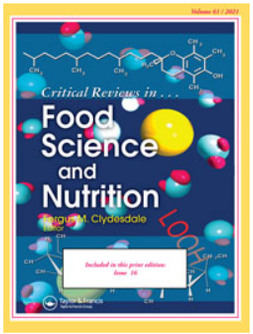

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REVIEW

Recent advances in β-galactosidase and fructosyltransferase immobilization technology

Maria Micaela Ureta[a], Gonçalo Nuno Martins[b], Onofre Figueira[b], Pedro Filipe Pires[b], Paula Cristina Castilho[b], and Andrea Gomez-Zavaglia[a]

[a]Center for Research and Development in Food Cryotechnology (CIDCA, CCT-CONICET La Plata), La Plata, Argentina; [b]CQM – Centro de Química da Madeira, Universidade da Madeira, Funchal, Portugal

ABSTRACT

The highly demanding conditions of industrial processes may lower the stability and affect the activity of enzymes used as biocatalysts. Enzyme immobilization emerged as an approach to promote stabilization and easy removal of enzymes for their reusability. The aim of this review is to go through the principal immobilization strategies addressed to achieve optimal industrial processes with special care on those reported for two types of enzymes: β-galactosidases and fructosyltransferases. The main methods used to immobilize these two enzymes are adsorption, entrapment, covalent coupling and cross-linking or aggregation (no support is used), all of them having pros and cons. Regarding the support, it should be cost-effective, assure the reusability and an easy recovery of the enzyme, increasing its stability and durability. The discussion provided showed that the type of enzyme, its origin, its purity, together with the type of immobilization method and the support will affect the performance during the enzymatic synthesis. Enzymes' immobilization involves interdisciplinary knowledge including enzymology, nanotechnology, molecular dynamics, cellular physiology and process design. The increasing availability of facilities has opened a variety of possibilities to define strategies to optimize the activity and re-usability of β-galactosidases and fructosyltransferases, but there is still great place for innovative developments.

KEYWORDS

β-galactosidase; immobilization methods; fructosyltransferase; supports

Introduction

Enzymes are biocatalysts responsible for specific chemical reactions, where a set of reactants (substrates) are converted into specific products during complex metabolic processes essential to sustain life (Illanes, Wilson, and Vera 2014). They are proteins that act as catalysts in living organisms, participating in biological processes, and regulating the rate at which chemical reactions proceed, without altering their equilibrium (Aehle 2007; Purich 2001). Furthermore, enzymes are integral parts of industrial processes since some of the reactions in which they participate would not be feasible without their aid. When enzymes are used in such applications, the overall cost of the process is lowered because enzymes are eco-friendly (reusable and biodegradable), obtained from renewable resources (microorganisms), and they increase the process's efficiency, requiring mild operating conditions (temperature, pH, energy), similar to physiological ones, and producing less waste and overall cleaner products (Schäfer et al. 2007; de Albuquerque et al. 2018).

Industrial chemical reactions can occur under extreme conditions, in terms of temperature, pH and presence of salts, surfactants, and organic solvents. These conditions may greatly lower the usefulness of enzymes due to their destabilization, so it is important from a technological and economical point of view to ensure enzyme stability (Silva et al. 2018). This can be achieved by implementing different methods, such as using innovative natural enzymes (screening the microbiota and metagenomics), the development of new enzymes (mutagenesis, directed evolution and enzyme engineering design), catalyst engineering (chemical modification, immobilization on solid matrices or auto-aggregation), medium engineering (use of non-conventional reaction media or the addition of cryoprotectants and surfactants, to aid during dehydration and storage), catalyst reactivation (reactivation of the enzyme after achieving activity exhaustion) and process engineering (design of scale-up processes that maintain or improve enzymes' stability, activity and specificity) (Behrens et al. 2011; Davids et al. 2013; Illanes, Wilson, and Vera 2014; Moehlenbrock and Minteer 2017).

During the last decades, enzyme immobilization had emerged as a suitable methodology having shown successful results (Illanes, Wilson, and Vera 2014; Moehlenbrock and Minteer 2017; de Albuquerque et al. 2018; Gonçalves et al. 2019; Sass and Jördening 2020). This strategy promotes enzyme stabilization and guarantees reusability of the catalyst, simplifying its removal from the reaction medium. This review will focus on immobilization strategies addressed to

CONTACT Maria Micaela Ureta micaelaureta@gmail.com Calle 47 y 116 La Plata, Buenos Aires 1900, Argentina.

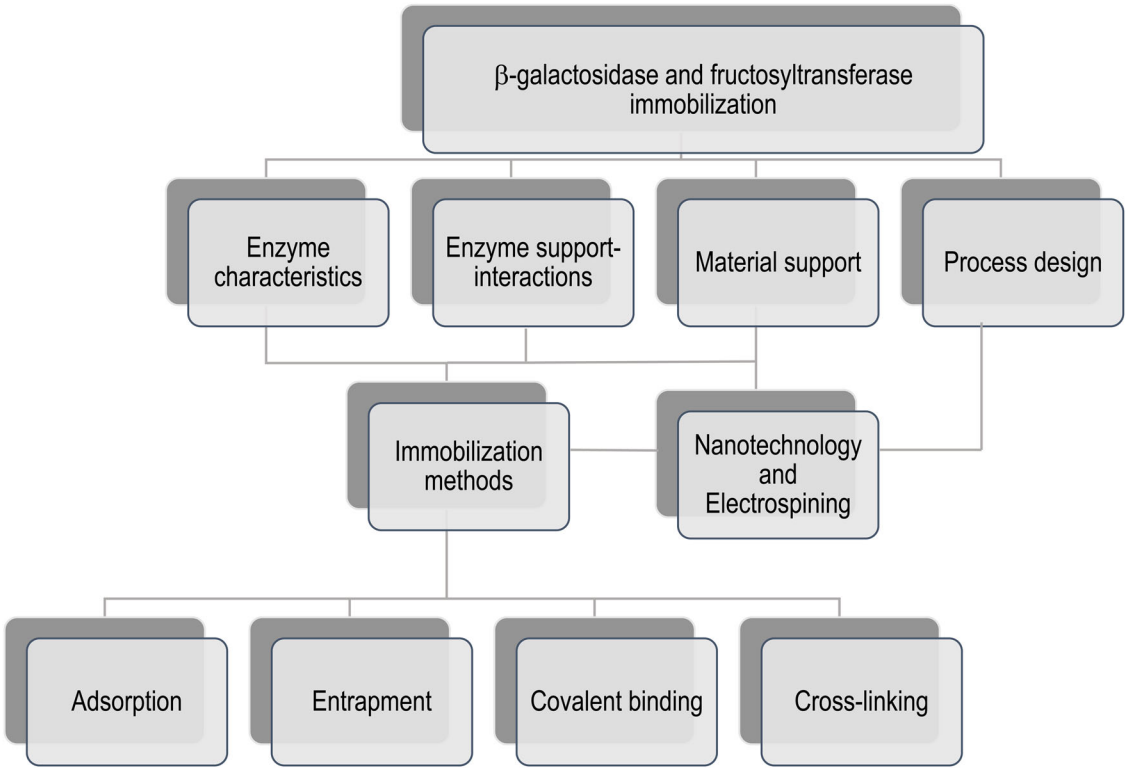
achieve optimal industrial processes. To this aim, special care was put on the physical and chemical fundamentals explaining the enzyme-support interaction, not forgetting the properties of the carrier materials, a key factor to optimize immobilization. All these aspects were specifically reported for two types of enzymes: β-galactosidases and fructosyltransferases, which are used to obtain GOS and FOS, respectively. These oligosaccharides are among the most widely employed prebiotic compounds with several applications in the food and pharmaceutical industries, indeed their demand has exponentially increased worldwide over time (Martins et al. 2019). Particularly, enzymes' cost is one of the most critical issues in the industrial production of prebiotics. For this reason, design processes need to consider both economic and technological aspects, and enzymes' immobilization emerges as a promising strategy to improve their stability and reusability. Scheme 1 provides a graphical representation of how the manuscript information was organized to guide the reader through the main discussed items.

