and pH have a great influence on the activity of physically adsorbed lipases. *Candida rugosa* lipase, immobilized on poly(N-methylolacrylamide) showed the highest activity at low temperatures and in alkaline environment (pH values of 7.5-8.0, whereas 90% of the highest activity retained at pH 8.5) (Santos, Nunes, Moreira, Perez, & de Castro 2007). The activity of *Candida antarctica* lipase B immobilized on polystyrene nanoparticles was much higher than that of the crude enzyme (Miletić, Abetz, Ebert, & Loos 2010).

3.1.2. *Electrostatic binding*

Enzymes can be attached onto the oppositely charged surface by electrostatic forces (ionic or polar interactions). Charge of the enzyme surface depends on the comparative difference between the isoelectric point of the enzyme and the pH value of the solution (Nguyen & Kim 2017). There are two electrostatic adsorption immobilization techniques: layer-by-layer deposition and electrochemical doping.

Layer-by-layer deposition is a simple and highly biocompatible route to immobilize enzymes. This thin-film fabrication technique consists of dipping a cationic/anionic charged substrate into an aqueous solution of oppositely charged polyelectrolyte. After washing, the coated substrate was immersed into a solution of the cationic/anionic enzyme. This procedure was repeated until the desired number of layers was obtained. A multilayer system, composed of oppositely charged layers, is formed due to electrostatic interactions, hydrogen bonding, coordination bonding, charge transfer, molecular recognition, hydrophobic interactions or a combination of these. This immobilization method requires mild conditions and it is a low-cost method. The layer thickness and structure can be easily controlled and modified. This system minimizes the enzyme denaturation. However, overcharging of surface, substrate or product may cause kinetics distortion due to partitioning or diffusion phenomena and consequently, may change the pH stability of the enzyme (Nguyen & Kim 2017).

Electrochemical doping refers to immobilization of an enzyme to a conductive polymer. This immobilization route requires oxidation/reduction of the polymer where the polymer carrier becomes charged and thus, the oppositely charged enzyme can interact with the conductive polymer. This system, where galactose was immobilized by electrochemical dipping, was used for development of biosensors for galactose monitoring (Shaolin 1994).

3.1.3. *Hydrophobic adsorption*

Hydrophobic adsorption allows for the enzyme immobilization through hydrophobic interactions between the enzyme and the carrier. The strength of these interactions depends on the hydrophobicity of both the carrier and the enzyme. Therefore, parameters that influence the hydrophobicity (pH, temperature, concentrations of salt etc.) also determine the interaction strength (Porath 1987; Sheldon 2007). β-amylase and amyloglucosidase were successfully immobilized by hydrophobic adsorption onto hexyl-agarose carriers (Caldwell, Axén, Bergwall, & Porath 1976; Caldwell, Axén, & Porath 1976).

3.2. Covalent bonding

Covalent bonding is the most common immobilization technique. In this method, the enzyme is immobilized onto a carrier through formation of a covalent bond between the carrier and the enzyme. Functional groups of the enzyme, which participate in bond formation, are amino groups, carboxylic groups, phenolic groups, sulfhydryl groups, thiol groups, imidazole groups, indole groups and hydroxyl groups (Novick & Rozzell 2005). The immobilization can occur through covalent attachment via long spacer arms or multipoint attachment. The first provides a moderate restrict of the enzyme configuration whereas the second, supposedly, higher stability.

Lysine is a quite common amino acid present in enzymes, which contains the ϵ-amino group. This group is suitable for bonding in the covalent immobilization since it is relatively reactive, positioned on the enzyme surface and it provides good bond stability (Křenková & Foret 2004; Miletić et al. 2012). On the other hand, the carriers with the epoxy groups are most commonly used in combination with this type of enzymes, since the epoxy groups can react with the amino groups under mild conditions and form a stable bond (Miletić et al. 2012).

Enzyme immobilization by covalent bonding is a two-step procedure. Prior to enzyme covalent attachment, the surface of the carrier is activated using linker molecules (glutaraldehyde or carbodiimide). These molecules act as a bridge between the surface and the enzyme. The type of the linker is determined by the type of the carrier

(carriers of inorganic materials, biopolymers or synthetic polymers) and immobilization protocols (Nguyen & Kim 2017).

The advantage of immobilization by covalent bonding is formation of a strong linkage between the carrier and the enzyme, which prevents the enzyme leakage. Moreover, this method provides increased enzyme stability and improved stereospecificity. E.g. *Pseudomonas fluorescens* lipase showed a significant 3-fold improvement in the enantioselectivity when immobilized on glyoxyl-agarose by multipoint covalent immobilization. The authors assumed that this was caused by distortion of the enzyme structure, since the catalytic activity was decreased, or/and by an increase in the rigidity of the enzyme, since the enzyme stability was improved (Fernández-Lorente et al. 2001).

The immobilization by covalent bonding has some disadvantages. This process is quite complex and needs longer incubation times than the immobilization by adsorption. It can require chemical modifications of the enzyme molecule to obtained functional groups for covalent bonding. This enzyme functionalization can cause its denaturation. Moreover, the amount of enzyme that can be immobilized onto the carrier is quite low ($\sim$ 0.02 grams per gram of carrier). Although the enzyme stability is increased by this type of immobilization, the activity is decreased in affinity reaction (Marrazza 2014). Lower activity is caused by the restricted mobility of enzyme chains that are covalently attached onto the carrier. The restricted mobility disables conformational changes required in catalysis. However, the immobilization by covalent bonding provides an increased enzyme stability, a good recovery and reuse of the enzyme, that can compensate lower activity of the enzyme and make it suitable for the industrial application.

Immobilization by covalent bonding was reported by several groups in literature. Prodanović et al. (2012) immobilized horseradish peroxidase (HRP) onto macroporous polymers of glycidyl methacrylate and ethylene glycol dimethacrylate. They used two different methods to immobilize the enzyme. In the first method, the polymer was aminated and glutaraldehyde was used as a linker molecule between the polymer and the enzyme. The authors also immobilized the enzyme by covalent bonding between periodate oxidized enzyme and aminated polymer. They discovered that HRP immobilized by the periodate method performed considerably better than that immobilized by glutaraldehyde. HRP, immobilized by periodate method, showed enhanced specific activity and a rather high thermal and organic-solvent stability (5.3-fold more stable in 80 % dioxane than HRP immobilized by glutaraldehyde and almost 3.5-fold than free enzyme) (Prodanović et al. 2012).

Dyal et al. (2003) immobilized *Candida rugosa* lipase onto γ-Fe_2O_3 magnetic nanoparticles. The activity of the immobilized enzyme was lower than that of the free enzyme. However, the immobilized *Candida rugosa* lipase showed a long-term stability. The authors observed constant activity over one month (Dyal et al. 2003).

Miletić et al. (2009) immobilized *Candida antarctica* lipase B onto macroporous polymers of glycidyl methacrylate and ethylene glycol dimethacrylate using glutaraldehyde and cyanuric chloride as linker molecules. They showed that with an increase in the amount of glutaraldehyde or cyanuric chloride, the activity of the immobilized enzyme primary increased, reaching the highest value for 0.66% and 0.050% w/w, respectively, and subsequently decreased. The authors concluded that immobilized *Candida antarctica* lipase B had higher activity than free enzyme powder (Miletić et al. 2009).

Spasojević at al. (2014) immobilized horseradish peroxidase onto alginate. In order to achieve covalent bonding, the enzyme was modified with ethylenediamine and the carrier was oxidized with sodium periodate to obtain reactive aldehyde groups into polysaccharide backbone. Immobilized enzyme showed high stability in the presence of organic solvents and increased tolerance to pH changes. The authors showed that immobilized HRP, in a batch reactor, could be used in 5 consecutive rounds for pyrogallol oxidation without significant loss of enzymatic activity (Spasojević et al. 2014)).

3.3. Entrapment

Enzymes can be immobilized by entrapment via inclusion, where they are not directly attached to the carrier but entrapped in a polymer network. In the immobilization process, an enzyme is added to the monomer solution and after mixing, monomer was polymerized. Since an enzyme is entrapped within a polymer network, there is no direct contact with the environment. This method allows for the optimization of microenvironment for enzyme (i.e. optimal pH, polarity) by the polymer network modification. The stability of enzyme can be improved by entrapment immobilization. The enzyme leakage is not completely prevented but it is considerably lower when compared to the adsorption. Drawbacks of entrapment are mass transfer limitations, low enzyme loading capacity and possibility of the carrier corruption. Also, the enzyme leakage can be substantial when the polymer network has large pores. Entrapment immobilization can be done via electropolymerization, photopolymerization, sol-gel process and microencapsulation.

3.3.1. *Entrapment by electropolymerization*

In the electrochemical polymerization a polymer network is formed at the electrode surface by applying an appropriate current and potential. The polymerization starts with monomer oxidation/reduction which results in radical formation. These radicals interact with other monomer molecules, form macroradicals that further react with the monomer molecules or each other and form a growing polymer chain. Enzymes in the electrode vicinity are firstly captured by the growing polymer. The advantages of this method are control over the polymer network thickness and homogeneity, and high enzyme activity. However, this method requires an oxygen-free environment, a proper polymerization solution (to maintain enzyme activity), and high concentrations of both enzyme (0.2–3.5 mg/ml) and monomer (0.05–0.5 M). Polymer networks obtained by this method are composed of polyaniline, polypyrrole or polythiophene, pyrroles, thiophenes and polyindole (Nguyen & Kim 2017).

3.3.2. *Entrapment by photopolymerization*

In the immobilization by photopolymerization an enzyme solution is mixed with liquid photopolymers and exposed to UV or visible light. Light initiates chain-growth and further light irradiation results in cross linking of photopolymer chains and network formation.

Ichimura at al. (1984) described the method to immobilize different enzymes photochemically with the use of photocrosslinkable poly(vinyl alcohol) bearing stilbazolium groups. It was found that the polymer with more than 0.9 mol% of the photofunctional group was suitable for the immobilization of enzymes with the molecular weights larger than that of hemoglobin. They concluded that the entrapped enzymes preserved their native properties. Also, the high activity was retained except for enzymes requiring large molecular weight carriers. The authors found that the simultaneous immobilization of plural enzymes was possible (Ichimura 1984).

Sirkar et al. (1998) immobilized glucose oxidase by its entrapment in a hydrogel formed from vinylferrocene and poly(ethylene glycol) diacrylate. The enzyme retained its activity under the high-intensity UV radiation. This system was promising for use as amperometric biosensors for glucose (Sirkar & Pishko 1998).

