Chapter 14
Enzyme Biocatalysis and Sustainability

Carminna Ottone, Oscar Romero, Paulina Urrutia, Claudia Bernal, Andrés Illanes, and Lorena Wilson

14.1 Introduction

Enzymes are protein catalysts bearing an active site where specific amino acid residues are capable to recognize a substrate and catalyze its chemical conversion into product. Enzyme catalyzed reactions go from the simple hydrolysis of a substrate into smaller molecules to reactions of synthesis of highly valuable products [1, 2]. In some cases, enzymes are made by more than one polypeptide chain that interacts with each other by electrostatic forces or covalent bonds, forming an active quaternary structure [3].

The most significant property of enzymes is their high specificity toward substrates since they can recognize and act upon a molecule from a pool of similar compounds [2, 4]. In addition, enzymes work well under mild conditions of temperature and pH [1]. Despite these interesting properties, enzymes are vulnerable to environmental conditions, leading to loss of activity in time. To tackle this problem, enzyme immobilization by attachment to a solid support has proven to be a good alternative to stabilize their three-dimensional structure and preserve their catalytic properties [5]. Thus, the term "biocatalyst" refers to a catalyst having enzymatic activity, and frequently it is used to denote an immobilized enzyme. Enzyme immobilization has not only the advantage of producing robust and stable biocatalysts, but also the benefits of biocatalyst reuse for several cycles in batch operation or prolonged continuous use, and the easy removal of the catalyst from the reaction medium delivering a catalyst-free product stream [4, 6, 7].

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M. Piumetti, S. Bensaid (eds.), *Nanostructured Catalysts for Environmental Applications*, https://doi.org/10.1007/978-3-030-58934-9_14

With regard to molecular biology, focus has been on the development of novel enzymes by means of directed evolution [8, 9] or genetic engineering techniques [10], that allow to enhance the stability and catalytic performance of the enzymes by altering the sequence of the corresponding structural gene. Recently, nanozymes [11, 12] have been also proposed as promising catalysts making use of bioinformatics tools, where small peptides are synthesized aiming to preserve only the catalytic site of the protein. Independent of the chosen strategy to increase stability, it will need to be coupled with an adequate support material and the correct selection of the immobilization method. In addition, even if a highly active biocatalyst is obtained, the conditions of the process must be taken into consideration in order to obtain the desired product at an affordable economic cost.

Enzyme biocatalysis has become a mature technology for some industrial processes, including the synthesis of fructose syrup, the production of antibiotic precursors and bulk chemicals, like acrylamide, among others [7]. Enzymes have been extensively used as additives in a wide variety of food products, in detergents and cleansing products, in textiles, in winemaking, and in fruit juice processing [13, 14]. With regard to sustainability and the environmental field, enzymes have attracted much attention due to their biodegradability, which makes them a sound option to replace chemical processes [15, 16]. In particular, great progress has been experienced in using biocatalysts for the remediation of polluted areas [17–20] and for the upgrading of agro-industrial residues [21–23], which is in line with a circular economy approach.

This chapter summarizes the fundamentals of heterogeneous enzyme catalysis with immobilized enzymes and their use in environmental applications and sustainable development. The most suitable support materials for the environmental applications of immobilized enzymes are enlisted, considering the most common immobilization methods. The use of enzyme biocatalysts in bioremediation, in biofuel production, and in the valorization of waste streams is reviewed.

14.2 Enzyme Immobilization Techniques

The immobilization of enzymes is one of the most powerful tools to address the main problems of the industrial application of enzymes, namely, the lack of long term-stability and the difficulty to recover and reuse them. A benefit of the immobilization of enzymes is the enhanced stability under storage and operational conditions [4, 24]. These advantages are in compliance with technical and economic requirements of most chemical processes, where a continuous use of the catalysts for a long period of time is often necessary.

14.2.1 Methods of Enzyme Immobilization

Nowadays, a great diversity of immobilization methods for enzymes are available. Despite the basic methods of enzyme immobilization can be classified in few categories, several variations based on the original methods have been developed. The fundamental methods of enzyme immobilization can be classified into a few categories, however, several variations have been developed from them.

There are no general guidelines for choosing a specific immobilization method. A proper selection of the immobilization method should take into consideration the properties of the enzyme and the intended application of the biocatalyst. A summary of enzyme immobilization techniques and their advantages and disadvantages is shown in Table 14.1.

14.2.1.1 Chemical Interaction

This category includes methods in which the enzyme molecules are bound to an inert carrier, by a covalent or non-covalent linkage, and also the methods where the enzyme molecules are covalently linked among themselves.

In covalent immobilization, the enzyme is linked to the support by a covalent bond between amino acid residues of the enzyme and functional groups on the support surface. Most frequent amino acid residues for enzyme immobilization are

Table 14.1 Main advantages and disadvantages of the immobilization methods

Immobilization method	Advantages	Disadvantages
Chemical interaction		
a. Carrier bound		
Covalent immobilization	Strong interaction High stabilization	Significant loss of activity Enzyme structure distortion Complex protocols
Non-covalent immobilization	Reversibility of enzyme-support binding (recoverable support) Inexpensive and simple High expression of activity	Weak enzyme-support interaction (enzyme leakage) Low stabilization
b. Carrier free	High specific activities Simple, easy, and cheap	Mass transfer limitation Conformational changes Small and variable particle size. Poor mechanical properties
Physical containment		
a. Entrapment	Inexpensive and simple Minimal enzyme structure modification	Low stabilization Enzyme leakagel Mass transfer limitations
b. Membrane retention		Requirement of expensive equipment Low stabilization Membranes fouling

lysine, aspartic acid, glutamic acid, tyrosine, and tryptophan (Fig. 14.1a). Regarding the support, several functionalities can be introduced on its surface, with amine, carboxylic, epoxide, and aldehyde groups being the most used. A scheme of the most applied immobilization chemistry is shown in Fig. 14.1b.

Among the functional groups mentioned, aldehydes stand out for being able to generate several points of attachment between the enzyme and the support, promoting a strong stabilization of the immobilized enzyme [25]. This methodology has been widely used with a large number of enzymes, obtaining high stabilization factors [26]. However, supports functionalized with amines and carboxylic acids are the most widespread carriers for enzyme immobilization. The covalent linkage is formed by the addition of a crosslinking agent, usually carbodiimide, which activates the carboxylic acid to form an amide bond [27].

In the case of non-covalent immobilization, the enzyme is bound to the support by relatively weak and reversible interactions, like hydrophobic interactions, electrostatic interactions, Van der Waals forces, and hydrogen bonding. The multiple interactions formed between enzyme and support have a cooperative effect that promotes the stabilization of the enzyme. Non-covalent immobilization methodologies can be divided into: ionic [28], hydrophobic [29–31], and affinity adsorption [32, 33].

In carrier-free systems, the enzyme is immobilized in its own protein structure, without the need of an inert support. The enzyme is first insolubilized and then crosslinked using bifunctional reagents, like glutaraldehyde. Insolubilization can be done either by protein crystallization or precipitation under non-denaturing condition. The former are termed crosslinked enzyme crystals (CLECs) and require the enzyme protein to be in a pure state [34]; the latter are termed crosslinked enzyme aggregates (CLEAs) and can be produced by non-denaturing protein precipitation even from crude protein mixtures [35, 36].