Enzymatic production of GOS and FOS: immobilization as a process design strategy

Immobilization proved to be a good alternative among the previously mentioned methods used to achieve enzyme stabilization mainly for being cheaper, simpler and providing a better chance of reusing and recovering the enzyme. In particular, when dealing with enzymes that are expensive, rare or difficult to purify, the reusability, stability over storage and transport, and ease of recovery are critical issues (Dwevedi 2016).

Immobilization involves the attachment or incorporation of enzymes into a support material. An insoluble, reusable, and more resistant form the enzyme is obtained, which can participate in chemical reactions with different process conditions, ideally without significant loss of stability or activity (Silva et al. 2018; Sirisha, Jain, and Jain 2016). Immobilized enzymes (IE) are used in several fields, such as in the food and pharmaceutical industries, in medicine, for the production of biofuels, detergents and cleaning products, and in bioremediation and waste waters' treatment. There are several reviews on this topic that delve into the use of several enzymes, the immobilization procedures and their applications (Basso and Serban 2019; DiCosimo et al. 2013; Dwevedi 2016; Franssen et al. 2013; Nguyen, Lee, et al. 2019; Sirisha, Jain, and Jain 2016).

Each active site of an enzyme is composed of a few amino acid residues and this part of the molecule is responsible for its catalytic activity. The rest of the enzyme is important for structural stability during catalysis (Illanes, Wilson, and Vera 2014) and environmental alterations (temperature, pH) can cause conformational changes and, consequently, loss of activity (Silva et al. 2018). This happens because the amino acid residues of the active site are very close in the folded protein, held in position by intermolecular forces, but can be very distant in the primary structure, and denaturation can lead to their separation, thus losing the catalytic activity (Illanes 2008). This effect, however, can be beneficially used, for example, when designing an immobilization procedure for controlling (stopping) the reaction. Immobilization should not block or hinder access to the active site of the enzyme, although it can be used for the steric exclusion of inhibitors. This can lead to an increase in



Scheme 1. Organization of the relevant issues discussed in this review.

activity, often mistaken for the obtaining of a more advantageous conformation of the enzyme after immobilization, when, in fact, there is loss of inhibition (Rodrigues et al. 2013; Nguyen, Styevkó, et al. 2019).

Reactions using free enzymes (FEs) are homogenous systems, i.e., the enzyme is in solution among the substrates, co-factors, products and other species relevant to the process. Working with IE implies heterogeneous systems since the enzyme exists in a different phase than the solutes (the enzyme is now insoluble). The kinetics of the two systems are not the same. Limitations in mass-transfer together with the reduction of enzymes' conformational mobility due to enzyme-carrier interactions often compromise enzyme activity and, consequently, the reactions' kinetics (Illanes, Wilson, and Vera 2014; Gonçalves et al. 2019). When designing new systems, the gain in stability *per se* may not compensate for slower reaction rates. There are no standardized concepts about the most appropriate immobilization technique for the different biocatalysts in the industry. In fact, this selection must be done with an optimization *via* trial and error comparing the activity, stability, and reusability of the FE with those of the IE.

What is more, industrial reactors can be designed to work in batch or in continuous mode. In the former, limited amounts of reactants are placed in a confined environment during the time needed for the reaction to be completed. Afterwards, the enzyme must be separated, often leading to its inhibition or inactivation. Eventually, substrate and enzyme are restocked, and the process is repeated. In this mode, soluble enzyme is commonly used, although IE can be used (i.e., in the recirculation batch reactor, IE is recovered and reused). In continuous processes, there is constant and simultaneous renewal of reactants and removal of products. The IE can be used until a significant loss of activity is observed so optimization is needed to determine the number of cycles the enzyme can perform. Continuous mode is more advantageous since it requires fewer steps, namely the preparation of the reaction medium, transformation, recovery of the medium post-reaction, purification/removal of enzyme and obtaining of the pure product can be done simultaneously and, consequently, at lower costs (Illanes, Wilson, and Vera 2014; Guerrero et al. 2019).

FOS and GOS enzymatic synthesis

Galacto- and fructo-oligosaccharides (GOS and FOS) are non-digestible oligosaccharides with prebiotic properties that can be incorporated into a wide number of products. From a nutritional point of view, they are low caloric sweeteners that give a feeling of satiety, contribute to body weight control, relieve constipation, have a low glycemic index and are not cariogenic (Moser and Wouters 2014). According to the latest definition, prebiotics are "substrates that are selectively used by host microorganisms conferring a health benefit" (Gibson et al. 2017). These compounds are used in the formulation of many food products, beverages, and especially in infant formula, to stimulate the development of newborn microbiota (Kumar, Sripada, and Poornachandra 2018; Martins et al. 2019). Both GOS and FOS can be obtained by hydrolysis or by enzymatic synthesis. The former consists in the hydrolysis of compounds naturally occurring in some plants or seeds (i.e.,, soybean, lupin, lentil, chickpea, pea and cowpea for GOS and roots of chicory, artichoke, yacon, dahlia or agave for FOS). This strategy promotes the obtaining of large molecular weight compounds (degree of polymerization, DP, higher than 8) and a mixture of different other compounds besides prebiotics. For this reason, this kind of processes need specific purification steps, depending on the natural source used to obtain GOS and FOS. Industrial enzymatic synthesis using a disaccharide as substrate (i.e., lactose and sucrose for GOS and FOS, respectively) is a strategy that allows the obtaining of short chain GOS and FOS (DP ranging from 3 to 7). Besides that, this is a versatile and easy way to control once the reaction mechanistic is well established, which depends on a large extent on understanding the enzyme activity characteristics (Martins et al. 2019).