3.3.3. *Entrapment by sol-gel process*

In the immobilization by sol-gel process, carriers are metal-oxides, silica and organosiloxane materials of defined porosity obtained from silane organic precursors such as metal alkoxides. Metal alkoxide (tetramethoxysilane or methyltrime-thoxysilane) is hydrolyzed and then activated by the addition of a base to initiate condensation reactions between silanol groups, generated by hydrolysis. The obtained siloxane polymer network is growing and aging until the gel is formed. The enzyme molecules are entrapped within this gel (Gupta & Chaudhury 2007; Jerónimo, Araújo, & Montenegro 2007). Entrapment by the sol-gel process preserves the enzyme activity and increases biosensor selectivity due to high encapsulation concentration. Also, it allows for the immobilization in stable carriers under mild conditions. Drawbacks of this method are carrier inhomogeneity and extra costs of organic precursors (alkoxides).

Vidinha et al. (2006) immobilized cutinase from *Fusarium solani pisi* into sol-gel carriers prepared with a combination of alkyl-alkoxysilane precursors of different chain-lengths. The authors showed that the type and combination of silica precursors had a great influence on the enzyme activity. When only tetrametoxysilane was used, the enzyme had no activity. However, when tetrametoxysilane was combined with a similar monoalkylated precursor, the enzyme activity increased with increasing alkyl chain up to a certain point, and then decreased (Vidinha et al. 2006).

Reetz, Zonta, and Simpelkamp (1996) developed a method to immobilize lipases by entrapment in sol-gel-derived hydrophobic silica. They used several different lipases and examined an influence of the type of silane precursor on the activity of sol-gel encapsulated lipases. The authors discovered that the lipase activity in gels composed of a mixture of tetramethoxysilane and alkyltrimethoxysilanes was dramatically enhanced with increasing the amount and the alkyl chain length of hydrophobic silanes. All sol-gel immobilized lipases showed substantially higher activities than those of the commercially available lipase powders (Reetz et al. 1996).

Erdemir et al. (2012) immobilized *Candida rugosa* lipase in sol–gel carriers using alkoxysilane precursors and calixarene polymer. They discovered that encapsulated lipases had better stability, enantioselectivity, adaptability and reusability than the free enzyme (Erdemir & Yilmaz 2012).

3.3.4. *Entrapment by microencapsulation*

Microencapsulation refers to entrapment of an enzyme in a semipermeable membrane (hollow fiber, polymeric network or microcapsule). Enzymes can be microencapsulated by coacervation or interfacial polymerization. The porosity of membrane should allow diffusion of small molecules and retain large molecules, such as enzymes, within. The immobilization by encapsulation preserves the enzyme integrity and activity. This system allows for

entrapment of more different enzymes inside the membrane (Nguyen & Kim 2017). The disadvantage of this method is a lack of precise control of the membrane porosity which would prevent the enzyme leakage.

Zhang et al. (2009) encapsulated β-glucuronidase in biomimetic alginate/protami-ne/silica capsules. These capsules were developed to improve their mechanical strength and increase recycling stability of encapsulated enzyme. The authors discovered that the pH and thermal stability of the immobilized enzyme was improved as well as the long-term storage and reusability (Zhang et al. 2009).

Betigeri et al. (2002) immobilized *Candida rugosa* lipase using various polymers (alginate, agarose and chitosan) in the form of beads. They determined that agarose beads were not suitable for immobilization due to its disintegration. The entrapment efficiency in alginate and chitosan were the same. However, lower enzyme activity and higher leakage was observed with alginate beads (Betigeri & Neau 2002).

Prodanovic et al. (2015) encapsulated horse radish peroxidase within hydrogel micro-beads formed by peroxidase catalyzed cross-linking of tyramine modified alginates. The immobilized enzyme showed substantially improved temperature and organic-solvent stability, and higher activity in operating conditions. After repeated use of 6 times in a batch reactor for pyrogallol oxidation immobilized HRP retained 45% of original activity (Prodanovic et al. 2015).

3.4. Cross-linking

Enzyme immobilization by cross-linking is a simple, carrier-free method where enzyme molecules are interconnected by covalent bonds. Intermolecular cross-linking of enzyme molecules is performed in a presence of linker molecules that act as a bridge between two enzyme molecules. Due to strong chemical binding between enzyme molecules, the enzyme leakage is minimal. This method allows for microenvironment modifications, which can increase the enzyme stability. The drawback of this method is the application of glutaraldehyde as a linker molecule. Glutaraldehyde can cause enzyme modifications and loss of activity as a consequence. However, the presence of inert proteins, such as gelatin and bovine serum albumin during the immobilization, can prevent the effect of cross-linker (Broun 1976).

There are several different approaches in immobilization by cross-linking, such as cross-linked dissolved enzymes (CLEs), cross-linked enzyme crystals (CLECs), cross-linked enzyme aggregates (CLEAs) and cross-linked spray-dried enzymes (CLSDs).

In the cross-linked dissolved enzymes systems (CLEs), surface amino groups of dissolved enzymes are connected using bifunctional chemical cross-linkers. For example, Quiocho et al. cross-linked carboxypeptidase-A, by treatment with glutaraldehyde to give an insoluble network (Quiocho & Richards 1966). This method has plenty disadvantages, such as low enzyme activity and poor mechanical stability. Therefore, CLEs is not in use anymore.

Cross-linked enzyme crystals (CLECs) refers to intermolecular cross linking of crystalline enzymes by glutaraldehyde. This system is stable, more resistant to heat and organic solvents and has higher efficiency when compared to the free enzyme powder (Jegan Roy & Emilia Abraham 2004). Cross-linked enzyme crystals are suitable for industrial application since they are reusable and easily recyclable. However, this system requires highly purified crystallisable enzymes and therefore, is expensive.

Cross-linked enzyme aggregates system (CLEAs) is a less-costly, improved version of CLECs. In this method, prior to cross linking, enzyme molecules form aggregates by adding salts, water miscible organic solvents or non-ionic polymers to an aqueous enzyme solution. This system, developed by Cao et al., is applicable to a wide variety of enzymes and allows for the preparation of 'combi CLEAs' that contain two or more enzymes (Cao, van Langen, & Sheldon 2003). Immobilization of penicillin acylase, *Pseudomonas putida* nitrilase, and *Trametes versicolor*, *Trametes villosa* and *Agaricus bisporus* laccases by cross-linked enzyme aggregates was reported in literature (S. Kumar, Mohan, Kamble, Pawar, & Banerjee 2010; Matijošytė, Arends, de Vries, & Sheldon 2010; Pchelintsev, Youshko, & Švedas 2009) Cross-linked spray-dried enzymes system (CLSDs) is abandoned since spray-drying reversibly deactivates the enzymes.

4. CARRIERS

As mentioned above, the most of immobilization techniques require a carrier. A wide variety of materials of both inorganic and organic origin have been used as carriers for enzyme immobilization.

4.1. Inorganic carriers

The inorganic materials are very stable and inert to the reaction conditions such as high pressures and temperatures but they have limited possibilities to create various geometrical shapes and are susceptible to abrasion while stirring. The most commonly used inorganic materials in the enzyme immobilization are silica and inorganic oxides, mineral materials, and carbon-based materials. Lately a new group of improved inorganic carriers was developed,

such as magnetic particles, ceramics, carbon-nanotubes, graphene and graphene oxide, etc.

4.1.1. *Silica and inorganic oxides*

Silica and inorganic oxides (titanium, aluminium and zirconium oxides) are thermally, mechanically and chemically stable materials with good sorption capacity. These inorganic materials are very hydrophilic since they have a large number of hydroxyl groups on the surface. This facilitates the enzyme immobilization and allows for surface modification to obtain strong binding of the enzyme onto the carrier. Silica and inorganic oxides were used for the immobilization of different enzymes (lipase, lignin, horseradish peroxidase, cysteine, urease, α-amylase, etc.) (Dezott, Innocentini-Mei, & Durán 1995; Foresti, Valle, Bonetto, Ferreira, & Briand 2010; Narwal, Saun, & Gupta 2014; Reshmi, Sanjay, & Sugunan 2007; Vallés, Furtado, Villadóniga, & Cantera 2011; Yang, Si, & Zhang 2008). Another material which belongs to this group of inorganic materials is glass. The use of glass for immobilization of nitrite reductase and urease was reported in literature (Rosa, Cruz, Vidal, & Oliva 2002; Sahney, Puri, & Anand 2005).

4.1.2. *Minerals*

Minerals are very economical immobilization materials due to their abundance in nature, easy accessibility and use without further purification or functionalization. These materials are also biocompatible. Due to the presence of different functional groups, such as hydroxyl, carboxyl, amino, thiol or carbonyl groups, surface properties of the mineral materials can easily be tailored to meet enzyme requirements. Bentonite, halloysite, kaolinite, montmorillonite, sepiolite and hydroxyapatite were used as carriers for immobilization of lipases, α-amylases, glucoamylase, invertase, alkaline phosphatase, protease, tyrosinases and glucose oxidases (An, Zhou, Zhuang, Tong, & Yu 2015; R. Prodanović, Simić, & Vujčić 2003; Sanjay & Sugunan 2008; Sedaghat, Ghiaci, Aghaei, & Soleimanian-Zad 2009a; 2009b; Xing, Li, Tian, & Ye 2000a; Zdarta et al. 2015). E.g. enzymes immobilized on minerals are used in biosensors, and for waste and wastewater treatment (Zdarta, Meyer, Jesionowski, & Pinelo 2018).

4.1.3. *Carbon-based materials*

Carbon-based materials are of great interest for the enzyme immobilization because of their well-developed porous structure which provides a large number of active sites for the enzyme adsorption. Besides high adsorption capacity, the presence of different functional groups is another benefit which makes these materials suitable for immobilization of various classes of enzymes. Immobilization of pancreatin, acid protease and acidic lipases on natural or modified activated carbon was reported in literature (A. G. Kumar, Perinbam, Kamatchi, Nagesh, & Sekaran 2010; Ramani et al. 2012; Silva, Missagia de Marco, Delvivo, Coelho, & Silvestre 2005). Charcoal carrier was used for the immobilization of amyloglucosidase which was applied in the starch hydrolysis in industry (Rani, Das, & Satyanarayana 2000).