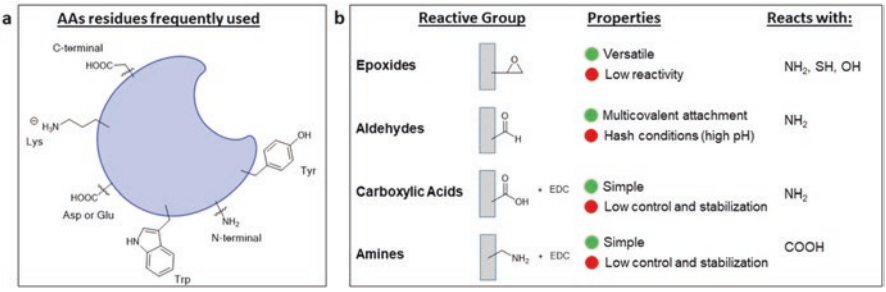

Fig. 14.1 Schemes of the most frequently used amino acid (AA) residues, (**a**), and functionalization groups of the support, (**b**), in covalent immobilization

14.2.1.2 Physical Containment

It corresponds to those methods in which the enzymes are retained in a confined space by physical means, and includes entrapment within polymer matrices and retention by permeable membranes.

In enzyme entrapment system, enzymes are embedded into a polymer matrix formed by chemical or physical means, like crosslinking or gelation. Usually the polymeric matrix is formed along with enzyme immobilization, so that the enzyme should be compatible with the matrix precursor (the corresponding monomer) and withstand the conditions required for polymerization. Polymeric matrices are quite flexible and can adopt different shapes, such as beads, films, fibers, and foams [37].

In membrane retention system, the enzyme is retained in a semipermeable membrane that allows the free passage of substrates and products [38].

14.2.2 Materials for Enzyme Support

Different materials have been used for the immobilization of enzymes, including a large variety of organic, inorganic, and hybrid materials [39]. The type of material used as support plays a crucial role having a strong effect on the performance of the immobilized enzyme. Table 14.2 shows some of the most frequently used materials for enzyme immobilization, including the commercial names of some. Although different companies supply these materials, few companies are manufacturing specially designed materials for the immobilization of enzymes and other biomolecules. Among the metal oxide materials, siliceous materials are the most reported. However, porous alumina [40] and titania nanoparticles have been also proposed as support materials for enzyme immobilization [41, 42].

Table 14.2 Classification of the most frequent materials used in enzyme immobilization, with the commercial name in parenthesis

Type of material	Examples
a. Organics	
Natural polymers	Agarose (Sepharose), chitosan (Chitopearl), alginate, dextran (Shephadex), cellulose (Cellufine)
Synthetic polymers	Polyacrylic acid (ChiralVision), poly(methacrylate) (ReliZyme), polystyrene—polyvinylbenzene (Purolite)
b. Inorganic	Silica, controlled pore glass (PureBiotech), zeolites, graphene, silico aluminates ceramics, titania, alumina

14.2.2.1 Organic Materials

The most traditional and frequently used polymeric matrices for enzyme encapsulation are: agarose, alginate, polyacrylamide, chitosan, and polyvinyl alcohol (PVA) [43]. Agarose beads activated with different functional groups have been extensively used for enzyme immobilization because they allow a high protein load. However, the use of agarose beads is limited to small-scale operations because of their small size and poor mechanical stability. Polymers, like alginate, chitosan, and PVA, have been extensity used for cell encapsulation with excellent results. However, the same encapsulation technique is not suitable for enzyme immobilization because of enzyme leakage, which has to be prevented by combining it with other immobilization technique. Enzyme leakage can be reduced by using a high concentration of polymer leading to small pores, but this will magnify mass transfer limitations. Other strategies to retain the enzymes molecules are the physical adsorption or chemical attachment of the enzyme to the polymer matrix by using functionalized polymers or by the addition of a crosslinking agent [44].

14.2.2.2 Siliceous Materials for Enzyme Immobilization

Siliceous materials are silica nets made of siloxane and Si-OH groups that exist as 3D polymers, whose units are regular SiO_4 tetrahedral structures with their vertices shared through Si-O-Si bonds [45]. Furthermore, these materials may contain some metallic oxides [40] and organic groups [46] providing suitable characteristics for specific enzyme immobilization processes. The synthesis of siliceous materials depends on the final requirements (e.g. morphology, porosity and chemical surface), the precursor type, and the enzyme to be immobilized, but usually the process involves hydrolysis and polycondensation reactions of siloxane groups [47]. Traditionally, a partially condensed silica source is used as precursor. The most used are tetraethyl orthosilicate (TEOS) and tetramethyl orthosilicate (TMOS), with which the hydrolysis and polycondensation reactions can be easily modulated. However, completely hydrolyzed precursor are also used, such as sodium silicate [48] and silicic acid [49].

Porosity is an important characteristic of siliceous materials that can be modulated, being possible to obtain hierarchical materials. Mesoporous and macroporous siliceous-based particles are most studied materials for enzyme immobilization. The pore size diameter ranges between 2 and 50 nm for mesoporous materials, and it is bigger than 50 nm for macroporous materials. The size of the pore is fundamental for achieving an effective enzyme immobilization: it needs to be higher than the enzyme molecule average size and provide enough space for a proper catalytic process in terms of substrates and products diffusion rates. In this regard, pore diameter, pore volume, and surface area have a strong effect on biocatalyst performance [50].

The other important characteristic is the chemical surface of the siliceous material, which can be modified with many functional groups, with the octyl, glyoxyl, epoxide, amino, and sulfonate groups being the most used for enzyme

immobilization [51]. Recently, there is the tendency of using supports with double chemical functionality, where covalent bond is formed with one functional group and non-covalent interaction is produced with the other. This heterofunctional strategy allows a proper enzyme orientation during the immobilization process, resulting in good biocatalyst performance [52]. Activation with glyoxyl or amino groups, combined with post-derivatization with glutaraldehyde, are the preferred options for covalent enzyme attachment to siliceous supports [53]. For non-covalent bonding the selection depends more on the enzyme to be immobilized. The immobilization of lipases is performed on silica activated with aliphatic groups, like octyl; for other enzymes, non-covalent immobilization is done using several functional groups and there is no specific trend, so that there is no general protocol to follow [10].

Conducting enzyme immobilization along with the formation of the siliceous materials is another way to obtain a heterogeneous biocatalyst. In this case, the enzyme is added during the sol-gel formation, which leads to siliceous precursors that interact with proteins forming complexes that after an ageing stage can produce an active and stable biocatalyst [54]. This strategy has many advantages, highlighting the possibility to have a one-step process of immobilization.

The applications of silica-based biocatalysts are quite diverse and are approached by multidisciplinary areas, including enzyme biocatalysis and materials science. There are still important challenges to be solved, including the reduction of diffusional restrictions that limit the enzymatic potential, the pore adaptation for big enzymes, and/or for enzyme acting on high molecular weight substrates, like proteases, polymer hydrolases, and synthetases. Moreover, finding a standard methodology that can be used for any enzyme to yield to an active and stable biocatalyst is a major challenge for future research.