Particularly, the enzymes used to obtain FOS and GOS, fructosyltransferases and β-galactosidases, have both transferase and hydrolase activities. These enzymes are capable of catalyzing the transfer of functional groups (glycosylic) and, at the same time, the hydrolysis of organic molecules (sucrose and lactose, respectively). From the one side, this double function considerably decreases the enzymes' costs. From the other side, the non-specificity represents a technological challenge because the products' yield is lower than that obtained using more specific enzymes. For this reason, the industry must implement production processes considering both economic and technological aspects. In line with this, industrial scale production of GOS and FOS by enzymatic synthesis can be performed through batch or continuous processes, either with soluble FE or with IE. The pros and cons of the different processing modes applied for obtaining GOS and FOS for enzyme immobilization studies are presented in this review and schematized in Figure 1.

β-Galactosidase

β-galactosidase, also called lactase, beta-gal or β-gal (EC 3.2.1.23), is the enzyme most commonly used to obtain glucose and galactose from lactose. This enzymatic reaction turns a wide variety of products adequate for lactose intolerant consumers (Nath et al. 2014). The reaction is sometimes also employed as a technological strategy to overcome the possibility of lactose deposition and to obtain a sweeter flavor (glucose and galactose are more soluble and sweeter than lactose) (Panesar et al. 2006).

As an advantage, β-galactosidase is widely distributed in nature and can be found in plants (especially in almonds, peaches, apricots, and apples) but often has low lactase activity (Mahoney 1998). On the contrary, β-galactosidase from microorganisms (yeast, bacteria, and fungi) (Richmond, Gray, and Stine 1981) and from mammalian's intestinal tract presents a high lactase activity. β-galactosidases used for the synthesis of GOS are usually from yeast (*Kluyveromyces lactis, Kluyveromyces fragilis, Rhodotorula minuta*), bacteria (*Escherichia coli*, *Bacillus circulans*, *Bacillus* sp., *Lactobacillus reuteri*) and fungi (*Aspergillus oryzae*) (Martins et al. 2019).

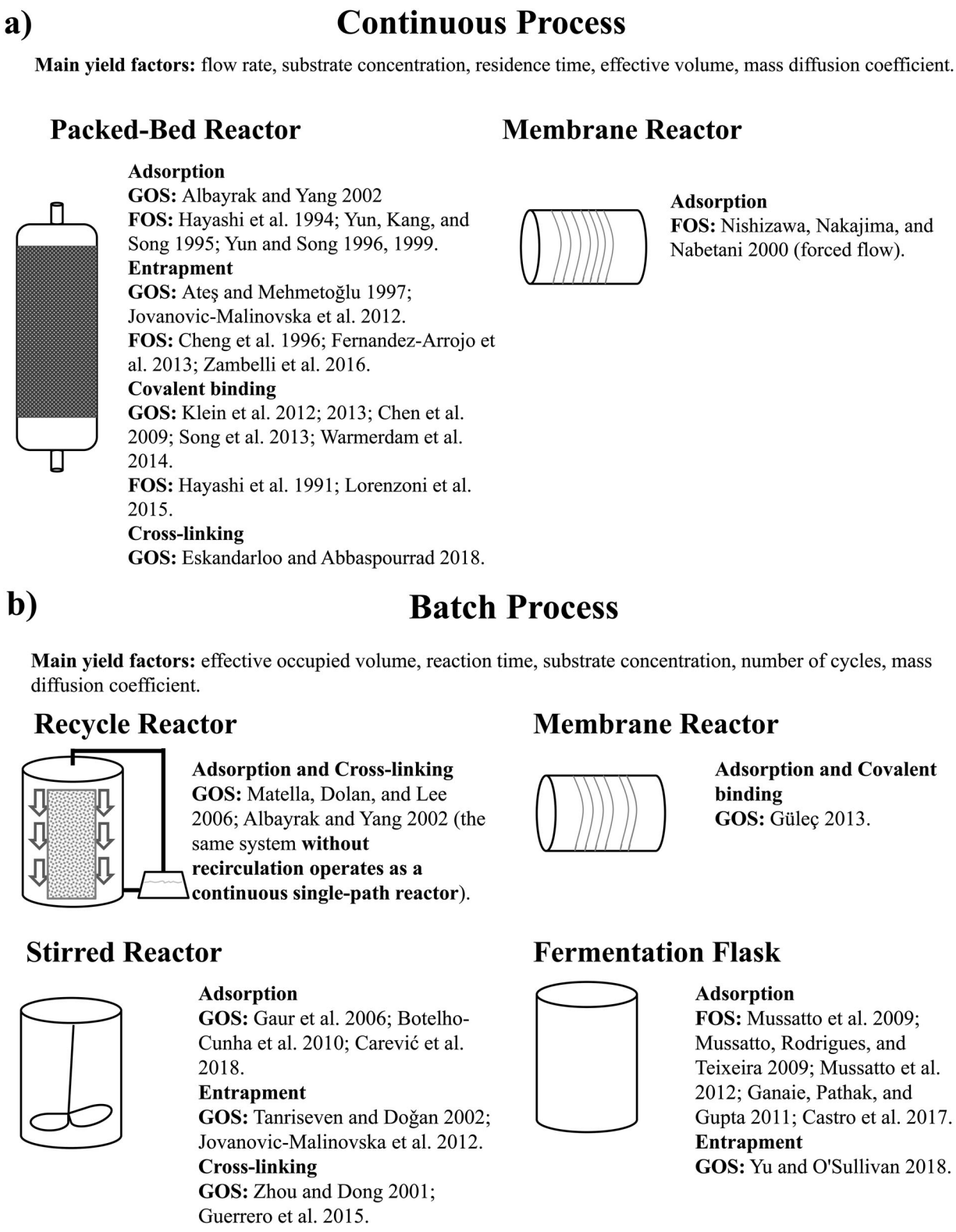


Figure 1. Process and reactor design used to perform GOS and FOS production with IE. (a) Continuous mode and (b) batch process.