4.1.4. *Magnetic materials*

Recently, magnetic materials have gained more attention in the enzyme immobilization since they can easily be manipulated by an external magnetic field. This behavior is of great interest because it allows for a simple separation of the immobilized enzyme from the rest of the biocatalytic system. Moreover, magnetic materials, such as iron oxide, have good mechanical stability, large surface area and many hydroxyl groups at the surface that allow for improvement of surface properties by chemical modifications and further, strong binding of the enzyme. *Candida antarctica* lipase immobilized on modified magnetic nanoparticles showed a high level of reusability when used for production of biodiesel from waste cooking oil (Mehrasbi, Mohammadi, Peyda, & Mohammadi 2017). High reusability was also observed when the immobilized glucose oxidase was used for the decolorization of acid yellow 12 (Aber, Mahmoudikia, Karimi, & Mahdizadeh 2016). The immobilization of trypsin on modified Fe_3O_4 magnetic nanoparticles for hydrolysis of bovine serum albumin was also reported in literature (Atacan, Çakıroğlu, & Özacar 2016).

4.1.5. *Ceramic materials*

Ceramic materials, such as alumina, zirconia, titania, silica, iron oxide and calcium phosphate, have been recently used as carriers in the enzyme immobilization. These materials are known to be mechanically stable, very inert to chemicals, temperature and pressure. The presence of the hydroxyl groups on their surface allows for the enzyme adsorption and chemical modifications. Enzymes such as β-galactosidase, horseradish peroxidase and *Candida antarctica* lipase were immobilized onto ceramic materials and used for synthesis of oligosaccharides, the oil removal from wastewater, and hydrolysis of butyl acetate in water and butyl laurate synthesis, respectively (Ebrahimi, Placido, Engel, Ashaghi, & Czermak 2010; Magnan, Catarino, Paolucci-Jeanjean, Preziosi-Belloy, & Belleville 2004; Wang, Li, Liu, & Wu 2012).

4.1.6. *Carbon nanotubes*

Carbon nanotubes have been receiving a lot of attention lately due to their excellent mechanical, chemical and thermal resistance, an ordered nanoporous structure, large surface area, biocompatibility, and the possibility of surface modifications to improve interactions with enzymes (Zdarta et al. 2018). These materials are unique since they enhance transfer of electrons between the carrier and enzyme as demonstrated by study of Liu, Wang, Zhao, Xu, and Dong (2005). They immobilized glucose oxidase on the carbon nanotubes/chitosan carriers and used this system as a glucose biosensor (Liu et al. 2005). Mohiuddin, Arbain, Islam, Ahmad, and Ahmad (2016) applied covalently immobilized α-glucosidase on carbon nanotubes as a biosensor for measuring the antidiabetic potential of medicinal plants (Mohiuddin et al. 2016).

4.1.7. *Graphene and graphene oxide*

Graphene and graphene oxide are biodegradable materials with high surface area, and good mechanical and chemical stability. Besides hydroxyl groups, the carboxylic and epoxy groups are also present in these materials, which allows for strong binding of enzymes without an additional chemical modification of the carrier. These materials can enhance the enzyme activity. Various lipases and esterases immobilized on amine-functionalized graphene oxide showed the enhanced catalytic behavior (Pavlidis et al. 2012). When horseradish peroxidase was covalently immobilized on functionalized reduced graphene oxide, the reusability and catalytic properties of enzyme were improved (Vineh, Saboury, Poostchi, Rashidi, & Parivar 2018).

4.1.8. *Mesoporous materials and nanoparticles*

Inorganic materials in a form of nanoparticles or mesoporous materials have recently been studied as promising materials for the enzyme immobilization. Mesoporous materials are water-insoluble, hydrophilic materials with good thermal and chemical stability, and well-defined structure and porosity that can be tailored during the synthesis. Since enzymes are captured within pores, the enzyme integrity and activity are preserved (Zdarta et al. 2018). Xing et al. (2000a) immobilized α-chymotrypsin and thermolysin on microporous Y and mesoporous dealuminized DAY zeolites and used them for peptide syntheses in organic media (Xing, Li, Tian, & Ye 2000b). *Candida antarctica* and *Candida rugosa* lipases immobilized on SBA 15 and MCM 41 mesoporous silica, respectively, were used as catalysts in organic (Cai, Gao, Liu, Zhong, & Liu 2016; Y. Chen, Xu, & Wu 2015). Nanoparticles, such as nanogold, graphene, titania and silica nanoparticles, provide high immobilization yields and improved catalytic activity (Zdarta et al. 2018).

4.2. Organic carriers

Organic carriers, widely used for immobilization of a variety of enzymes, can be divided into two groups: carriers made of biopolymers and carriers made of synthetic polymers. Organic materials used in the form of single particles, nanofibers or membranes have increased performance, better efficiency and provide higher stability of immobilized enzymes.

4.2.1. *Biopolymers*

Biopolymers are natural materials obtained from sustainable resources. They are biocompatible, not-toxic and biodegradable. Due to their natural origin, they have excellent affinity to enzymes, and preserve the enzyme structure and properties. The presence of hydroxyl, amine and carbonyl functional groups allows for surface modifications and the enzyme immobilization by covalent bonding or adsorption. Unlike inorganic materials, biopolymer materials can form a variety of geometrical shapes. These materials gel easily, which allows for the enzyme immobilization by microencapsulation or entrapment. Most commonly used biopolymers are alginate, chitosan and chitin, collagen, cellulose, starch, pectin, agarose etc.

Alginates are natural, unbranched polysaccharides mainly isolated from brown algae. They gel easily in the presence of divalent cations and without involvement of any toxic compounds. Alginate gels are flexible and pliable but their mechanical stability is quite low. Also, alginate gels are quite porous which can cause diffusion of encapsulated enzymes out of the gel. *Candida rugosa* lipase was immobilized by entrapment in calcium alginate with good entrapment efficiency. However, the reusability was quite low due to enzyme leaching from the gels (Betigeri & Neau 2002). Horse radish peroxidase immobilized by microencapsulation in tyramine modified alginates had significantly improved temperature and organic-solvent stability, and enhanced activity in operating conditions (Prodanovic et al. 2015).

Chitosan is a linear polysaccharide derived from chitin, polysaccharide that is found in the cell wall of fungi and also in the exoskeletons of crustaceans. The enzyme immobilization in the chitosan carriers is a simple, non-toxic, versatile method which can be applied to various enzymes. Moreover, this immobilization system provides improved thermal stability and good reusability. Betigeri and Neau (2002) immobilized *Candida rugosa* lipase in

both alginate and chitosan. Lipase immobilized in chitosan had substantially higher *activity than that* immobilized in alginate (Betigeri & Neau 2002). The immobilization of nuclease p1 and glucose isomerase on chitosan beads and surface-modified chitosan beads, respectively, were reported in literature (Cahyaningrum, Herdyastusi, & Maharani 2014; Shi et al. 2011). Invertase, immobilized by covalent bonding on chitosan, had improved both activity and thermal stability (Hsieh, Liu, & Liao 2000). Xylanase from *Bacillus pumilus* strain MK001 was immobilized on chitin by physical adsorption. Catalytic activity, thermal and pH stability and reusability of immobilized enzyme were improved when compared to those of the free enzyme (Kapoor & Kuhad 2007).

Collagen is the most abundant protein in all animals and is a component of connective tissue. Immobilization of *Saccharomyces cerevisiae* using the polyethyleneimine grafted collagen fiber and its fermentation performance was reported in literature (D. Zhu, Li, Liao, & Shi 2018). When catalase was immobilized on Fe (III) modified collagen fibers the thermal and storage stability, and reusability of the enzyme were considerably improved after immobilization (S. Chen, Song, Liao, & Shi 2011).

Cellulose is the most abundant biopolymer on Earth. It is a linear polysaccharide and the main structural component of cell walls of plants. Cellulose is also synthesized by algae, tunicates and some bacteria. Cellulose is robust, dual hydrophilic/hydrophobic, nontoxic, and chemically inert under physiological conditions and therefore suitable for enzyme immobilization. A wide variety of enzymes was immobilized by different immobilization techniques on the cellulose-based carriers (Liu & Chen 2016). Laccase immobilized on the bacterial cellulose particles showed improved stability against heat denaturation and very successful reusability (Frazão et al. 2014). When i*mmobilized* on the electrospun cellulose nanofiber membrane, *Candida rugosa* lipase exhibited significantly higher both thermal stability and durability than free enzyme (Huang et al. 2011).

Starch, composed of two polysaccharides (linear amylase and branched amylopectin), is another biomaterial which has been used as an enzyme carrier. *Aspergillus niger* glucoamylase was immobilized by using a combined method (adsorption of the enzyme to gelatinized corn starch and subsequent alginate fiber entrapment) and used to produce glucose from maltodextrin (Tanriseven, Uludağ, & Doğan 2002). Bitter gourd peroxidase was immobilized by either entrapment or surface immobilization. Entrapped peroxidase was considerably more stable than the surface immobilized form of enzyme, whereas surface immobilized peroxidase had better activity (Matto & Husain 2009).

Pectin, a water-soluble heteropolysaccharide present in ripe fruits, has also been used as a carrier for the enzyme immobilization. Chemically modified Saccharomyces cerevisiae invertase, immobilized on the pectin-coated chitin carrier via polyelectrolyte complex formation showed excellent thermal, storage and operational stability properties (Gomez, Ramírez, Neira-Carrillo, & Villalonga 2006). Calcium-alginate pectin entrapped bitter gourd peroxidase was used for the treatment of disperse dyes: Disperse Brown 1 and Disperse Red 17. The immobilized enzyme had improved stability as a consequence of the specific and strong binding between the carrier and peroxidase, that prevented denaturation of the enzyme (Satar & Husain 2011).

Agarose is a linear polysaccharide, generally extracted from agar or agar-bearing marine algae. It forms gel easily by decreasing temperature of an agarose solution below 35°C without addition of any ions. Agarose gels are highly porous, mechanically and chemically inert and resistant to organic solvents. They can form various shapes, such as beads, capsules and fibers. α-amylase from soybean seeds was immobilized by entrapment on agarose and agar carriers and used in removal of starch stain from clothes. The reusability of agarose and agar immobilized enzyme was found to be up to 5 cycles (Prakash & Jaiswal 2011). The immobilization of β-glucosidase on the agarose carrier modified with different reactive groups, e.g. polyethylenimine (PEI), glyoxyl (linear aliphatic aldehydes) and amine-epoxy groups was reported in literature. The best results were obtained when β-glucosidase was immobilized on amino-epoxy agarose: 80% of its activity was preserved and the enzyme was ca. 200 times more stable than its soluble form (Vieira et al. 2011). Laccase immobilized onto thiolsulfinate-agarose was used for colour removal from textile industrial effluents. The immobilized enzyme showed good thermal and pH stability and high capacity for dye decolouration (Gioia, Rodríguez-Couto, Menéndez, Manta, & Ovsejevi 2015).