14.2.2.3 Hybrid Matrices

Silica-organic hybrid matrices have gained importance as enzyme supports, since this kind of materials bring the best of two worlds together: the high surface area and stability of silica and the high enzyme-support compatibility of organic materials [55]. Several silica-hybrid carriers have been used for enzyme immobilization, by constructing silica-chitosan [56], silica-cellulose [57], silica-alginate [58], and silica-lignin composites [59]. In all cases, the catalytic performance of immobilized enzymes on hybrid silica materials is better than with the silica counterpart biocatalysts.

14.2.3 Coimmobilization of Enzymes

Enzyme coimmobilization is a recent feature in enzyme biocatalysis that consists in the immobilization of more than one enzyme in a single support particle, allowing the development of cascade reactions. In principle, coimmobilization allows a more

efficient catalysis approaching the conditions of a metabolic route inside a cell. Having several enzymes in close proximity may increase the efficiency of cascade reactions, reducing product or substrate inhibition and mass transfer limitations [60, 61].

The proportion of each enzyme in the so-called combi catalyst is quite important for the proper balancing of the corresponding reaction rates [62]. Therefore, the proportion of the enzymes offered to the support should be determined considering their respective kinetic parameters [63].

Some recent illustrative examples are: the coimmobilization of dehydrogenases for the conversion of CO_2 into methanol; the coimobilization of glycerol dehydrogenase, NADH oxidase and catalase [64]; the coimmobilization of glucose oxidase and horseradish peroxidase [65]; the coimmobilization of pyruvate kinase and lactic dehydrogenase [66]; and the coimmobilization of glucose dehydrogenase and malate dehydrogenase [67]. Many multi-enzyme processes involve coenzyme-requiring enzymes and therefore coenzyme regeneration is necessary; in such cases it is possible to coimmobilize cofactors and enzymes in the same support [68].

CLEA technology is well suited for enzyme coimmobilization. In this case, the resulting catalyst is termed combi-CLEA [69–71]. In this case, two or more enzymes are precipitated and crosslinked forming a combined catalytic particle. Combi-CLEAs have the same advantages and constraints of CLEAs: they have very high specific activities and are easy to prepare, while their mechanical properties may not be robust enough and particle size is difficult to control. In the case of combi-CLEAs, an additional problem is the non-uniform distribution of the enzymes within the catalyst particle.

14.2.4 Assessment of the Immobilization Process

Enzyme immobilization is a multivariable process, in which several factors influence the final result. In the last decades substantial efforts and progress have been made in understanding the immobilization process, but until now their molecular and physicochemical bases have not been fully elucidated [5].

Main parameters for evaluating the immobilization process are:

- Immobilization Yield (Y_A), which is the percentage of the contacted enzyme that is expressed in the biocatalyst, being calculated by a simple balance of activity. The determination of the residual activity in the supernatant allows knowing how much enzyme has been immobilized. Y_A reflects the deactivation of the enzyme due to the immobilization conditions (pH, temperature), conformational changes induced by the support, and the reduction in the activity by mass transfer limitations.
- Protein Immobilization Yield (Y_P), which is the percentage of the contacted protein that is immobilized in the biocatalyst. The most frequently used protein determination methods are not suitable for insoluble protein, so that the

immobilized protein is determined by the difference between the contacted protein and the residual protein in the supernatant.
- Specific activity (a_{sp}), which represents the enzyme activity per unit of mass of the resulting biocatalyst, being a very relevant parameter of enzyme immobilization.

14.3 Applications of Enzyme Biocatalysis for Sustainability

The most efficient systems for the reintegration of contaminant compounds to the natural cycles are biological systems, hence the efforts to develop different biotechnologies aiming to set up a sustainable development. In particular, enzymatic biocatalysis proposes several solutions to achieve this goal.

Enzyme biocatalysts can be used in different applications within a sustainability approach. Such applications can be classified into three main groups: bioremediation, biorefinery, and biofuels. Figure 14.2 shows a scheme of the main enzymes involved in each process.

In the field of green chemistry, new applications are being reported in the literature about the use of immobilized enzymes for the synthesis of different chemical compounds [72–74]. However, this type of application is beyond the scope of this chapter and will not be reviewed here.

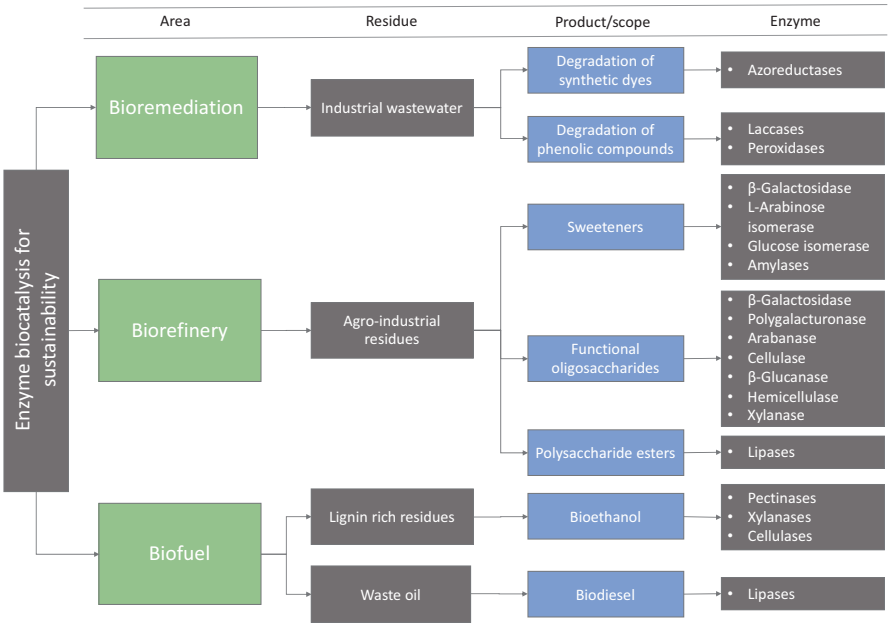

Fig. 14.2 Scheme of the main applications of enzymatic biocatalysis for sustainability

Different examples of the use of immobilized enzymes for bioremediation, biofuel production, and revalorization of carbohydrate-rich residues will be summarized.

14.3.1 Enzymatic Bioremediation

Bioremediation is a technique that goes hand in hand with the environmentally responsible industrial growth. Depending on the degree of pollution, type, and concentration of the contaminant, bioremediation can be carried out with plants, microorganisms, or enzymes. The type and number of water and soil pollutants that are degraded by enzymes increase along with research and innovation in the enzyme biocatalysis field.

The discovery of an enzyme that is able to hydrolyze polyethylene terephthalate, better known as PET, has been recently reported [75]. This finding has a paramount importance for our current lifestyle, where huge amounts of plastics are being disposed into the environment, with PET being one of the most abundant.

The use of enzymes is gaining relevance for the degradation of micropollutants that go through the microbiological waste treatments. Several hazardous endocrine disrupting chemicals (EDCs) coming from human activities, including fertilizers, dyes, and pharmaceutical bioactive components, are released into natural environments.