The most studied β-galactosidase is that produced by *E. coli*, but because of its origin (coliforms), it is not a suitable option for food applications if it is not appropriately purified (Nath et al. 2014). Therefore, β-galactosidase is produced from recognized safe microorganism sources [yeasts (*Kluyveromyces*) and fungus (*Aspergillus*)] at industrial scale. Although enzyme molecular size varies depending on the enzyme source, there is an estimation of its molecular size since early 70s. Melchers and Messer (1973) defined that *E. coli* β-galactosidase is tetrameric, being composed of four identical subunits of 135,000 daltons. Also, Yang, Marchio, and Yen (1994) reported that it has an average protein diameter of 12 nm, but when exposed

to low concentrations of salts aggregation was observed, reaching particle sizes of around 1000 nm and 2000 nm.

In general terms, the dynamics of this enzyme was described by Mahoney (1998) as a simple three steps mechanistic: first, thanks to the enzyme active site, the lactose-enzyme complex is formed; then, galactosyl transfer occurs, resulting in the formation of a galactosyl-enzyme complex, while glucose is released; finally, the enzyme will transfer galactose to a nucleophilic acceptor containing a hydroxyl group. When the acceptor is water, galactose is formed, and when the acceptor is another sugar, the product is an oligosaccharide.

Fructosyltransferases

Fructosyltransferases (β-fructofuranosidase, invertase, EC 3.2.1.26 or β-D-fructosyltransferase, EC 2.4.1.9) are the biocatalysts for the transfructosylation reactions leading to the obtaining of FOS. They can be extracted from plants, yeasts and molds and from bacteria (GH32 and GH68 families from CAZy classification, respectively) (Cantarel et al. 2009). Depending on the enzyme source, some characteristics like molecular weight will differ from each other. In this sense, Sangeetha, Ramesh, and Prapulla (2005) provides a detailed study including several fructosyltransferase sources.

The activity of this enzyme consist on the cleavage of the β-2,1-glycosidic bond and the transfer of fructosyl moieties from carbohydrates acting as donors onto any acceptor other than water (Vega and Zuniga-Hansen 2014). Synthesis and hydrolysis occur simultaneously both in parallel and in series (Martins et al. 2019): FOS (DPn) synthesized in the first steps act as fructosyl donors and acceptors leading simultaneously to the production of FOS with DP immediately higher (DPn + 1) and lower (DPn-1); mixtures of short chain FOS (DP ranging from 3 to 6, i.e., DP3, DP4, DP5 and DP6), together with glucose (secondary product), are obtained (Jung et al. 1989; Martins et al. 2019).

β-Galactosidase and fructosyltransferases: their mechanisms of enzymatic action

Understanding the mechanism of the kinetics involved in the catalytic enzyme performance is crucial for process design. In particular, for immobilization, considering all these mechanisms is of great importance to choose both an appropriate immobilization method and an appropriate support. β-galactosidase and fructosyltransferase belong to the glucosyl hydrolases family (GH-A superfamily). This family of enzymes has been widely studied with the objective of unraveling their catalytic mechanism (Davies and Henrissat 1995; St John, González, and Pozharski 2010). The enzymatic hydrolysis of glycosidic bonds is carried out with retention or inversion of the anomeric configuration, thus hydrolases are classified as either retaining or inverting (Withers 2001). Particularly, β-galactosidase is a retaining hydrolase and fructosyltransferase is an inverting.

β-Galactosidase

Using x-ray diffraction data together with directed site mutations, kinetic experiments and *in silico* studies, the *E. coli* β-galactosidase structure, binding sites and catalytic mechanism are well understood (Brás et al. 2008; Hrmova and Fincher 2007; Juers et al. 2001; Zhang et al. 2018). The enzyme has two binding modes: a shallow mode, with weak interactions and poor specificity that allows weak binding from several different substrates; and a deep mode, at the catalytic site, with strong interactions and high specificity. As it is shown in Figure 2, in Following Davies' nomenclature (Davies, Wilson, and Henrissat 1997), the deep mode corresponds to subsites −1 and +1, and the shallow mode, to subsites +2 and higher.

For the catalytic process, the lactose substrate is captured in the deep mode at the catalytic pocket with its binding pose controlled mainly by the β-D-galactosyl moiety, which is pinned down at the −1 subsite by a hydrophobic stacking with a tryptophan residue and a complex network of hydrogen bonds between its hydroxyl groups and six polar residues (among them, GLU461, GLU537, Figure 2). The glucosyl moiety is stabilized at subsite +1, by a hydrophobic interaction with a tryptophan residue. The weak interactions at subsite +1 result on higher mobility and less specificity than subsite −1. Both kinetic and quantum mechanics/molecular mechanics *in silico* studies indicate that an Mg^{2+} ion located at the catalytic pocket, complexed with three water molecules and three residues, including GLU461, increases the specificity toward the β-D-galactosyl moiety (Brás, Fernandes, and Ramos 2010).

The proposed catalytic mechanism for cleavage of the lactose glycosidic bond involves several steps. The first step is the formation of two hydrogen bonds, first between the carboxylate of the GLU537 residue below the galactosyl ring and the hydroxyl at C2, and second, between the carboxylic acid of GLU461 residue, above the ring, and the glycosidic oxygen (Figure 2). On the second step, the second hydrogen bond results in proton donation after the nucleophilic attack by the GLU537 carboxylate on the anomeric carbon, forcing the glycosidic bond cleavage. The Mg^{2+} ion modulates the acidity of GLU461, facilitating the bond cleavage. After the glycosidic bond has been cleaved, the weak interactions at subsite +1 allow the glucose molecule to leave. Calculations also suggest that a change on the galactosyl ring to a half-chair conformation is also important for the glycosidic bond cleavage (Brás, Fernandes, and Ramos 2010). This ring conformation is consistent on all GH-A enzymes, although differences between families have been found (Kumar, Henrissat, and Coutinho 2019).