4.2.2. *Synthetic polymers*

Unlike biopolymers, synthetic polymers are chemically synthesized materials with a high degree of purity, defined chemical structure and lot-to-lot uniformity. A large number of identical monomer units (small organic molecules) bonded together form homopolymer, whereas copolymers are composed of two or more different repeating units. Synthetic polymers can be tailored by a careful choice of monomer units to fulfill certain requirements directed by the application (specific mechanical properties, hydrophilicity/hydropho-bicity, stability, porosity etc.). Also, these materials can contain different

functional groups, such as carbonyl, carboxyl, hydroxyl, epoxy, amine and diol groups, which allow for the surface modification to adjust properties of a material to the requirements of the application. Therefore, synthetic polymers are widely used as immobilization carriers (Miletić et al. 2010; Prodanović et al. 2012; Prokopijevic et al. 2014). However, the synthesis of a polymer with the desired properties and functional groups is usually a time-consuming and costly process.

Cyclodextrin glucosyltransferase was immobilized on aminated polyvinylchloride by covalent binding in the presence of glutaraldehyde and used to catalyze the cyclodextrin formation. The enzyme properties were improved when compared to the free form. The immobilized enzyme retained about 85% of the initial catalytic activity after being used for 14 cycles (Abdel-Naby 1999). Lipase immobilization onto glutaraldehyde activated Nylon-6 by covalent binding, preserved good enzyme activity at pH 7.5 and temperature 5°C and enhanced the activity when exposed to organic solvents (Pahujani, Kanwar, Chauhan, & Gupta 2008). α-amylase was immobilized by physical adsorption and covalent binding onto polyanilines. The covalently immobilized enzyme had good immobilization efficiency, enhanced thermal stability, reusability and storage stability, whereas the adsorbed one encountered the problem of leaching and activity loss (Ashly, Joseph, & Mohanan 2011). *Trametes versicolor* laccase was immobilized onto polyvinyl alcohol microspheres activated with aldehyde and used as a biosensor. The immobilized enzymes exhibited good chemical and storage stability and reusability (Bai et al. 2014). Horseradish peroxidase was immobilized, covalently through its lysine residues, on a *N*-hydroxysuccinimidyl carbonate-activated poly(2-hydroxyethyl methacrylate) (PHEMA) brush (Lane et al. 2011). Polystyrene nanoparticles, obtained by nanoprecipitation, were used as immobilization carriers for *Candida antarctica* lipase B. The immobilized enzyme catalyzed hydrolysis of *p*-nitrophenyl acetate. It was found that immobilized enzyme had higher hydrolytic activity in buffer solution pH 6.8 than crude enzyme powder (Miletić et al. 2010).

Eupergit® is a copolymer of methacrylamide, *N*,*N*′-methylen-bis(acrylamide) and a monomer carrying oxirane groups. In the shape of macroporous beads, it is applied for the enzyme immobilization. The immobilization can be achieved without the presence of any additional reagents, since eupergit® reacts directly with an enzyme by forming strong covalent linkages like amino, hydroxy or mercapto functional groups. Eupergit® is hydrophilic, stable over a wide range of pH and has good mechanical and chemical properties. It is compatible with a wide range of different enzymes, and therefore, frequently used for the immobilization of enzymes not only in academics but also in industry. Oxidoreductases, transferases, hydrolases, lyases, lipases and aldolases were successfully immobilized on Eupergit® (Boller, Meier, & Menzler 2002; Knezevic, Milosavic, Bezbradica, Jakovljevic, & Prodanovic 2006; Knežević-Jugović et al. 2017; Tibhe et al. 2013).

The enzyme immobilization on diblock copolymers have gained attention of many researcher groups since properties of these carriers can easily be tailored during synthesis. Mechanical properties, hydrophilicity/hydrophobicity, porosity and the amount of desired functional groups are determined by the type of comonomers and the ratio between them. Porosity of the carrier plays an important role in enzyme immobilization since it influences the enzyme activity and stability (R. M. Prodanović et al. 2006). The porous properties, such as pore size distribution, specific pore volume, specific surface area and average pore diameter are determined by the type and the amount of crosslinking monomer, and the type and the amount of the inert component (pore-forming agent) (Horák et al. 2003; Jovanovic, Nastasović, Jovanovic, & Jeremic 1996; Jovanović, Nastasović, Jovanović, Jeremić, & Savić 1994). Macroporous polymers, used for the enzyme immobilization, can be obtained in the shape of spherical beads by suspension polymerization or monoliths in an unstirred mold.

Prodanović et al. (2012) immobilized horseradish peroxidase (HRP) on macroporous poly(glycidyl methacrylate-co-ethylene glycol dimethacrylate) beads obtained by suspension polymerization (Prodanović et al. 2012). By varying the amount of the inert component (10, 15 or 20 wt%) and its type (butanol, dodecanol and hexadecanol) different diblock copolymers were obtained with the mean pore diameters of 44, 53, 120 and 200 nm. The highest specific activity was observed when the enzyme was immobilized on the carriers with the largest pores. The carriers with small pores had larger total surface area but the pore size seemed to be the limiting factor since it restricted mass transfer and enzyme penetration. The thermal stability of the immobilized enzyme also depended on the mean pore diameter. The most stable system was obtained when the enzyme was immobilized on the methacrylate carriers with 120 nm large pores. The authors attributed the lower thermal stability of HRP immobilized on the carriers with the largest pores (200 nm) to a finite number of binding sites which prevented multipoint attachment of the enzyme, crucial for stability (Prodanović et al. 2012).

Miletić et al. (2009) immobilized *Candida antarctica* lipase B on poly(glycidyl methacrylate-co-ethylene glycol dimethacrylate) with identical chemical structure (60% of glycidyl methacrylate) but different average pore sizes

(from 30 to 560 nm) and different particle sizes (Miletić et al. 2009). The carrier was modified by diamines, glutaraldehyde and cyanuric chloride and the enzyme was immobilized by adsorption and covalent bonding. When the enzyme was immobilized on the 630–300 μm beads (average pore diameters: 30 and 90 nm) modified with diamines, a slight increase in the enzyme hydrolytic activity was obtained. However, modification of the 300–150 μm (average pore diameters: 87, 270 and 560 nm), 150–100 μm (59 nm) and < 100μm (48 nm) beads, resulted in the decrease of enzyme activity. Changes in porous properties caused by amination and the type of binding between the carrier and enzyme influenced the enzyme hydrolytic activity. The authors discovered that the enzyme activity was significantly better when the carrier was modified by glutaraldehyde and cyanuric chloride (Miletić et al. 2009).

Soybean hull peroxidase was also immobilized on poly(glycidyl methacrylate-co-ethylene glycol dimethacrylate) with various surface properties and pore size diameters ranging from 44 to 200 nm (Prokopijevic et al. 2014). Specific activities of the enzyme immobilized on carriers with larger pore sizes (120 and 200 nm) were the highest. The best thermo stability at 85∘C had the enzyme immobilized on the carrier with 120 nm large pores. However, the authors determined higher organic-solvent stability of the enzyme attached to copolymers with smaller pore sizes of 44 and 50 nm. The immobilized enzyme had higher activity than the native enzyme at both basic and acidic conditions (Prokopijevic et al. 2014).

Another example is the immobilization of α-glucosidase on macroporous poly(glycidyl methacrylate-co-ethylene glycol dimethacrylate) with pore sizes ranging from 44 nm to 270 nm (R. M. Prodanović et al. 2006). The immobilized enzyme showed similar specific activities and immobilization yields, except for the enzyme immobilized on the copolymer with the largest pores (270 nm). This sample had higher both specific activity and yield. The organic-solvent stability depended on the type of the used solvent and the type of the carrier. α-glucosidase, immobilized on the macroporous carrier with the 48 nm large pores had the highest stability in methanol, whereas the enzyme immobilized on the methacrylate carrier with the largest pores (270 nm) was, among all samples, the most stable in DMSO (R. M. Prodanović et al. 2006).

Knezevic-Jugovic et al. (2016) investigated the influence of the shape of the carrier on enzyme properties. For that purpose, penicillin G acylas was immobilized on macroporous poly(glycidyl methacrylate-co-ethylene glycol dimethacrylate) in the shape of microbeads, and rod-like and plates-like monoliths (Knežević-Jugović et al. 2016). Despite higher porosity of monoliths, the enzyme immobilized on these carriers had lower activity yields than that immobilized on beads. The authors attributed these findings to difference in the surface of the interface between the carrier and the enzyme. Monoliths had lower interfaces than beads (Knežević-Jugović et al. 2016).

Properties of organic carriers are determined not only by the chemical composition but also the material form. Electrospun nanofibers are long fibers with a uniform diameter easily produced by electrospinning. They can be made of different materials with properties tailored to meet the enzyme needs. The nanofibers have high surface areas and porosity that allows for high enzyme loading, reduces both diffusional limitations and barriers to mass transfer. They are biocompatible, non-toxic, biodegradable, hydrophilic and mechanically stable (Zdarta et al. 2018). Poly(vinyl alcohol), polystyrene, polyacrylamide, polyurethanes, poly(ϵ-caprolactone), chitin, chitosan, alginate and cellulose can be used to produce electrospun nanofibers for the enzyme immobilization (Canbolat, Savas, & Gultekin 2017; Ramakrishna et al. 2006; Weiser et al. 2016; Zdarta et al. 2018). Polymeric membranes are known for their high mechanical stability, good porosity, well-defined pore size and structure. Their properties can easily be tailored to fulfill the enzyme requirements. The enzyme can be immobilized not only on the surface of polymeric membranes but also inside the pores. The membranes can be produced in different shapes. When used as an enzyme carrier in catalytic processes, no additional separation and purification of reaction mixture is required. The polymeric membranes can be made of polyamide, polyurethanes, poly(vinylidene fluoride), chitosan, cellulose etc. (Donato, Algieri, Rizzi, & Giorno 2014; Konovalova et al. 2016; Sen et al. 2011; Vitola et al. 2016).