The addition of the enzymes into contaminated places can be designed according to the requirements of the place to be remediated, and care must be taken that no undesired byproducts are formed. Unlike microorganisms, enzymes do not require the presence of nutrients in the contaminated place. In addition, the advances achieved in enzyme immobilization allow the biocatalysts to be reused for several cycles, reducing the impact of the enzyme cost on bioremediation.

Extracellular oxidoreductases highlight for their capacity to degrade organic pollutants such as insecticides, herbicides, phenolic compounds, and hydrocarbons into less toxic compounds [76]. In particular, peroxidases [77, 78] and laccases [76, 79] are the two most studied enzymes in bioremediation processes. In addition, the study of the four groups of ligninolytic enzymes (i.e., lignin peroxidase, manganese-dependent peroxidase, versatile peroxidase, and laccase) coming from rot fungi (e.g., *Phanerochaete chrysosporium* and *Trametes versicolor*) is gaining increasing importance for bioremediation [76]. These enzymes are involved in the natural degradation pathway of lignin and cellulose, which are structurally and chemically similar to many organic pollutants.

The most popular enzymes used in bioremediation processes are enlisted below:

- Laccases (Lac) belong to a family of copper-containing polyphenol oxidases having a multicopper center in their active sites that catalyze the redox reactions [76].
- Peroxidases are oxidoreductases that utilize hydrogen peroxide to catalyze oxidative reactions [77]. Among the non-fungal enzymes, horseradish peroxidase

(HRP) has been widely studied for bioremediation purposes [80–82]. Versatile peroxidase (VP) oxidizes a wide range of molecules thanks to a surface catalytic tryptophan present in the active site that oxidizes low-redox, and, more significantly, high-redox potential substrates through a long-range electron transfer pathway to the heme complex, where Mn^{2+} is oxidized to Mn^{3+} [77, 83].

- Azoreductases are able to decolorize azo dyes into their corresponding colorless aromatic amines via the hydrolysis of the azo bond [55]. The reaction occurs only in the presence of the reduced form of nicotinamide-based coenzymes [19, 84].
- Other enzymes, like monooxygenase [18] and tyrosinases [85] are also reported in the literature for bioremediation purposes.

As far as the immobilization techniques is regarded, almost all of them have been used with peroxidases and laccases, where a wide variety of supports, going from polymeric beads [79, 86] to different metal oxides [87–90], have been used. A thorough review of the different immobilization techniques used for such enzymes can be found elsewhere [91, 92]. A selection of the most significant works found in the literature for bioremediation of wastewater containing synthetic dyes and phenolic compounds is presented below.

14.3.1.1 Degradation of Synthetic Dyes

Synthetic dyes are important pollutants. Leather, paper, cosmetics, and pharmaceutical industries employ over ten million tons of synthetic dyes per year in their industrial processes [19]. Half of them correspond to azo dyes, which are aromatic compounds with one or more –N=N– groups, many of them having carcinogenic effects [93]. Immobilized azoreductases [84], laccases [79, 91, 94], and horseradish peroxidase [95, 96] have shown great potential for the degradation or decolorization of azo dyes [19]. Some examples are shown below, as well as the immobilization methods and supports used for the preparation of the biocatalysts for the degradation of synthetic dyes.

A CLEA of HRP from *Armoracia rusticana* was used for the degradation of different synthetic dyes (i.e., basic red 9, indigo, methyl orange, rhodamin B, and rhodamine 6G) in a packed bed reactor [97]. The authors observed different decolorization percentages, varying from 73% to 95% according to the dye type and a residual activity of 60% after seven consecutive cycles.

The effective removal of methylene blue and orange II was studied with a laccase-based biocatalyst [94]. Lac was immobilized on a polymethacrylate/carbon nanotubes hybrid material by glutaraldehyde crosslinking. The maximum decolorization yields observed with methylene blue and orange II were 96% and 74%, respectively. The biocatalysts showed a high operational stability, with only 10% of activity loss after 10 successive reaction cycles.

Mesoporous silica particles with two different pore structures: ordered mesoporous silica (SBA-15) and mesocellular foams (MCF) were used to immobilize an

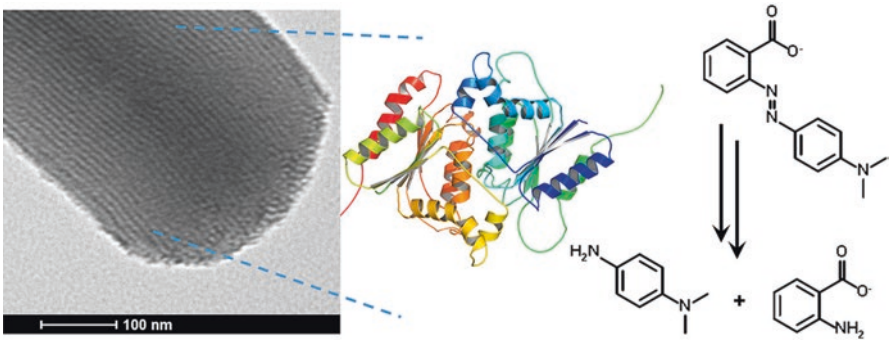

Fig. 14.3 Scheme of the degradation of an azo dye catalyzed by a biocatalyst made of azoreductase from *Rhodococcus opacus* 1CP immobilized on mesoporous silica. (Reprinted from [84])

azoreductase from *Rhodococcus opacus* 1CP [84]. Enzyme immobilization was performed by the functionalization of the support with epoxy and amino groups. Both biocatalysts showed a higher stability in acidic conditions with respect to the free enzyme. The latter was completely inactivated after 35 h of incubation at pH 4, whereas the immobilized enzymes retained 30% of the initial activity after 60 h of incubation. These biocatalysts were studied in the degradation of azo dyes. A scheme of the system is presented in Fig. 14.3.

14.3.1.2 Degradation of Phenolic Compounds

Phenolic compounds are aromatic molecules containing a hydroxyl group attached to the benzene ring structure that are found in several industrial effluents, such as in petrochemical and pharmaceutical plants, pulp mills, mines, and wood preservation plants [82]. The release of these hazardous compounds to the environment may cause serious health effects on the aquatic flora and fauna, and in humans [98]. Because of this, the US Environmental Protection Agency (EPA) has included phenol in the list of priority pollutants [99].

Different immobilized horseradish peroxidases have been studied for the degradation of phenolic compounds [92, 100, 101]. For instance, the removal of 2,4-dichlorophenol was studied with an HRP-based biocatalyst, where the enzyme was immobilized on polyacrylonitrile-based beads, modified with ethanediamine and chitosan, and activated with glutaraldehyde [100]. The results showed a 90% removal of the phenolic compounds by using this enzyme biocatalyst, which was reused up to three cycles with no activity loss, which confirms the robustness of the HRP-based biocatalyst that makes it suitable for large-scale application. A scheme of the biocatalyst preparation method is reported in Fig. 14.4.