After the glycosidic bond cleavage, two fates await the covalent enzyme-galactosyl complex. The complex can suffer hydrolysis, following a nucleophilic attack by a water molecule, or a new glycosidic bond can be formed with the freed glucose or another substrate resulting on the transglycosylation. On the second case, a lactose or longer GOS can be captured by the shallow binding mode placing the galactosyl end at the subsite +1. For the *E. coli* β-galactosidase, the quantum mechanics/molecular mechanics study allowed

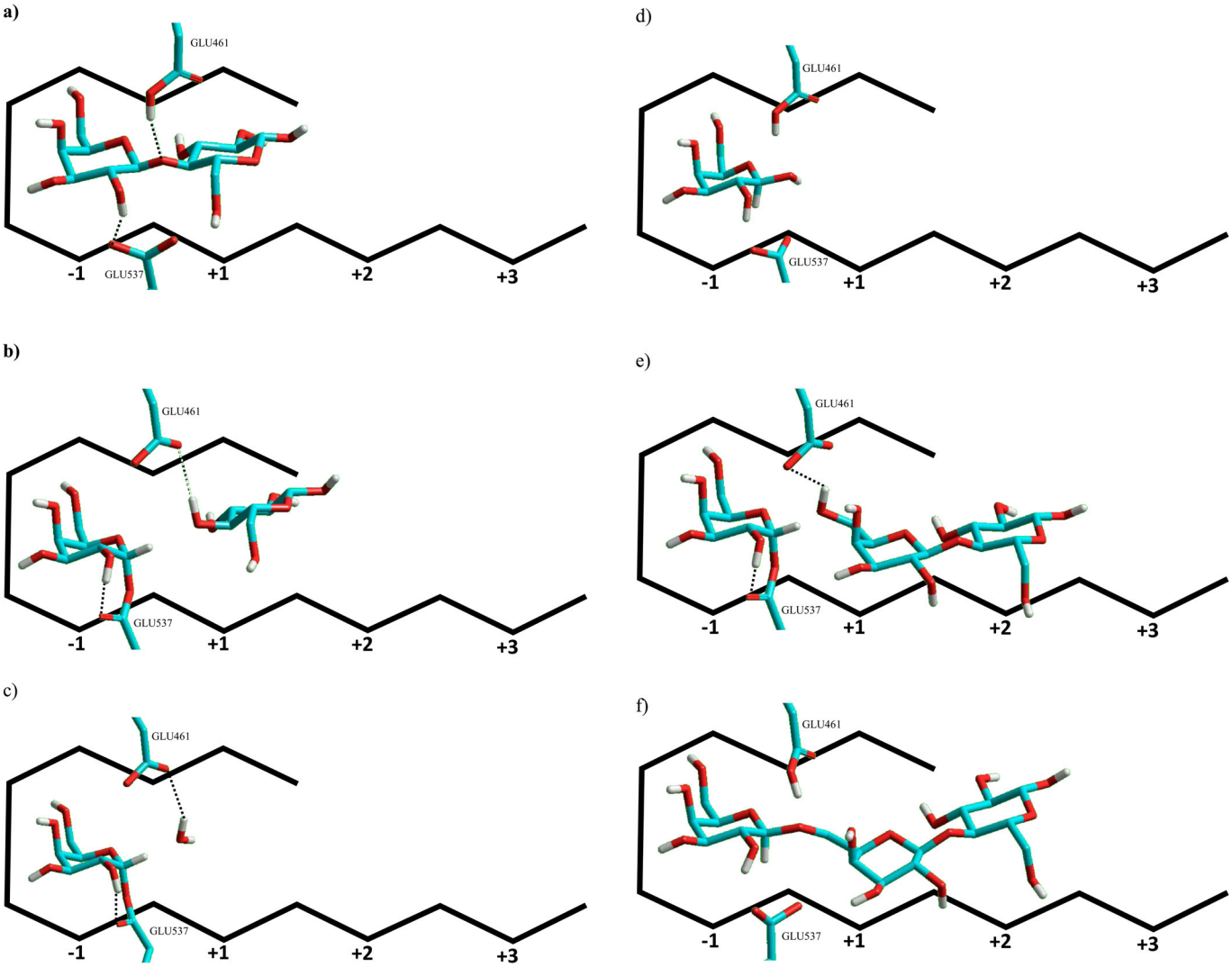


Figure 2. Diagram representing subsites −1 to +3 and 6 different situations from *β*-galactosidase catalytic mechanism: (a) binding of lactose on subsites −1 and +1; (b) glycosidic bond cleavage; (c) enzyme-galactosyl plus water at subsite −1; (d) galactose ready to leave subsite −1; (e) enzyme-galactosyl plus lactose at subsites +1 and +2; and (f) GOS ready to leave.

the elucidation of the reaction path showing that transglycosylation leads *β* (1-6) oligosaccharides as the thermodynamically favored products, galactosyl-*β* (1-6)-glucose (allolactose) being the preferred one (Brás, Fernandes, and Ramos 2010). The catalytic mechanisms for both hydrolysis and transglycosylation are also represented by the diagrams on Figure 2.

The true nature of the catalytic mechanism is relevant for the establishment of a proper kinetic model. Experimental data led to the conclusion that glucose acts as a competitive inhibitor. *In silico* studies with human *β*-galactosidase (also a retaining hydrolase) suggest that a similar catalytic mechanism is the origin for the inhibiting effect (Guce et al. 2010), since glucose can enter the catalytic site but the absence of an hydroxyl in the adequate position prevents the formation of the covalent bond to the enzyme (Brás et al. 2008).

β-galactosidase from Aspergylus oryzae belongs to GH-35 family, together with human galactosidase, while the enzyme from E. coli belongs to the GH-2 family (Maksimainen et al. 2013). A comparison of several enzymes has shown that all members of the GH-A superfamily have the same two glutamic acid residues at the catalytic site, one to act as a nucleophile and the other, as a Brönsted proton donor. Therefore, the mechanism should be the same as that for other enzymes from the superfamily (Davies and Henrissat 1995; Henrissat et al. 1995; Irague et al. 2013; Kumar et al. 2011; Thongpoo et al. 2013; Vukić et al. 2015). Crystallographic and kinetic studies confirm this finding (Zechel and Withers 2000). All enzymes from the GH-A super family are structurally and mechanistically related (Kumar, Henrissat, and Coutinho 2019).

Fructosyltransferase

Fructosyl hydrolases and transferases belong to the GH-32 family, including retaining and inverting enzymes. The general mechanism has been elucidated with data from X-ray diffraction, directed site mutations, kinetic experiments and *in silico* studies. It follows a similar path than that of galactosidases, but with three instead of two key residues: one aspartic acid acts as a nucleophile and an aspartic acid, as