Hybrid and composite materials have gained attention of many research groups since they allow for obtaining the desired properties of a carrier by choosing and combining individual components. These carriers are designed to meet the enzyme requirements. The hybrid and composite materials have improved properties when compared to their precursors. They have higher mechanical, thermal and chemical stability than their composite units (Zdarta et al. 2018). These materials provide an appropriate microenvironment for immobilized enzymes and therefore, high catalytic activities. Enzymes immobilized on the hybrid and composite nanomaterials can have higher reusability and storage stability. Combination of different materials allows for immobilization of enzymes belonging to all catalytic classes. The hybrid materials can be composed of:

1. organic-organic precursors, where the enhanced properties of a carrier are achieved by combining

two organic compounds: two synthetic polymers (e.g. polyethyleneimine with epoxy-activated acrylate copolymer, poly(acrylic acid) and polyvinyl alcohol) (Rajdeo, Harini, Lavanya, & Fadnavis 2016; Sui, Wang, Wei, & Wang 2016), two biopolymers (e.g. chitosan and alginate, cellulose and dextran, alginate-pectin) (Celia Monteiro de Paula, Pessoa Andrade Feitosa, Paula, & Haroldo 2015; Satar & Husain 2011; Zdarta et al. 2018) or synthetic and biopolymer (e.g. poly(acrylic acid) and cellulose, polyanilin and chitosan) (Riccardi, Kasi, & Kumar 2017; Yavuz, Uygun, & Bhethanabotla 2010);

2. organic-inorganic precursors, where the properties of organic materials (e.g. polyacrylonitrile, polyethyleneimine, polyvinyl alcohol, chitosan, lignin, alginate, cellulose) were combined with the properties of inorganic compounds (e.g. silica, inorganic oxides, minerals, carbon, magnetic materials) (J. Li et al. 2012; Namdeo & Bajpai 2009; Zdarta et al. 2016; 2018) and

3. inorganic-inorganic precursors, where better pH stability, thermal, mechanical and chemical inertness were achieved by combing different inorganic compounds (e.g. combination of silica and carbon nanotubes, silica-coated magnetic nanoparticles, calcium carbonate and gold nanoparticles etc.) (Lee, Doan, Won, Ha, & Koo 2010; F. Li et al. 2010; Y.-T. Zhu et al. 2014).

5. CONCLUSIONS

Enzymes allow for the preparation of a wide range of products under mild and environmentally friendly conditions. The enzyme application in industry lacks a continual production, easy separation and purification of the product. Also, enzymes lose their properties under industrial conditions. Enzyme immobilization is beneficial since it improves the process economics by enabling enzyme reuse and enhancing its thermal, chemical and storage stability and overall productivity. Among all immobilization methods, covalent bonding is the most appropriate for the industrial application due to the increased enzyme stability, an absence of enzyme leakage, and good recovery and reuse of the enzyme. The inorganic carriers are very stable and inert to various reaction conditions but can not be processed into different geometrical shapes. Biopolymers are good candidates for immobilization carries due to their abundance in nature, excellent affinity to enzymes, and ability to preserve the enzyme structure and properties. However, biopolymer carriers are not mechanically stable. Synthetic polymers are the most widely used immobilization carriers since their properties (mechanical stability, porosity, geometrical shape, hydrophilicity/hydrophobicity) can be tailored precisely by a careful choice of the synthetic method and composition units.

The application of immobilized enzymes is still at low commercial level due to their high costs and storage problems. New immobilization routes and carriers should be designed to overcome these issues and allow for large scale production.

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REFERENCES

Abdel-Naby, M. A. (1999). Immobilization of paenibacillus macerans nrrl b-3186 cyclodextrin glucosyltransferase and properties of the immobilized enzyme. *Process Biochemistry*, *34*(4), 399–405.

Aber, S., Mahmoudikia, E., Karimi, A., & Mahdizadeh, F. (2016). Immobilization of glucose oxidase on fe 3 o 4 magnetic nanoparticles and its application in the removal of acid yellow 12. *Water, Air, & Soil Pollution*, *227*(3), 93.

An, N., Zhou, C. H., Zhuang, X. Y., Tong, D. S., & Yu, W. H. (2015). Immobilization of enzymes on clay minerals for biocatalysts and biosensors. *Applied Clay Science*, *114*, 283–296. doi: https://doi.org/10.1016/j.clay.2015.05.029

Ashly, P., Joseph, M., & Mohanan, P. (2011). Activity of diastase α-amylase immobilized on polyanilines (panis). *Food Chemistry*, *127*(4), 1808–1813. doi: https://doi.org/10.1016/j.foodchem.2011.02.068

Atacan, K., Çakıroğlu, B., & Özacar, M. (2016). Improvement of the stability and activity of immobilized trypsin on modified fe3o4 magnetic nanoparticles for hydrolysis of bovine serum albumin and its application in the bovine milk. *Food chemistry*, *212*, 460–468. doi: https://doi.org/10.1016/j.foodchem.2016.06.011

Bai, X., Gu, H., Chen, W., Shi, H., Yang, B., Huang, X., & Zhang, Q. (2014). Immobilized laccase on activated poly (vinyl alcohol) microspheres for enzyme thermistor application. *Applied biochemistry and biotechnology*, *173*(5), 1097–1107. doi: 10.1007/s12010-014-0913-3

Betigeri, S. S., & Neau, S. H. (2002). Immobilization of lipase using hydrophilic polymers in the form of hydrogel beads. *Biomaterials*, *23*(17), 3627–3636. doi: https://doi.org/10.1016/S0142-9612(02)00095-9

Boller, T., Meier, C., & Menzler, S. (2002). Eupergit oxirane acrylic beads: how to make enzymes fit for biocatalysis. *Organic Process Research & Development*, *6*(4), 509–519. doi: 10.1021/op015506w

Broun, G. B. (1976). [20] chemically aggregated enzymes. In *Methods in enzymology* (Vol. 44, pp. 263–280). Elsevier.

Cabrera-Padilla, R. Y., Lisboa, M. C., Fricks, A. T., Franceschi, E., Lima, A. S., Silva, D. P., & Soares, C. M. (2012). Immobilization of candida rugosa lipase on poly (3-hydroxybutyrate-co-hydroxyvalerate): a new eco-friendly support. *Journal of industrial microbiology & biotechnology*, *39*(2), 289–298. doi: 10.1007/s10295-011-1027-3

Cahyaningrum, S., Herdyastusi, N., & Maharani, D. (2014). Immobilization of glucose isomerase in surface-modified chitosan gel beads. *Res. J. Pharm. Biol. Chem. Sci*, *5*, 104–111.

Cai, C., Gao, Y., Liu, Y., Zhong, N., & Liu, N. (2016). Immobilization of candida antarctica lipase b onto sba-15 and their application in glycerolysis for diacylglycerols synthesis. *Food chemistry*, *212*, 205–212. doi: 10.1016/j.foodchem.2016.05.167

Caldwell, K. D., Axén, R., Bergwall, M., & Porath, J. (1976). Immobilization of enzymes based on hydrophobic interaction. ii. preparation and properties of an amyloglucosidase adsorbate. *Biotechnology and bioengineering*, *18*(11), 1589–1604. doi: 10.1002/bit.260181108

Caldwell, K. D., Axén, R., & Porath, J. (1976). Reversible immobilization of enzymes to hydrophobic agarose gels. *Biotechnology and Bioengineering*, *18*(3), 433–438. doi: 10.1002/bit.260180313

Canbolat, M. F., Savas, H. B., & Gultekin, F. (2017). Improved catalytic activity by catalase immobilization using γ-cyclodextrin and electrospun pcl nanofibers. *Journal of Applied Polymer Science*, *134*(4). doi: 10.1002/app.44404

Cao, L., van Langen, L., & Sheldon, R. A. (2003). Immobilised enzymes: carrier-bound or carrier-free? *Current opinion in Biotechnology*, *14*(4), 387–394. doi: https://doi.org/10.1016/S0958-1669(03)00096-X

Celia Monteiro de Paula, R., Pessoa Andrade Feitosa, J., Paula, B., & Haroldo, C. (2015). Polysaccharide based copolymers as supramolecular systems in biomedical applications. *Current drug targets*, *16*(14), 1591–1605.

Chen, B., Miller, E. M., Miller, L., Maikner, J. J., & Gross, R. A. (2007). Effects of macroporous resin size on candida a ntarctica lipase b adsorption, fraction of active molecules, and catalytic activity for polyester synthesis. *Langmuir*, *23*(3), 1381–1387. doi: 10.1021/la062258u

Chen, S., Song, N., Liao, X., & Shi, B. (2011). Immobilization of catalase on fe (iii) modified collagen fiber. *Sheng wu gong cheng xue bao= Chinese journal of biotechnology*, *27*(7), 1076–1081.

Chen, Y., Xu, Y., & Wu, X.-m. (2015). Efficient improving the activity and enantioselectivity of candida rugosa lipase for the resolution of naproxen by enzyme immobilization on mcm-41 mesoporous molecular sieve. *International Journal Bioautomation*, *19*(3).

Cowan, D. A., & Fernandez-Lafuente, R. (2011). Enhancing the functional properties of thermophilic enzymes by chemical modification and immobilization. *Enzyme and Microbial Technology*, *49*(4), 326–346. doi: 10.1016/j.enzmictec.2011.06.023

Cunha, A., Fernández-Lorente, G., Bevilaqua, J., Destain, J., Paiva, L., Freire, D., … Guisán, J. (2008). Immobilization of yarrowia lipolytica lipase–a comparison of stability of physical adsorption and covalent attachment techniques. *Applied biochemistry and biotechnology*, *146*(1-3), 49. doi: 10.1007/s12010-007-8073-3

Dezott, M., Innocentini-Mei, L. H., & Durán, N. (1995). Silica immobilized enzyme catalyzed removal of chlorolignins from eucalyptus kraft effluent. *Journal of biotechnology*, *43*(3), 161–167. doi: https://doi.org/10.1016/0168-1656(95)00110-7

Donato, L., Algieri, C., Rizzi, A., & Giorno, L. (2014). Kinetic study of tyrosinase immobilized on polymeric membrane. *Journal of Membrane Science*, *454*, 346–350. doi: https://doi.org/10.1016/j.memsci.2013.12.029

Dyal, A., Loos, K., Noto, M., Chang, S. W., Spagnoli, C., Shafi, K. V., … Gross, R. A. (2003). Activity of candida rugosa lipase immobilized on γ-fe2o3 magnetic nanoparticles. *Journal of the American Chemical Society*, *125*(7), 1684–1685. doi: 10.1021/ja021223n

Ebrahimi, M., Placido, L., Engel, L., Ashaghi, K. S., & Czermak, P. (2010). A novel ceramic membrane reactor system for the continuous enzymatic synthesis of oligosaccharides. *Desalination*, *250*(3), 1105–1108. doi: https://doi.org/10.1016/j.desal.2009.09.118

Erdemir, S., & Yilmaz, M. (2012). Catalytic effect of calix [n] arene based sol–gel encapsulated or covalent immobilized lipases on enantioselective hydrolysis of (r/s)-naproxen methyl ester. *Journal of Inclusion Phenomena and Macrocyclic Chemistry*, *72*(1-2), 189–196.