Besides, a multienzyme approach was evaluated to study the possible synergistic effect between two or more enzymes. Vishnu et al. [90] studied the co-immobilization of Lac and VP on magnetic silica nanospheres activated with amine and vinyl

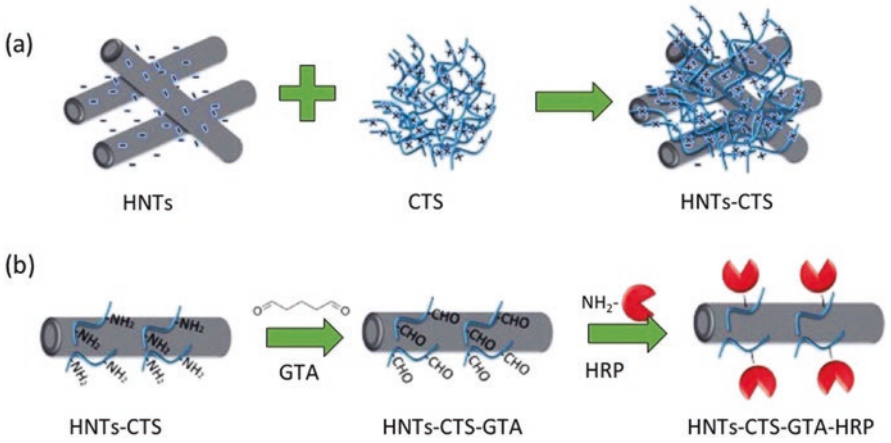

Fig. 14.4 Schematic illustration of preparation and immobilization mechanism. *HNT* halloysite nanotube, *CTS* chitosan, *GTA* glutaraldehyde, *HRP* horseradish peroxidase. (Reprinted from [100])

functional groups, obtaining an immobilization yield of 61% and 76% of retained activity for Lac and VP, respectively. Free enzymes and individually immobilized enzymes showed identical catalytic activity in terms of the degradation of the phenolic compounds in biorefinery wastewater, with 80% of phenols removal in 5 days, while the same reduction was achieved by the co-immobilized biocatalyst after only 1 day.

14.3.2 Biorefinery

An integral waste management is proposed in the context of circular economy that is based on the concept of biorefinery and the approach to reduce, reuse, and recycle waste [75]. Most of the agro-industrial residues are intendent for landfill or are disposed causing environmental damage and economic loss. However, agro-industrial residues are a good source of carbohydrates, proteins, and lipids, thus holding significant potential for enzymatic biotransformation into a variety of high-value compounds.

Food processing wastes rich in carbohydrates can be considered within a biorefinery concept, through their enzymatic transformation into value-added products, such as sweeteners and prebiotics, by the action of hydrolases and isomerases. The upgrading of agro-industrial wastes rich in carbohydrates varies according to their composition; therefore, opportunities for valorization are classified according to the carbohydrate chain length.

Significant amounts of lactose-containing dairy waste streams are generated every year [102, 103]. Whey is the main by-product of the dairy industry, especially

in cheese and casein production, and contains many underused nutrients, including 4.5–4.9% lactose [104]. Since a high quantity of lactose is discharged in whey as a waste, the use of immobilized enzymes has been mainly focused on lactose valorization by hydrolysis, transfructosylation, transglycosylation, transgalactosylation, isomerization, and epimerization [105].

Polysaccharides that are present in waste streams from the processing of vegetable, fruit, and crustacean products are considered as attractive substrates for enzymatic transformations. Enzymes such as amylases, cellulases, xylanases, and chitinases have the potential to convert these waste polysaccharides into different products, such as bioplastics, prebiotics, biofuels, and sweeteners [23].

14.3.2.1 Production of Sweeteners

Sweeteners such as fructose and rare sugars may be produced from lactose using different enzymatic biocatalysts, as illustrated in Fig. 14.5.

In the case of the production of fructose syrup from lactose using a bi-enzymatic system, a mixture of fructose, glucose, galactose, and some residual lactose in the final product results in an attractive sweetener for dairy products. The first reaction involved in this process is the hydrolysis of lactose into glucose and galactose by a β-galactosidase (GAL), which is followed by glucose isomerization into fructose by a glucose isomerase (GI) [69, 106, 107–111]. The fructose syrup produced enzymatically from lactose has been applied as a sweetener in ice-cream [107] and yoghurt [112]. Production of fructose syrup from lactose by immobilized forms of GAL and GI has also been reported. Arndt and Wehling [107] immobilized GAL by adsorption in microporous plastic sheets and used the commercial catalyst Maxazyme GI-Immob (Gist-Brocades), where GI is entrapped within gelatin particles and then crosslinked with glutaraldehyde [113]. A trienzymatic system for the

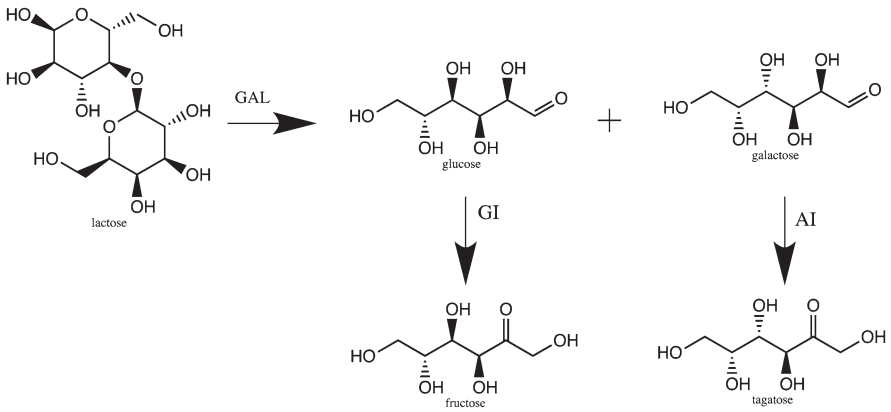

Fig. 14.5 Enzymatic synthesis of fructose and D-tagatose from lactose. *GAL* β-galactosidase, *AI* L-arabinose isomerase, *GI* glucose isomerase

conversion of lactose into fructose and tagatose was reported [111] using GAL, L-arabinose isomerase (AI) and GI immobilized separately on the commercial support Eupergit C. Recently, Araya et al. [69] investigated the co-immobilization of GAL and GI in crosslinked enzyme aggregates (combi-CLEAs) for the production of fructose syrup from lactose. The presence of amino groups on the enzyme surface is a key factor for CLEAs formation, since they are necessary for the crosslinking of the precipitated molecules. Due to the lack of sufficient amine groups on the GI surface, its carboxylic groups were chemically aminated to favor the crosslinking process. Combi-CLEAs preparation was optimized, finding that using a GI-GAL activity ratio of 0.2 and a glutaraldehyde-protein mass ratio of 1.67 resulted in a biocatalyst with good mechanical properties, high expressed activity of both enzymes, and the highest reaction rates of hydrolysis and isomerization among the biocatalysts tested. The selected biocatalyst was utilized in five sequential batch operations obtaining a lactose conversion close to 90% in all batches, with a glucose-fructose conversion close to 45%, which represents approximately 90% of the equilibrium conversion. Results showed that the combi-CLEAs can be used at least for five sequential batches (equivalent to 50 h of operation) with no loss in product quality. Fructose content in the product was around 22% of the total carbohydrates.