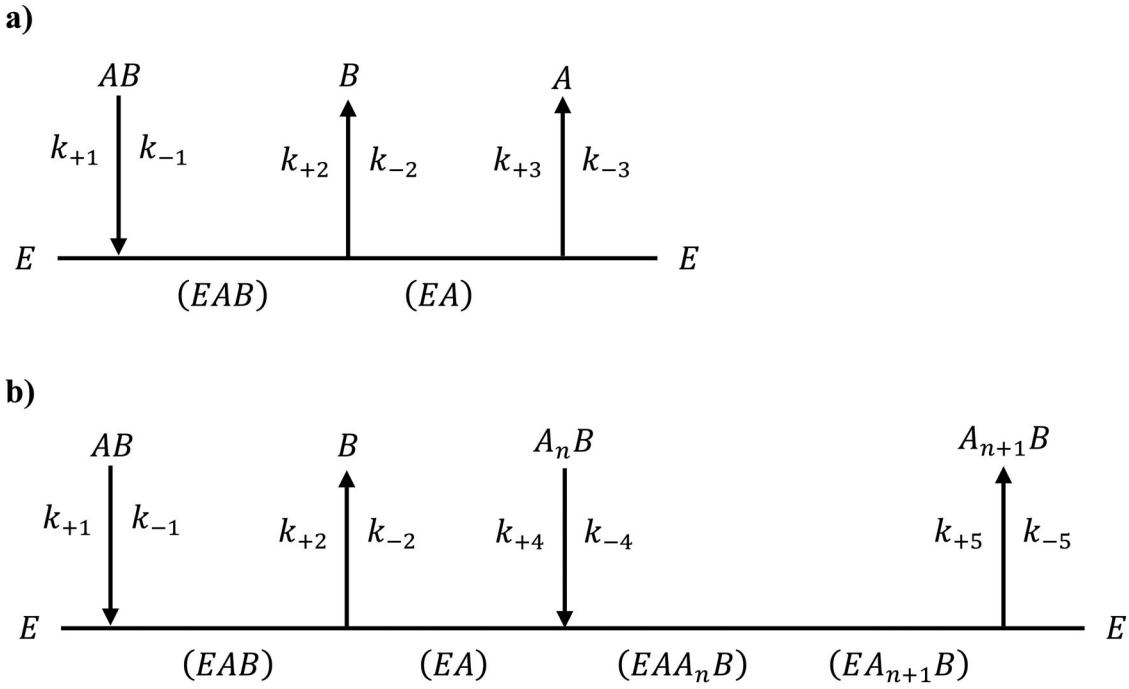


Figure 3. Proposed mechanism for: (a) hydrolysis and (b) transglycosylation of a disaccharide. *A* is a galactoside or fructoside, *B* is a glucoside, and *E* is the enzyme.

acid/base donor; a second aspartic acid modulates the nucleophile with a stabilizing effect on the covalent enzyme-fructosyl intermediate. The presence of a Ca^{2+} ion has been noted and its role might be the same as the Mg^{2+} ion discussed before (Alvaro-Benito et al. 2010; Alberto et al. 2004; Chuankhayan et al. 2010; Lafraya et al. 2011; Martínez-Fleites et al. 2005; Meng and Fütterer 2003; Ozimek et al. 2006). As with *β*-galactosidase, quantum mechanics/molecular mechanics calculations for transfructosylase from *Aspergillus japonicus* allowed the calculation of the reaction path, through a retaining mechanism, and the prediction of the most stable products to come from transfructosylation instead of sucrose hydrolysis (Jitonnom, Ketudat-Cairns, and Hannongbua 2018). The results confirmed those previously obtained, suggesting that transfructosylation and sucrose inversion are performed by different enzymes (L'Hocine et al. 2000).

The modeling of glucosidases' and fructosyltransferases' kinetics has been widely studied. Invertase, also a hydrolase, was used by Michaelis and Menten on their studies leading to the general enzyme kinetics model (Cornish-Bowden 2015). Due to their industrial importance, hydrolases' kinetics have been studied in the free form, immobilized and with several types of reactors (Alvarado-Huallanco and Maugeri Filho 2011; Detofol et al. 2015; Díez-Municio et al. 2014; Duan, Chen, and Sheu 1994; Guio et al. 2012; Jung et al. 1989; Khandekar et al. 2014; L'Hocine et al. 2000; Lorenzoni et al. 2014; Sen, Bhattacharjee, and Bhattacharya 2016; Surin et al. 2012; Vega and Zuniga-Hansen 2014; Wong et al. 2015). Several authors have proposed empirical based kinetic models with support from mechanistic conclusions, adapted to every experimental setup fitting the models' parameters (Michaelis constants or kinetic constants) to existing experimental data. Figure 3 represents the hydrolysis (Figure 3a) and the transglycosylation of a disaccharide (Figure 3b).

Contribution of immobilization technology for *β*-galactosidase and fructosyltransferase

Different classifications for the various types of immobilization can be found in the literature. The most common one is the distinction between physical or chemical methods (Guo 2019). The first group includes the methods involving *physical* interaction between the enzyme and the support or those in which the enzyme is *physically* restrained by the carrier, hindering its release to the medium. The chemical methods depend mostly on the establishment of covalent bonds (Dwevedi 2016; Mohamad et al. 2015). Other classifications distinguish the presence or absence of carriers/supports (Illanes 2008) or those that differentiate the reversible or irreversible nature of the interactions (Homaei et al. 2013). There are also authors that do not classify them, and simply discuss the different methods (Aehle 2007; Moehlenbrock and Minteer 2017; Nguyen, Lee, et al. 2019; Sheldon 2007; Sirisha, Jain, and Jain 2016). This latter perspective is that assembling most with the focus of this review. In this line, although there are many immobilization methods and types of enzymes in the food industry, we will discuss those particularly used for fructosyltransferase and *β*-galactosidase. In this sense, adsorption, entrapment, covalent coupling, cross-linking and aggregation are the main methods used to immobilize these type of enzymes, all of them having pros and cons. Figure 4 gives a general outline of the mechanisms of each method.

Adsorption is often considered a simple method consisting on physical interactions such as van der Waals forces, ionic interactions and hydrogen bonding, between the