Faber, K., et al. (2011). *Biotransformations in organic chemistry (6th ed.)*. Berlin; Heidelberg: Springer-Verlag,.

Fernández-Lorente, G., Terreni, M., Mateo, C., Bastida, A., Fernández-Lafuente, R., Dalmases, P., … Guisán, J. M. (2001). Modulation of lipase properties in macro-aqueous systems by controlled enzyme immobilization: enantioselective hydrolysis of a chiral ester by immobilized pseudomonas lipase. *Enzyme and microbial technology*, *28*(4-5), 389–396.

Foresti, M., Valle, G., Bonetto, R., Ferreira, M., & Briand, L. (2010). Ftir, sem and fractal dimension characterization of lipase b from candida antarctica immobilized onto titania at selected conditions. *Applied Surface Science*, *256*(6), 1624–1635. doi: https://doi.org/10.1016/j.apsusc.2009.09.083

Frazão, C. J., Silva, N. H., Freire, C. S., Silvestre, A. J., Xavier, A. M., & Tavares, A. P. (2014). Bacterial cellulose as carrier for immobilization of laccase: Optimization and characterization. *Engineering in Life Sciences*, *14*(5), 500–508. doi: 10.1002/elsc.201400054

Gioia, L., Rodríguez-Couto, S., Menéndez, M. d. P., Manta, C., & Ovsejevi, K. (2015). Reversible covalent immobilization of trametes villosa laccase onto thiolsulfinate-agarose: An insoluble biocatalyst with potential for decoloring recalcitrant dyes. *Biotechnology and applied biochemistry*, *62*(4), 502–513. doi: 10.1002/bab.1287

Gomez, L., Ramírez, H. L., Neira-Carrillo, A., & Villalonga, R. (2006). Polyelectrolyte complex formation mediated immobilization of chitosan-invertase neoglycoconjugate on pectin-coated chitin. *Bioprocess and Biosystems Engineering*, *28*(6), 387–395. doi: 10.1007/s00449-005-0043-y

Gupta, R., & Chaudhury, N. (2007). Entrapment of biomolecules in sol–gel matrix for applications in biosensors: Problems and future prospects. *Biosensors and Bioelectronics*, *22*(11), 2387–2399. doi: 10.1016/j.bios.2006.12.025

Horák, D., Švec, F., Ilavskỳ, M., Bleha, M., Baldrián, J., & Kálal, J. (2003). Reactive polymers, xxxvi. the effect of polymerization conditions on the porosity and mechanical properties of macroporous suspension copolymers from glycidylmethacrylate-ethylenedimethacrylate. *Die Angewandte Makromolekulare Chemie: Applied Macromolecular Chemistry and Physics*, *95*(1), 117–127.

Hsieh, H.-J., Liu, P.-C., & Liao, W.-J. (2000). Immobilization of invertase via carbohydrate moiety on chitosan to enhance its thermal stability. *Biotechnology letters*, *22*(18), 1459–1464.

Huang, X.-J., Chen, P.-C., Huang, F., Ou, Y., Chen, M.-R., & Xu, Z.-K. (2011). Immobilization of candida rugosa lipase on electrospun cellulose nanofiber membrane. *Journal of Molecular Catalysis B: Enzymatic*, *70*(3-4), 95–100. doi: https://doi.org/10.1016/j.molcatb.2011.02.010

Ichimura, K. (1984). A convenient photochemical method to immobilize enzymes. *Journal of Polymer Science: Polymer Chemistry Edition*, *22*(11), 2817–2828. doi: 10.1002/pol.1984.170221108

Jegan Roy, J., & Emilia Abraham, T. (2004). Strategies in making cross-linked enzyme crystals. *Chemical Reviews*, *104*(9), 3705–3722. doi: 10.1021/cr0204707

Jerónimo, P. C., Araújo, A. N., & Montenegro, M. C. B. (2007). Optical sensors and biosensors based on sol–gel films. *Talanta*, *72*(1), 13–27. doi: 10.1016/j.talanta.2006.09.029

Jovanovic, S., Nastasović, A., Jovanovic, N., & Jeremic, K. (1996). Targeted porous structure of macroporous copolymers based on glycidyl methacrylate. In *Materials science forum* (Vol. 214, pp. 155–162).

Jovanović, S. M., Nastasović, A., Jovanović, N. N., Jeremić, K., & Savić, Z. (1994). The influence of inert component composition on the porous structure of glycidyl methacrylate/ethylene glycol dimethacrylate copolymers. *Die Angewandte Makromolekulare Chemie: Applied Macromolecular Chemistry and Physics*, *219*(1), 161–168. doi: 10.1002/apmc.1994.052190113

Kapoor, M., & Kuhad, R. C. (2007). Immobilization of xylanase from bacillus pumilus strain mk001 and its application in production of xylo-oligosaccharides. *Applied biochemistry and biotechnology*, *142*(2), 125–138.

Katchalski-Katzir, E. (1993). Immobilized enzymes—learning from past successes and failures. *Trends in biotechnology*, *11*(11), 471–478. doi: https://doi.org/10.1016/0167-7799(93)90080-S

Knezevic, Z., Milosavic, N., Bezbradica, D., Jakovljevic, Z., & Prodanovic, R. (2006). Immobilization of lipase from candida rugosa on eupergit® c supports by covalent attachment. *Biochemical Engineering Journal*, *30*(3), 269–278. doi: 10.1016/j.bej.2006.05.009

Knežević-Jugović, Z. D., Grbavčić, S. Ž., Jovanović, J. R., Stefanović, A. B., Bezbradica, D. I., Mijin, D. Ž., & Antov, M. G. (2017). Covalent immobilization of enzymes on eupergit® supports: Effect of the immobilization protocol. In *Enzyme stabilization and immobilization* (pp. 75–91). Springer. doi: 10.1007/978-1-4939-6499-4_7

Knežević-Jugović, Z. D., Žuža, M. G., Jakovetić, S. M., Stefanović, A. B., Džunuzović, E. S., Jeremić, K. B., & Jovanović, S. M. (2016). An approach for the improved immobilization of penicillin g acylase onto macroporous poly (glycidyl methacrylate-co-ethylene glycol dimethacrylate) as a potential industrial biocatalyst. *Biotechnology progress*, *32*(1), 43–53. doi: 10.1002/btpr.2181

Kobayashi, S., Uyama, H., & Kimura, S. (2001). Enzymatic polymerization. *Chemical Reviews*, *101*(12), 3793–3818. doi: 10.1021/cr9901211

Konovalova, V., Guzikevich, K., Burban, A., Kujawski, W., Jarzynka, K., & Kujawa, J. (2016). Enhanced starch hydrolysis using α-amylase immobilized on cellulose ultrafiltration affinity membrane. *Carbohydrate polymers*, *152*, 710–717. doi: 10.1016/j.carbpol.2016.07.065

Křenková, J., & Foret, F. (2004). Immobilized microfluidic enzymatic reactors. *Electrophoresis*, *25*(21-22), 3550–3563. doi: 10.1002/elps.200406096

Kumar, A. G., Perinbam, K., Kamatchi, P., Nagesh, N., & Sekaran, G. (2010). In situ immobilization of acid protease on mesoporous activated carbon packed column for the production of protein hydrolysates. *Bioresource technology*, *101*(4), 1377–1379. doi: https://doi.org/10.1016/j.biortech.2009.09.014

Kumar, S., Mohan, U., Kamble, A. L., Pawar, S., & Banerjee, U. C. (2010). Cross-linked enzyme aggregates of recombinant pseudomonas putida nitrilase for enantioselective nitrile hydrolysis. *Bioresource technology*, *101*(17), 6856–6858. doi: 10.1016/j.biortech.2010.03.084

Lane, S. M., Kuang, Z., Yom, J., Arifuzzaman, S., Genzer, J., Farmer, B., … Vaia, R. A. (2011). Poly (2-hydroxyethyl methacrylate) for enzyme immobilization: impact on activity and stability of horseradish peroxidase. *Biomacromolecules*, *12*(5), 1822–1830. doi: 10.1021/bm200173y

Lee, S. H., Doan, T. T. N., Won, K., Ha, S. H., & Koo, Y.-M. (2010). Immobilization of lipase within carbon nanotube–silica composites for non-aqueous reaction systems. *Journal of Molecular Catalysis B: Enzymatic*, *62*(2), 169–172. doi: https://doi.org/10.1016/j.molcatb.2009.10.004

Li, F., Feng, Y., Wang, Z., Yang, L., Zhuo, L., & Tang, B. (2010). Direct electrochemistry of horseradish peroxidase immobilized on the layered calcium carbonate–gold nanoparticles inorganic hybrid composite. *Biosensors and Bioelectronics*, *25*(10), 2244–2248. doi: 10.1016/j.bios.2010.03.006

Li, J., Wu, H., Liang, Y., Jiang, Z., Jiang, Y., & Zhang, L. (2012). Facile fabrication of organic–inorganic hybrid beads by aminated alginate enabled gelation and biomimetic mineralization. *Journal of Biomaterials Science, Polymer Edition*, *24*(2), 119–134.