Rare sugars synthesized from lactose represent another option to valorize a waste stream. Rare sugars are monosaccharides and their derivatives that rarely exist in nature and are not easily metabolized by the living organisms, though possessing beneficial health effects. Rare sugars such as D-psicose, D-allose, and D-tagatose are quite interesting due to their high relative sweetness (70–92% of sucrose) and low caloric value (0–2 kcal/g) [114, 115]. After a first step of lactose hydrolysis, rare sugars may be produced from glucose or galactose using additional enzymes [114]. In spite of the attractive properties of rare sugars, many of the enzymes that have been utilized for their production are not commercial; therefore, extensive research is still needed [23]. Until now, investigation about the synthesis of rare sugar by immobilized enzymes is limited. In the case of D-tagatose, research has been carried out mainly using permeabilized and immobilized cells [116, 117]. Recently, Torres and Batista-Viera [111] have reported the synthesis of D-tagatose and fructose from lactose using three immobilized enzymes: GAL for the hydrolysis of lactose, AI for the isomerization of galactose to D-tagatose, and GI for the isomerization of glucose into fructose. L-arabinose isomerase was produced from *Enterococcus faecium* and purified by affinity chromatography. Each enzyme was immobilized in Eupergit C and Eupergit C 250 L. Sequential application in separate bio-reactors of immobilized GAL, L-arabinose isomerase, and D-xylose isomerase in the biotransformation of 4.6% lactose in phosphate buffer pH 7.0 resulted in 31% of D-tagatose conversion after 6 h of operation at 50 °C. Under similar operation conditions, lower productivity and conversion were obtained with the soluble enzymes than with the corresponding immobilized biocatalysts.

Starch-rich waste streams are originated from the processing of rice, corn, potato, and sweet potato. This polysaccharide may be hydrolyzed by the use of several enzymes obtaining saccharides of different polymerization degree (Fig. 14.6).

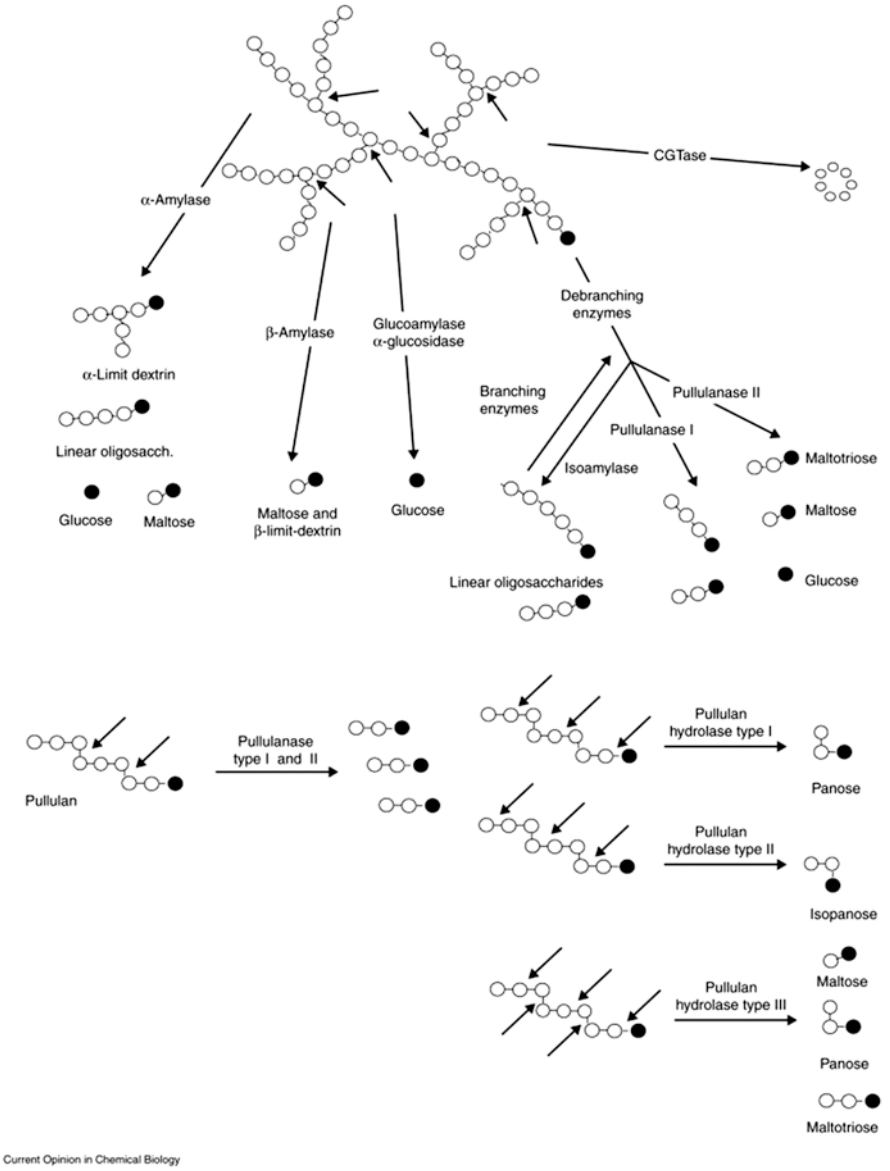

Fig. 14.6 Schematic representation of the action of amylases. Black circles indicate reducing sugars. (Reprinted from [118])

The most common utilization of starch is through its hydrolysis into monosaccharides, as in the case of glucose syrup production. Glucose syrup may be enzymatically produced from starch in a two-step process: α-amylase hydrolyses the α (1–4)glycosidic bonds in starch by a so-called liquefaction process, which is

followed by saccharification, in which glucoamylase breaks α (1–6) as well as α (1–4) glycosidic bonds to generate glucose [119]. Both enzymes have been immobilized in order to increase yield and process efficiency. In most of the investigations α-amylase and glucoamylase are immobilized independently using organic or inorganic supports [120–123]. Co-immobilization is another alternative to carry out multi-step cascade reactions but in a single pot. This one-pot strategy has several advantages, such as smaller reactor volumes, fewer unit operations, less solvent usage, shorter reaction time, higher volumetric and space time yields, and less waste generation [124]. Co-immobilization of α-amylase and glucoamylase for their application in starch hydrolysis has been reported using silica gel and DEAE-cellulose entrapped in alginate beads [121] and metal organic frameworks [124]. Edama et al. [125] reported the co-immobilization of α-amylase, glucoamylase, and also cellulase in calcium alginate clay beads for using such biocatalyst in the saccharification of starch. After 7 cycles with 1 h of reaction time each, the biocatalyst still retained 33% of its activity (measured in terms of the release of reducing sugars). In other investigations, α-amylase and glucoamylase were co-immobilized with pullulanase. Pullulanase is a debranching enzyme that has been included since glucoamylase is slower in hydrolyzing α (1–6) bonds. The three enzymes were co-immobilized in the form of combi-CLEAs [71] and in magnetic nanoparticles using glutaraldehyde as crosslinker [126]. In both investigations, the o-immobilizationc of the three enzymes allowed a higher conversion than using the free enzymes in a one-pot reaction.

14.3.2.2 Production of Functional Health-Promoting Oligosaccharides

GAL may also be used as catalyst for the synthesis of galacto-oligosaccharides (GOS) and lactulose (Fig. 14.7), that are recognized as prebiotic [127, 128].