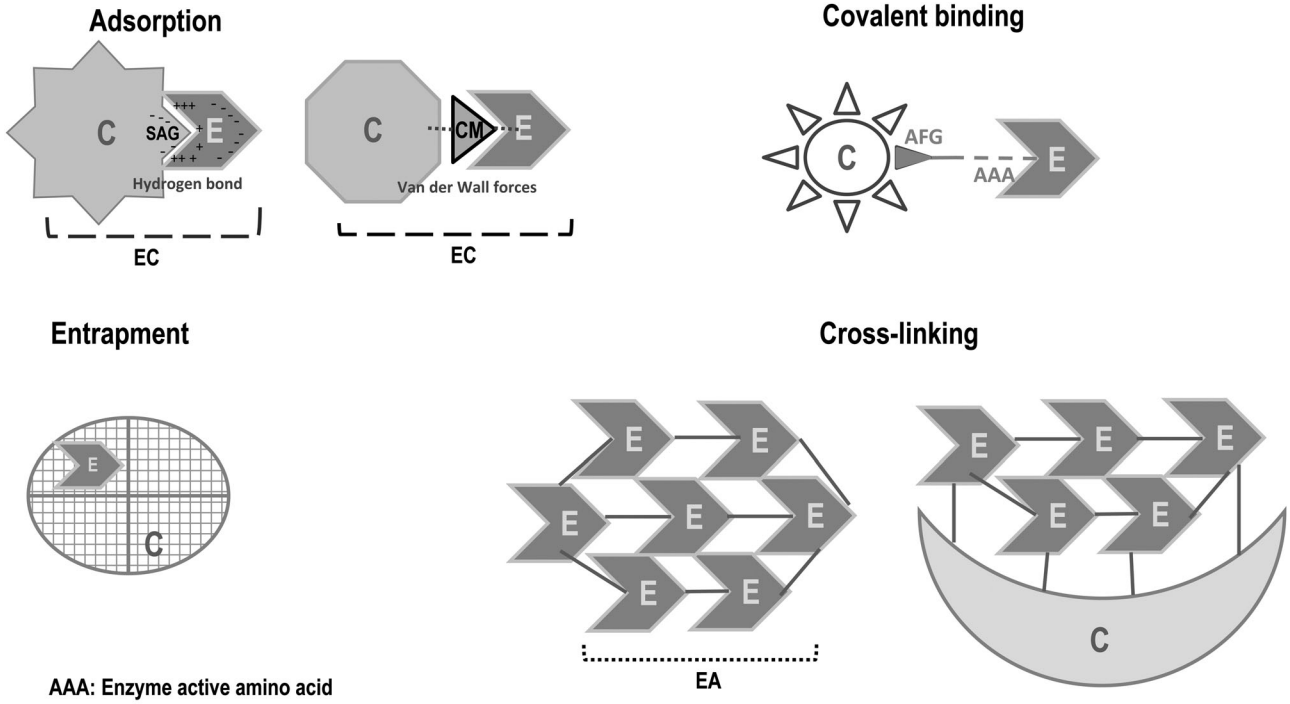


Figure 4. Simple representation of the principal immobilization methods (adsorption, entrapment, covalent binding and cross-linking) used to immobilize β-galactosidases and fructosyltransferases.

carrier and the enzyme without modifying the natural structure of this last one (Gonçalves et al. 2019; Nisha, Karthick, and Gobi 2012; Panesar et al. 2006). For this type of immobilization, hydrophobicity/hydrophilicity character and charge (isoelectric point) of the enzyme are very important for the selection of the support (Moehlenbrock and Minteer 2017). The carrier needs to provide specific functional groups (SAG in Figure 4) on the surface for the conformation of the enzyme-carrier binding. If this interaction does not occur spontaneously, intermediates (carrier modifiers) can be applied (CM in Figure 4). Silica, silica gel, alumina, alumina gel, activated charcoal, ion exchange resins, hydroxyapatite (inorganic), chitosan, calcium alginate, agarose gel, cellulose, synthetic polymers, fiber membranes, cotton cloth (organic) are common supports to immobilize enzymes by adsorption (Jesionowski, Zdarta, and Krajewska 2014; Nisha, Karthick, and Gobi 2012).

Entrapment involves the occlusion of an enzyme in a synthetic or natural polymeric network (gel, fiber or microcapsule) that act as a permeable membrane to substrates and products, while the enzyme is retained inside (Guo 2019; Nisha, Karthick, and Gobi 2012; Sheldon 2007). This is a fast, cost-effective and feasible strategy of immobilization but it has some drawbacks, the main one being the mass transfer limitation due to diffusional problems between the substrates, products and the carrier (Aehle 2007; Nisha, Karthick, and Gobi 2012). Additionally, physical retain is generally weak and does not prevent enzyme leakage. Therefore, very often additional treatments with specific reactants are required to effectively retain the catalyst, usually by covalent attachment. Consequently, the difference between entrapment and covalent binding is not clear. In this sense, Sheldon (2007) proposes that the term entrapment involves the synthesis of the polymeric network during the immobilization process. As a drawback, this last strategy makes the supports not renewable.

Enzyme immobilization via covalent binding is one of the first methods implemented, therefore, one of the most widely investigated. In brief, it consists in a chemical interaction between the aminoacids from the active site of the enzyme with the active functional groups from the carrier surface (AAA and AFG in Figure 4) (Cao 2005). Carriers need often to be activated, and this process involves two stages: the addition of a reactive compound and the modification of the polymer backbone to activate the matrix (Nisha, Karthick, and Gobi 2012; Nguyen, Styevkó, et al. 2019). Covalent bonding generates strong and stable linkages and it usually prevents leakage of the enzyme from the carrier's matrix (Cao 2005; Nisha, Karthick, and Gobi 2012). However, the main drawback is the fact that both the

enzyme and the support remain unavailable if the enzyme is irreversibly deactivated during either the immobilization or the synthesis processes (Sheldon 2007).

Cross-linking consists in intermolecular linking of molecules by the use of bifunctional reagents (substances that contain two identical or two different functional groups or groups of different reactivities) (Sheldon, Schoevaart, and van Langen 2006). The principal bifunctional cross-linking reagent used for enzyme immobilization is glutaraldehyde (GA), which has the ability to react with different enzyme moieties (primary amino groups, thiols, phenols, imidazoles) and also, the capacity to polymerize (Barbosa et al. 2014). In this sense, there are two main approaches: to use GA as an intermolecular enzyme crosslinker in support-free methods, and to use GA to activate the support and prepare the IE. The first one can overcome the diffusional limitations of mass transfer by using different supports, it does not require purified enzyme, and it is highly specific. Nevertheless, its main disadvantage is that it can modify the enzyme's configuration, leading to a loss of activity. This may condition the selectivity of the enzyme for a certain substrate or the formation of side-products (Boudrant, Woodley, and Fernandez-Lafuente 2020).

In the next paragraphs the reader will find a chronological description of the evolution to immobilize β-galactosidases and fructosyltransferases, with special emphasis on those works which gave a useful insight in the field during the last decades. Although this section is divided considering each method of immobilization, it must be mentioned that in many works authors compared different immobilization methods or used a combination of them. Hence, the sequence selected for presentation is just for a matter of organization rather than a strict classification. The examples mentioned in this section are listed in both Table 1 and Table 2 and are discussed with greater detail below. It is worth mentioning that in the case of β-galactosidase immobilization, most of the works are focused on the hydrolysis reaction rather than on its transferase activity. For this reason, the table includes a column indicating the application procured by each author to design the immobilization process.