Liu, Y., & Chen, J. Y. (2016). Enzyme immobilization on cellulose matrixes. *Journal of Bioactive and Compatible Polymers*, *31*(6), 553–567. doi: 10.1177/0883911516637377

Liu, Y., Wang, M., Zhao, F., Xu, Z., & Dong, S. (2005). The direct electron transfer of glucose oxidase and glucose biosensor based on carbon nanotubes/chitosan matrix. *Biosensors and Bioelectronics*, *21*(6), 984–988. doi: 10.1016/j.bios.2005.03.003

Magnan, E., Catarino, I., Paolucci-Jeanjean, D., Preziosi-Belloy, L., & Belleville, M.-P. (2004). Immobilization of lipase on a ceramic membrane: activity and stability. *Journal of Membrane Science*, *241*(1), 161–166. doi: https://doi.org/10.1016/j.memsci.2004.05.023

Marrazza, G. (2014). Piezoelectric biosensors for organophosphate and carbamate pesticides: a review. *Biosensors*, *4*(3), 301–317. doi: 10.3390/bios4030301

Marzadori, C., Miletti, S., Gessa, C., & Ciurli, S. (1998). Immobilization of jack bean urease on hydroxyapatite: urease immobilization in alkaline soils. *Soil Biology and Biochemistry*, *30*(12), 1485–1490. doi: https://doi.org/10.1016/S0038-0717(98)00051-0

Mateo, C., Grazú, V., Pessela, B., Montes, T., Palomo, J., Torres, R., … Guisán, J. (2007). Advances in the design of new epoxy supports for enzyme immobilization-stabilization. *Biochemical Society transactions*, *35*(Pt 6), 1593–1601. doi: 10.1042/bst0351593

Mateo, C., Palomo, J. M., Fernandez-Lorente, G., Guisan, J. M., & Fernandez-Lafuente, R. (2007). Improvement of enzyme activity, stability and selectivity via immobilization techniques. *Enzyme and microbial technology*, *40*(6), 1451–1463. doi: https://doi.org/10.1016/j.enzmictec.2007.01.018

Matijošytė, I., Arends, I. W., de Vries, S., & Sheldon, R. A. (2010). Preparation and use of cross-linked enzyme aggregates (cleas) of laccases. *Journal of Molecular Catalysis B: Enzymatic*, *62*(2), 142–148. doi: https://doi.org/10.1016/j.molcatb.2009.09.019

Matto, M., & Husain, Q. (2009). Calcium alginate–starch hybrid support for both surface immobilization and entrapment of bitter gourd (momordica charantia) peroxidase. *Journal of Molecular Catalysis B: Enzymatic*, *57*(1-4), 164–170. doi: https://doi.org/10.1016/j.molcatb.2008.08.011

Mehrasbi, M. R., Mohammadi, J., Peyda, M., & Mohammadi, M. (2017). Covalent immobilization of candida antarctica lipase on core-shell magnetic nanoparticles for production of biodiesel from waste cooking oil. *Renewable Energy*, *101*, 593–602. doi: https://doi.org/10.1016/j.renene.2016.09.022

Miletić, N., Abetz, V., Ebert, K., & Loos, K. (2010). Immobilization of candida antarctica lipase b on polystyrene nanoparticles. *Macromolecular rapid communications*, *31*(1), 71–74. doi: 10.1002/marc.200900497

Miletić, N., Nastasović, A., & Loos, K. (2012). Immobilization of biocatalysts for enzymatic polymerizations: possibilities, advantages, applications. *Bioresource Technology*, *115*, 126–135. doi: https://doi.org/10.1016/j.biortech.2011.11.054

Miletić, N., Rohandi, R., Vuković, Z., Nastasović, A., & Loos, K. (2009). Surface modification of macroporous poly (glycidyl methacrylate-co-ethylene glycol dimethacrylate) resins for improved candida antarctica lipase b immobilization. *Reactive and Functional Polymers*, *69*(1), 68–75. doi: https://doi.org/10.1016/j.reactfunctpolym.2008.11.001

Mohamad, N. R., Marzuki, N. H. C., Buang, N. A., Huyop, F., & Wahab, R. A. (2015). An overview of technologies for immobilization of enzymes and surface analysis techniques for immobilized enzymes. *Biotechnology & Biotechnological Equipment*, *29*(2), 205–220. doi: 10.1080/13102818.2015.1008192

Mohiuddin, M., Arbain, D., Islam, A. S., Ahmad, M., & Ahmad, M. (2016). Alpha-glucosidase enzyme biosensor for the electrochemical measurement of antidiabetic potential of medicinal plants. *Nanoscale research letters*, *11*(1), 1–12. doi: 10.1186/s11671-016-1292-1

Namdeo, M., & Bajpai, S. (2009). Immobilization of α-amylase onto cellulose-coated magnetite (ccm) nanoparticles and preliminary starch degradation study. *Journal of Molecular Catalysis B: Enzymatic*, *59*(1-3), 134–139. doi: https://doi.org/10.1016/j.molcatb.2009.02.005

Narwal, S. K., Saun, N. K., & Gupta, R. (2014). Characterization and catalytic properties of free and silica-bound lipase: a comparative study. *Journal of Oleo Science*, *63*(6), 599–605.

Nguyen, H. H., & Kim, M. (2017). An overview of techniques in enzyme immobilization. *Applied Science and Convergence Technology*, *26*(6), 157–163. doi: 10.5757/ASCT.2017.26.6.157

Novick, S. J., & Rozzell, J. D. (2005). Immobilization of enzymes by covalent attachment. In J. L. Barredo (Ed.), *Microbial enzymes and biotransformations* (pp. 247–271). Totowa, NJ: Humana Press.

Pahujani, S., Kanwar, S. S., Chauhan, G., & Gupta, R. (2008). Glutaraldehyde activation of polymer nylon-6 for lipase immobilization: enzyme characteristics and stability. *Bioresource Technology*, *99*(7), 2566–2570. doi: https://doi.org/10.1016/j.biortech.2007.04.042

Pavlidis, I. V., Vorhaben, T., Tsoufis, T., Rudolf, P., Bornscheuer, U. T., Gournis, D., & Stamatis, H. (2012). Development of effective nanobiocatalytic systems through the immobilization of hydrolases on functionalized carbon-based nanomaterials. *Bioresource technology*, *115*, 164–171. doi: 10.1016/j.biortech.2011.11.007

Pchelintsev, N., Youshko, M., & Švedas, V. (2009). Quantitative characteristic of the catalytic properties and microstructure of cross-linked enzyme aggregates of penicillin acylase. *Journal of Molecular Catalysis B: Enzymatic*, *56*(4), 202–207. doi: https://doi.org/10.1016/j.molcatb.2008.05.006

Porath, J. (1987). Salting-out adsorption techniques for protein purification. *Biopolymers: Original Research on Biomolecules*, *26*(S0), S193–S204. doi: 10.1002/bip.360260017

Prakash, O., & Jaiswal, N. (2011). Immobilization of a thermostable"-amylase on agarose and agar matrices and its application in starch stain removal. *World Applied Sciences Journal*, *13*(3), 572–577.

Prodanović, O., Prokopijević, M., Spasojević, D., Stojanović, Ž., Radotić, K., Knežević-Jugović, Z. D., & Prodanović, R. (2012). Improved covalent immobilization of horseradish peroxidase on macroporous glycidyl methacrylate-based copolymers. *Applied biochemistry and biotechnology*, *168*(5), 1288–1301. doi: 10.1007/s12010-012-9857-7

Prodanovic, O., Spasojevic, D., Prokopijevic, M., Radotic, K., Markovic, N., Blazic, M., & Prodanovic, R. (2015). Tyramine modified alginates via periodate oxidation for peroxidase induced hydrogel formation and immobilization. *Reactive and Functional Polymers*, *93*, 77–83. doi: https://doi.org/10.1016/j.reactfunctpolym.2015.06.004

Prodanović, R., Simić, M., & Vujčić, Z. (2003). Immobilization of periodate oxidized invertase by adsorption on sepiolite. *Journal of the Serbian Chemical Society*, *68*(11), 819–824.

Prodanović, R. M., Milosavić, N. B., Jovanović, S. M., Ćirković-Veličković, T., Vujčić, Z. M., & Jankov, R. M. (2006). Stabilization of α-glucosidase in organic solvents by immobilization on macroporous poly (gma-co-egdma) with different surface characteristics. *Journal of the Serbian Chemical Society*, *71*(4), 339–347.

Prokopijevic, M., Prodanovic, O., Spasojevic, D., Stojanovic, Z., Radotic, K., & Prodanovic, R. (2014). Soybean hull peroxidase immobilization on macroporous glycidyl methacrylates with different surface characteristics. *Bioprocess and biosystems engineering*, *37*(5), 799–804. doi: 10.1007/s00449-013-1050-z

Quiocho, F. A., & Richards, F. M. (1966). The enzymic behavior of carboxypeptidase-a in the solid state. *Biochemistry*, *5*(12), 4062–4076.

Rajdeo, K., Harini, T., Lavanya, K., & Fadnavis, N. W. (2016). Immobilization of pectinase on reusable polymer support for clarification of apple juice. *Food and Bioproducts Processing*, *99*, 12–19. doi: https://doi.org/10.1016/j.fbp.2016.03.004

Ramakrishna, S., Fujihara, K., Teo, W.-E., Yong, T., Ma, Z., & Ramaseshan, R. (2006). Electrospun nanofibers: solving global issues. *Materials today*, *9*(3), 40–50. doi: https://doi.org/10.1016/S1369-7021(06)71389-X

Ramani, K., Karthikeyan, S., Boopathy, R., Kennedy, L. J., Mandal, A., & Sekaran, G. (2012). Surface functionalized mesoporous activated carbon for the immobilization of acidic lipase and their application to hydrolysis of waste cooked oil: isotherm and kinetic studies. *Process biochemistry*, *47*(3), 435–445.

Rani, A., Das, M., & Satyanarayana, S. (2000). Preparation and characterization of amyloglucosidase adsorbed on activated charcoal. *Journal of Molecular Catalysis B: Enzymatic*, *10*(5), 471–476. doi: https://doi.org/10.1016/S1381-1177(99)00116-2

Reetz, M. T., Zonta, A., & Simpelkamp, J. (1996). Efficient immobilization of lipases by entrapment in hydrophobic sol-gel materials. *Biotechnology and Bioengineering*, *49*(5), 527–534. doi: 10.1002/(sici)1097-0290(19960305)49:5<527::Aid-bit5>3.0.Co;2-l

Reshmi, R., Sanjay, G., & Sugunan, S. (2007). Immobilization of α-amylase on zirconia: a heterogeneous biocatalyst for starch hydrolysis. *Catalysis Communications*, *8*(3), 393–399. doi: https://doi.org/10.1016/j.catcom.2006.07.009

Riccardi, C. M., Kasi, R. M., & Kumar, C. V. (2017). Nanoarmoring of enzymes by interlocking in cellulose fibers with poly (acrylic acid). In *Methods in enzymology* (Vol. 590, pp. 475–500). Elsevier. doi: 10.1016/bs.mie.2017.01.009

Rosa, C. C., Cruz, H. J., Vidal, M., & Oliva, A. G. (2002). Optical biosensor based on nitrite reductase immobilised in controlled pore glass. *Biosensors and Bioelectronics*, *17*(1-2), 45–52.