The production of GOS occurs by the transgalactosylation of lactose, which is a kinetically controlled reaction. In the first step of reaction, β-gal forms a galactosyl-enzyme complex after attacking the anomeric center of the galactose residue in lactose, releasing a glucose molecule to the medium [129, 130]. The second step of the reaction depends on the acceptor substrate: if it is water, the galactosyl-enzyme complex undergoes hydrolysis, releasing a galactose molecule; if the acceptor is lactose, transgalactosylation occurs resulting in the production of GOS [130–132]. The predominance of synthesis over hydrolysis depends mainly on the origin of the β-gal [130, 133, 134], the initial sugar concentration [135], and the water thermodynamic activity [136, 137]. GALs of different sources have been immobilized in organic and inorganic carriers for GOS synthesis. Among organic carriers, agarose and chitosan have been reported for the immobilization of GAL from *Aspergillus oryzae* [138–143], *Aspergillus niger* [144], *Bacillus circulans* [145, 146] and *Kluyveromyces lactis* [147, 148]. The enzyme has been covalently bound to both organic carriers activated with aldehyde groups [138–140, 142, 146, 148] or through a two-step process using heterofunctional supports where the enzyme is first adsorbed and then covalently linked [139, 143, 146]. In the case of inorganic sup-

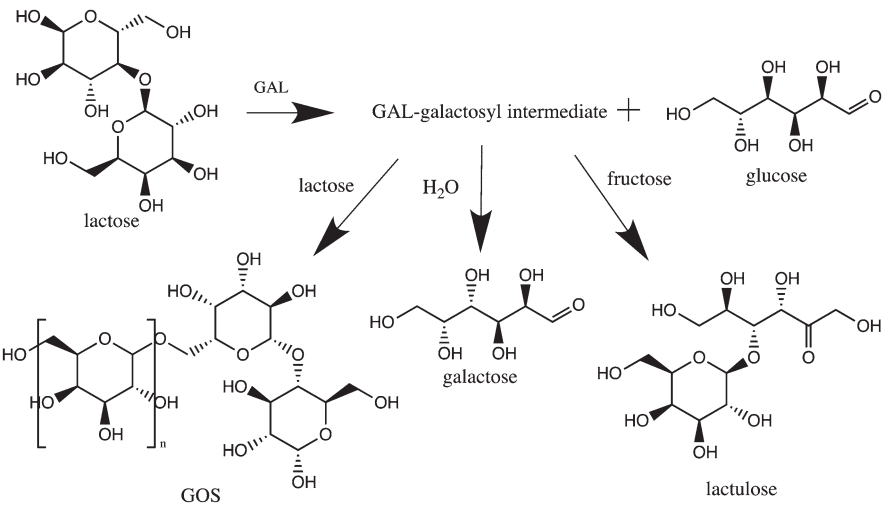

Fig. 14.7 Schematic representation of the enzymatic synthesis of GOS and lactulose from lactose. *GAL* β-galactosidase

ports, synthesis of GOS has been reported for *B. circulans* GAL immobilized in silica supports [149, 150]. It has also been reported for GALs from *A. oryzae* [151], *A. aculeatus* [152] and *K. lactis* [153]. In the case of this siliceous carrier, immobilization is mostly done by adsorption [151, 153] and covalent binding [151, 152]. Mozzafar et al. [149, 150] carried out a two-step process, where the enzyme was first adsorbed and then glutaraldehyde was added to covalently bind the adsorbed enzyme. Among these studies, the investigations of Misson et al. [153] and Banjanac et al. [151] stand out since the utilization of the immobilized enzyme resulted in a production of GOS two or three times higher than obtained with the soluble enzyme at the same conditions, indicating that transgalactosylation was favored over hydrolysis when using the immobilized enzyme. In both cases the best biocatalyst performance was obtained with nanoparticles of silica functionalized with amino groups.

β-galactosidases can be used also in the synthesis of lactulose from lactose through a transgalactosylation reaction, using fructose as galactosyl acceptor [154]. Lactulose is a synthetic ketose disaccharide that is widely used as a drug against constipation and hepatic encephalopathy and also as a prebiotic food additive [155]. Lactulose synthesis using immobilized enzymes has been carried out using mainly organic supports such as agarose [139, 156, 157, 158–160] and chitosan [161]. Immobilization occurred by the covalent binding of GAL, using a one- or two-step process as in GOS synthesis. Only one investigation has been reported for the synthesis of lactulose in silica supports, where GAL from *K. lactis* was covalently immobilized in silica gel functionalized with glutaraldehyde; the immobilized enzyme was reutilized and 52.9% of the initial activity was retained after 10 cycles of use. Continuous synthesis of lactulose was also performed in a packed-bed

reactor operated at a flow rate of 0.5 mL/min producing a product stream with 19.1 g/L of lactulose [162].

Pectic substances (polygalacturonic acid, methyl-esterified polygalacturonic acids) extracted from vegetable and fruit wastes can be upgraded to produce fillers, texturizers, thickeners, and glazes. Additionally, the enzymatic hydrolysis of pectin results in the production of pectic oligosaccharides (POS), compounds with potential health benefits. POS are quite variable in structure and degree of polymerization, and include arabinose, xylose, rhamnose and galactose as sugar units [163]. Baldassarre et al. [164] carried out the production of POS from onion skins in order to valorize this agricultural waste. The hydrolysis was carried out using the commercial enzyme preparation Viscozyme L (including carbohydrases such as arabanase, cellulase, β-glucanase, hemicellulase, and xylanase) using a cross-flow continuous membrane enzyme bioreactor. A stable POS production was obtained at a volumetric productivity of 22 g/L/h and 4.5 g/g POS/monosaccharides. Recently, Ramírez-Tapias et al. [165] reported the saccharification of citrus wastes by polygalacturonase immobilized by encapsulation in an alginate matrix. Orange peel represents a large fraction of the by-products generated from citrus processing, and the polysaccharide composition of its albedo is rich in pectin, so it has great potential as raw material for the production of oligogalacturonides. Different bacterial strains of *Streptomyces* were immobilized in alginate gel and the best results in terms of activity and stability were obtained with *Streptomyces halstedii* ATCC 10897 immobilized in the alginate matrix. The hydrolysis of albedo from orange peels with this cell biocatalyst was maximum at 2 h of reaction, generating 1.54 g/L of reducing sugars and decreasing the viscosity of polygalacturonic acid by 98.9%. This immobilized cell biocatalyst with polygalacturonase activity allowed obtaining a product with 9% (w/w) of valuable sugars on a dry basis, which could be used as a nutraceutical food ingredient and as fermentable sugars.

14.3.2.3 Production of Polysaccharide Esters

Starch acylation represents another opportunity for the valorization of starch-containing waste streams. The acylation of starch hydroxyl groups results in different types of polysaccharide esters having a wide range of applications. Acetylated starch with low degree of substitution is used in the food industry to control and adjust the rheological behavior of pastes [166], while succinylated starches reinforce the swelling capacity at lower temperature [167]. Immobilized enzyme catalysts have been utilized to improve enzyme stability in the solvents that are required to solubilize both the starch and the acyl donor substrates. Chakraborty et al. [168] investigated the regioselective modification of starch nanoparticles with *Candida antartica* lipase B in its immobilized (Novozym 435) and free (SP-525) forms. Starch nanoparticles reacted with vinyl stearate, ε-caprolactone, and maleic anhydride using Novozym 435 at 40 °C for 48 h to give starch esters with of 0.8, 0.6, and 0.4 degrees of substitution (DS), respectively. Horchani et al. [169] reported the use

of a non-commercial $CaCO_3$-immobilized lipase from *Staphylococcus aureus* (SAL3) to catalyze the esterification reaction between pure oleic acid and starch using microwave heating followed by liquid state esterification. A 76% conversion with a DS of 2.86 was obtained after optimization of the reaction conditions.

14.3.3 Biofuel

The European Commission defines biofuels as liquid or gaseous transport fuels that are made from biomass. Biofuels represent a renewable alternative to fossil fuels in the transport sector and a sound technology to reduce greenhouse gas emissions. If industrial waste is considered as raw material, the production of biofuels by means of enzymatic technology meets all the criteria for environmental sustainability.

14.3.3.1 Production of Bioethanol

Lignocellulose or cellulosic-based waste materials are other raw materials for the production of valuable bioproducts, including rare sugars, surfactants, and biofuels. Lignocelluloses are complex heterogeneous natural composites that comprise three main biopolymers: lignin, cellulose, and hemicellulose. Due to the recalcitrant chemical nature of this material, its valorization requires a multi-enzyme system. Perwez et al. [170] prepared and characterized magnetic combi-CLEAs of pectinases, xylanases and cellulases for the saccharification of wheat straw prior to the fermentation of the resulting sugars. The catalyst was produced adding amino-functionalized magnetic nanoparticles into a mixture of pectinases, xylanases, and cellulases. Using this biocatalyst for the saccharification step, bioethanol concentration was 1.82-fold higher than obtained with free enzymes and could be efficiently reused for 12 cycles, after which pectinase, xylanase, and cellulase retained 86.5%, 90.3%, and 88.6% of activity, respectively. These results show that combi-CLEA methodology can be used for a variety of industrial applications, like food processing, textiles, and bioethanol production. Similarly, Periyasamy et al. [171] reported the immobilization of cellulase, xylanase, and β-1,3-glucanase in silica-amine functionalized iron oxide magnetic nanoparticles and their application in the depolymerization of cellulosic biomass into monomeric sugars. The enzymes were adsorbed and glutaraldehyde was utilized for their crosslinking. The biocatalyst was reused for at least eight consecutive cycles retaining over 70% of its initial activity and the resulting product exhibited approximately 15% increase in carbohydrate digestibility on sugarcane bagasse and eucalyptus pulp with respect to the one obtained with the free enzyme.

14.3.3.2 Biodiesel Production

The replacement of alkaline transesterification for biodiesel production by an enzymatic technology has attracted increasing interest because of its advantages over chemical catalysis. Biodiesel can be produced from fresh and waste oil. The valorization of the latter gives an additional input in the sustainable development direction.

Lipases from different sources and different immobilization methods have been utilized for conducting lipids transesterification, since the cost of the enzyme is a main obstacle for industrial biodiesel production. Badoei-dalfard et al. [22] reported the covalent bonding of Km12 lipase CLEAs in amino-coated magnetite nanoparticles. Covalent linkage of CLEAs to the carrier was conducted by contacting the immobilized enzyme with nanoparticles in the presence of glutaraldehyde. Biodiesel production from waste cooking oils by the immobilized biocatalyst increased about 20% with respect to the free enzyme, and the immobilized biocatalyst remained fully active up to 6 cycles, indicating that crosslinking of lipase and amino-coated magnetite nanoparticles produced operationally stable CLEAs. Another interesting strategy recently reported for lipase immobilization was the use of 5-aminoisophthalic acid as a novel metal-chelating ligand. This acid was successfully grafted onto magnetic nanoparticles (MNP) for the Co^{2+}-chelated affinity immobilization of *Pseudomonas fluorescens* lipase [172]. The MNP-lipase was used for the production of biodiesel from waste cooking oil, and 95% yield was achieved. Biodiesel yield was still 83% after 10 cycles of repeated use, showing a good operational stability. The chelated support could be regenerated and reused after enzyme activity exhaustion, which can reduce the costs associated to the synthesis of the support. This newly designed strategy has great potential in biotechnological applications [172]. A different alternative is the use of a multi-enzyme system for biodiesel production. Babaki et al. [21] studied the production of biodiesel from waste cooking oil using a co-immobilized biocatalyst of lipase from *Rhizomucor miehei* and lipase B from *Candida antarctica* covalently bound onto epoxy-silica. This biocatalyst allowed removing the acyl-migration step, which is the rate-determining step in biodiesel production. The effect of different factors such as enzyme to substrate ratio, *t*-butanol to oil ratio, adsorbed water content, and reaction duration was studied and optimized. A high yield of fatty acid methyl esters (91.5%) was obtained after 10 h of reaction. Zhang et al. [173] analyzed the impact of support characteristics on enzyme performance. The authors compared mesoporous silica supports of varying channel sizes (1.8, 14.0, and 28.0 nm) for lipase (Lipase LVK-S200 from LEVEKING Co. Ltd.) immobilization and reported an optimal 80.1% yield of biodiesel from unrefined waste cooking oil using the enzyme immobilized in the mid-range 14 nm channel size support. The increase of the channel size increased the specific activity of the biocatalyst to a point representing an optimal channel dimension. It was also observed that the need for channel size optimization was conditioned by the nature of the feedstock: the more complex nature of waste cooking oil (with insoluble materials which may block smaller channels) benefited from channel size optimization, while pure olive oil was less sensitive to channel size.

14.4 Conclusions

The impact of heterogeneous enzyme biocatalysts in sustainable development has been highlighted. Different strategies for the attachment of the protein structure of the enzyme to a porous matrix were presented showing the advantages and disadvantages of each one. The procedure of synthesis and the properties of silica, which is one of the most used support materials, was also described. However, the selection of the support and the immobilization strategy will strictly depend on the final application of the biocatalyst. On the one hand, the cost of the biocatalyst is an important issue in environmental applications, which can limit their field implementation. On the other hand, the applications related to the environment and sustainability are quite diverse; thus, they have to comply with different types of regulations and require the use of different enzyme biocatalysts. For bioremediation, oxidoreductases, like laccases and peroxidases, offer good opportunities for the recovery of areas contaminated with different organic pollutants. For biodiesel production, immobilized lipases have been utilized for lipids transesterification. Pectinases, xylanases, and cellulases contribute to bioethanol production by hydrolyzing long-chain carbohydrate polymers into fermentable sugars. β-Galactosidase has a great potential for the revalorization of lactose, as catalyst for the synthesis of prebiotics, such as GOS and lactulose. In addition, when combined with another enzyme, such as L-arabinose isomerase, it can be used to produce rare sugars, like D-tagatose, a sugar that has both sweetening and health-promoting properties.

Despite the difficulties that still have to be overcome, there is no doubt that enzyme biocatalysis will help in achieving a circular economy model and an environmentally sustainable industry.

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