Sahney, R., Puri, B., & Anand, S. (2005). Enzyme coated glass ph-electrode: Its fabrication and applications in the determination of urea in blood samples. *Analytica chimica acta*, *542*(2), 157–161. doi: https://doi.org/10.1016/j.aca.2005.03.069

Sanjay, G., & Sugunan, S. (2008). Acid activated montmorillonite: an efficient immobilization support for improving reusability, storage stability and operational stability of enzymes. *Journal of Porous Materials*, *15*(3), 359–367. doi: 10.1007/s10934-006-9089-8

Santos, J. C., Nunes, G. F., Moreira, A. B., Perez, V. H., & de Castro, H. F. (2007). Characterization of candida rugosa lipase immobilized on poly (n-methylolacrylamide) and its application in butyl butyrate synthesis. *Chemical Engineering & Technology: Industrial Chemistry-Plant Equipment-Process Engineering-Biotechnology*, *30*(9), 1255–1261. doi: 10.1002/ceat.200700153

Satar, R., & Husain, Q. (2011). Catalyzed degradation of disperse dyes by calcium alginate-pectin entrapped bitter gourd (momordica charantia) peroxidase. *Journal of Environmental Sciences*, *23*(7), 1135–1142. doi: https://doi.org/10.1016/S1001-0742(10)60525-6

Sedaghat, M., Ghiaci, M., Aghaei, H., & Soleimanian-Zad, S. (2009a). Enzyme immobilization. part 3: Immobilization of α-amylase on na-bentonite and modified bentonite. *Applied clay science*, *46*(2), 125–130. doi: https://doi.org/10.1016/j.clay.2009.07.023

Sedaghat, M., Ghiaci, M., Aghaei, H., & Soleimanian-Zad, S. (2009b). Enzyme immobilization. part 4. immobilization of alkaline phosphatase on na-sepiolite and modified sepiolite. *Applied clay science*, *46*(2), 131–135. doi: https://doi.org/10.1016/j.clay.2009.07.021

Sen, D., Sarkar, A., Gosling, A., Gras, S. L., Stevens, G. W., Kentish, S. E., … Bhattacharjee, C. (2011). Feasibility study of enzyme immobilization on polymeric membrane: A case study with enzymatically galacto-oligosaccharides production from lactose. *Journal of Membrane Science*, *378*(1-2), 471–478. doi: https://doi.org/10.1016/j.memsci.2011.05.032

Shaolin, M. (1994). Bioelectrochemical response of the polyaniline galactose oxidase electrode. *Journal of Electroanalytical Chemistry*, *370*(1-2), 135–139. doi: https://doi.org/10.1016/0022-0728(93)03166-M

Sheldon, R. A. (2007). Enzyme immobilization: the quest for optimum performance. *Advanced Synthesis & Catalysis*, *349*(8-9), 1289–1307. doi: 10.1002/adsc.200700082

Shi, L., Tang, Z., Yi, Y., Chen, J., Xiong, W., & Ying, G. (2011). Immobilization of nuclease p1 on chitosanmicro-spheres. *Chemical and biochemical engineering quarterly*, *25*(1), 83–88.

Silva, V., Missagia de Marco, L., Delvivo, F., Coelho, J., & Silvestre, M. (2005). Immobilization of pancreatin in activated carbon and in alumina for preparing whey hydrolysates. *Acta Scientiarum - Health Sciences*, *27*, 163-169.

Sirkar, K., & Pishko, M. V. (1998). Amperometric biosensors based on oxidoreductases immobilized in photopolymerized poly (ethylene glycol) redox polymer hydrogels. *Analytical Chemistry*, *70*(14), 2888–2894. doi: 10.1021/ac971236x

Spasojević, D., Prokopijević, M., Prodanović, O., Pirtea, M. G., Radotić, K., & Prodanović, R. (2014). Immobilization of chemically modified horse radish peroxidase within activated alginate beads. *Hemijska industrija*, *68*(1), 117–122.

Sui, C.-h., Wang, Z.-y., Wei, Y.-q., & Wang, C. (2016). Immobilization of glucoamylase onto electrospun paa/pva microfibrous membrane by active ester method. *Chin. J. Process Eng.*, *16*(3), 494–499. doi: 10.12034/j.issn.1009-606X.215417

Tanriseven, A., Uludağ, Y. B., & Doğan, Ş. (2002). A novel method for the immobilization of glucoamylase to produce glucose from maltodextrin. *Enzyme and Microbial Technology*, *30*(3), 406–409. doi: https://doi.org/10.1016/S0141-0229(02)00004-2

Tibhe, J. D., Fu, H., Noël, T., Wang, Q., Meuldijk, J., & Hessel, V. (2013). Flow synthesis of phenylserine using threonine aldolase immobilized on eupergit support. *Beilstein journal of organic chemistry*, *9*(1), 2168–2179. doi: 10.3762/bjoc.9.254

Vallés, D., Furtado, S., Villadóniga, C., & Cantera, A. M. B. (2011). Adsorption onto alumina and stabilization of cysteine proteinases from crude extract of solanum granuloso-leprosum fruits. *Process biochemistry*, *46*(2), 592–598. doi: https://doi.org/10.1016/j.procbio.2010.10.016

Vidinha, P., Augusto, V., Almeida, M., Fonseca, I., Fidalgo, A., Ilharco, L., … Barreiros, S. (2006). Sol–gel encapsulation: an efficient and versatile immobilization technique for cutinase in non-aqueous media. *Journal of biotechnology*, *121*(1), 23–33. doi: 10.1016/j.jbiotec.2005.06.018

Vieira, M. F., Vieira, A. M. S., Zanin, G. M., Tardioli, P. W., Mateo, C., & Guisán, J. M. (2011). β-glucosidase immobilized and stabilized on agarose matrix functionalized with distinct reactive groups. *Journal of Molecular Catalysis B: Enzymatic*, *69*(1-2), 47–53. doi: https://doi.org/10.1016/j.molcatb.2010.12.009

Vineh, M. B., Saboury, A. A., Poostchi, A. A., Rashidi, A. M., & Parivar, K. (2018). Stability and activity improvement of horseradish peroxidase by covalent immobilization on functionalized reduced graphene oxide and biodegradation of high phenol concentration. *International journal of biological macromolecules*, *106*, 1314–1322. doi: 10.1016/j.ijbiomac.2017.08.133

Vitola, G., Mazzei, R., Fontananova, E., Porzio, E., Manco, G., Gaeta, S., & Giorno, L. (2016). Polymeric biocatalytic membranes with immobilized thermostable phosphotriesterase. *Journal of Membrane Science*, *516*, 144–151. doi: https://doi.org/10.1016/j.memsci.2016.06.020

Wang, W., Li, Z., Liu, W., & Wu, J. (2012). Horseradish peroxidase immobilized on the silane-modified ceramics for the catalytic oxidation of simulated oily water. *Separation and purification technology*, *89*, 206–211. doi: https://doi.org/10.1016/j.seppur.2012.01.031

Weiser, D., Sóti, P. L., Bánóczi, G., Bódai, V., Kiss, B., Gellért, Á., … others (2016). Bioimprinted lipases in pva nanofibers as efficient immobilized biocatalysts. *Tetrahedron*, *72*(46), 7335–7342. doi: https://doi.org/10.1016/j.tet.2016.06.027

Wohlgemuth, R. (2010). Biocatalysis—key to sustainable industrial chemistry. *Current opinion in biotechnology*, *21*(6), 713–724. doi: 10.1016/j.copbio.2010.09.016

Xing, G.-W., Li, X.-W., Tian, G.-L., & Ye, Y.-H. (2000a). Enzymatic peptide synthesis in organic solvent with different zeolites as immobilization matrixes. *Tetrahedron*, *56*(22), 3517–3522. doi: https://doi.org/10.1016/S0040-4020(00)00261-1

Xing, G.-W., Li, X.-W., Tian, G.-L., & Ye, Y.-H. (2000b). Enzymatic peptide synthesis in organic solvent with different zeolites as immobilization matrixes. *Tetrahedron*, *56*.

Yang, Z., Si, S., & Zhang, C. (2008). Study on the activity and stability of urease immobilized onto nanoporous alumina membranes. *Microporous and mesoporous materials*, *111*(1-3), 359–366. doi: https://doi.org/10.1016/j.micromeso.2007.08.009

Yavuz, A. G., Uygun, A., & Bhethanabotla, V. R. (2010). Preparation of substituted polyaniline/chitosan composites by in situ electropolymerization and their application to glucose sensing. *Carbohydrate Polymers*, *81*(3), 712–719. doi: https://doi.org/10.1016/j.carbpol.2010.03.045

Zdarta, J., Budzinska, K., Kolodziejczak-Radzimska, A., Siwinska-Stefanska, K., Bartczak, P., Piasecki, A., … others (2015). Hydroxyapatite as a support in protease immobilization process. *Physicochemical Problems of Mineral Processing*, *51*(2), 633–646.

Zdarta, J., Klapiszewski, L., Jedrzak, A., Nowicki, M., Moszynski, D., & Jesionowski, T. (2016). Lipase b from candida antarctica immobilized on a silica-lignin matrix as a stable and reusable biocatalytic system. *Catalysts*, *7*(1), 14.

Zdarta, J., Meyer, A. S., Jesionowski, T., & Pinelo, M. (2018). A general overview of support materials for enzyme immobilization: characteristics, properties, practical utility. *Catalysts*, *8*(2), 92.

Zhang, Y., Ge, J., & Liu, Z. (2015). Enhanced activity of immobilized or chemically modified enzymes. *ACS Catalysis*, *5*(8), 4503–4513. doi: 10.1021/acscatal.5b00996

Zhang, Y., Wu, H., Li, L., Li, J., Jiang, Z., Jiang, Y., & Chen, Y. (2009). Enzymatic conversion of baicalin into baicalein by β-glucuronidase encapsulated in biomimetic core-shell structured hybrid capsules. *Journal of Molecular Catalysis B: Enzymatic*, *57*(1-4), 130–135. doi: https://doi.org/10.1016/j.molcatb.2008.08.005

Zhu, D., Li, X., Liao, X., & Shi, B. (2018). Immobilization of saccharomyces cerevisiae using polyethyleneimine grafted collagen fibre as support and investigations of its fermentation performance. *Biotechnology & Biotechnological Equipment*, *32*(1), 109–115. doi: 10.1080/13102818.2017.1389302

Zhu, Y.-T., Ren, X.-Y., Liu, Y.-M., Wei, Y., Qing, L.-S., & Liao, X. (2014). Covalent immobilization of porcine pancreatic lipase on carboxyl-activated magnetic nanoparticles: characterization and application for enzymatic inhibition assays. *Materials Science and Engineering: C*, *38*, 278–285. doi: 10.1016/j.msec.2014.02.011