Adsorption

Adsorption of β-galactosidase with polyethyleneimine on cotton cloth has been applied by Albayrak and Yang (2002). The immobilization process consisted in a combination of adsorption (polyethyleneimine solution to cotton cloth and exposing it to the enzyme solution) and cross-linking (polyethyleneimine-enzyme aggregates with GA). The procedure performed in monolayer presented an enhancement of the immobilization yield when increasing the enzyme concentration up to a maximum loading value. In contrast, the maximum enzyme immobilization yield, in multilayer mode, was achieved when the polyethyleneimine to enzyme ratio was near 1/20–1/25. When testing GOS formation from lactose with the multilayered polyethyleneimine-IE technique in a packed-bed reactor, high productivity was achieved and GOS formation kinetic was not affected compared with soluble enzyme. However, Matella, Dolan, and Lee et al. (2006), who applied the same approach to immobilize β-galactosidase from *Aspergillus oryzae*, claimed that adsorption was not effective and inactivation occurred, but their enzyme contained significant amounts of dextrin (neutral charge), which could avoid the electrostatic interaction between the enzyme and the polyethyleneimine-cloth. Therefore, when applying this type of configuration, special care must be paid in the presence of substances that can alter the electrochemical nature of the interaction between enzyme and the carrier.

Gaur et al. (2006), immobilized the enzyme through physical adsorption on celite, and compared with other immobilization methods, finding that adsorption was the method with less enzyme recovery (only 2% of activity yield).

Güleç (2013) immobilized β-galactosidase from *Kluyveromyces lactis*, comparing simple adsorption and covalent attachment onto cellulose acetate membrane surface. For adsorption, authors used plain and oxygen-plasma modified membrane, applying radio frequency and low-pressure, finding that plasma activated surface membrane was able to immobilize higher amounts of the initial enzyme concentration since surface hydrophilicity increased with oxygen activation. The IE via adsorption caused higher lactose conversion than the soluble enzyme system but the initial GOS yields (30%–34%) of the IE was lower than that of the soluble enzyme (39%), so for GOS synthesis IE demanded higher reaction time to reach the same yield than operating with soluble enzyme.

These references confirm that charge (isoelectric point) of the enzyme and hydrophobicity/hydrophilicity characters are crucial for the efficiency of adsorption as immobilization method. Additionally, according to our exhaustive search, more updated bibliography promoted this method in combination with entrapment (Souza, Garcia-Rojas, and Favaro-Trindade 2018) or covalent binding and cross-linking (Urrutia et al. 2018), both discussed in the next paragraphs. Also, Carević et al. 2018 applied this immobilization technology, but given that this work was focused on the study of different resins as supports, it will be discussed later, in *Support materials and approaches for enzymatic production of GOS and FOS*.

Immobilization of fructosyltransferase *via* ion exchange was early investigated using different supports. Hayashi et al. (1994) evaluated the immobilization of β-fructofuranosidase with diethylaminoethyl (DEAE) cellulose in a continuous reaction. They found that IE was less susceptible to inhibition by metal ions and the temperature stability was comparable with that of FE. The long-term stability of IE enabled the continuous production of FOS. Yun, Kang, and Song (1995) and Yun and Song (1996, 1999) used a porous styrene-derived ion exchange resin and packed it into a glass column to reproduce an industrial system. The combination of a high porous support and the column system allowed a high volumetric activity, more stability against pH changes, and long term continuous operation, while product composition was very similar with FE and IE. Additionally, authors compared the same system design with IE and immobilized whole cells,

Table 1. Characteristics of the principal innovative strategies to immobilize β-galactosidase.

Immobilization method	Support	Application	Enzyme source	Results/observations	Reference
Adsorption and cross-linking of enzyme aggregates.	Cotton cloth	GOS synthesis	*A. oryzae* (Genencor Int.l) Albayrak and Yang 2002 (Sigma and Bio-Cat); Matella, Dolan, and Lee 2006.	Multilayer immobilization was achieved. The method required highly concentrated and pure enzymes (i.e., 100,000 U/mg). If not, it could cause enzyme inactivation	Albayrak and Yang 2002; Matella, Dolan, and Lee 2006
1. Physical adsorption 2. Covalent binding 3. Aggregation by cross-linking	1. Celite 2. Chitosan 3. —	Oligosaccharide synthesis	*A. oryzae*	Covalent coupling to chitosan was more appropriate for oligosaccharide synthesis, as compared to adsorption or aggregation. Enzyme aggregates appear to be more suitable for lactose hydrolysis applications	Gaur et al. 2006
Adsorption	Duolite A-568 (resin beads)	GOS synthesis	Commercial *K. lactis* (*Lactozym 3000 L HP-G*, Novo Nordisk A/S)	Higher the concentration of enzyme, higher the loading obtained, but with loss of enzyme activity and leading to substrate hydrolysis over GOS synthesis	Botelho-Cunha et al. 2010
Adsorption and covalent binding	Cellulose acetate membranes	GOS synthesis	*K. lactis*	The IE via adsorption caused higher lactose conversion but lower initial GOS yields than the soluble enzyme system. The most efficient strategy was covalent bonding with plasma polymerization of 2-mercaptoethanol modification, which achieved high immobilization yield and high enzyme activity	Güleç 2013
Adsorption by ionic interaction	Resins with different functional groups.	GOS synthesis	*L. acidophilus ATCC 4356*	Resin carriers with amino groups and larger pores and longer spacer were selected in order to enhance enzyme activity yield	Carević et al. 2018
Entrapment	Cobalt alginate beads	Lactose hydrolysis	*A. oryzae*	GA enabled to avoid enzyme leakage. The hydrolysis products contained higher amounts of Co^{2+} than the limits allowed in foods so, removal is needed	Ateş and Mehmetoğlu 1997
Entrapment	Alginate and gelatin fibers	Lactose hydrolysis	Commercial *A. oryzae* (Sigma)	More stability at high pH and temperatures than FE. Activity did not decrease for 35 days	Tanriseven and Doğan 2002
Entrapment	PVA lenses and sol–gel carriers	GOS synthesis	Commercial *A. oryzae*	PVA IE was better biocatalyst than sol–gel enzyme in terms of lactose conversion and operational stability	Jovanovic-Malinovska et al. 2012
Entrapment	Chitosan beads and alginate beads	GOS synthesis	*Lc lactis*	First the recombinant DNA was degraded in the whole cells using UV treatment. Chitosan was more appropriate for immobilization than alginate for GOS production at high temperature (85 °C).	Yu and O'Sullivan 2018
Entrapment and adsorption by ionic interaction	Sodium alginate or ι -carrageenan	Lactose hydrolysis	Commercial *K. lactis* (Lactomax, Prozyn)	Immobilization of the enzyme might negatively affect its activity, however, is able to reduce the denaturation rate of the enzyme exposed to low pH	Souza, Garcia-Rojas, and Favaro-Trindade 2018
Entrapment and adsorption by ionic interaction	Sodium alginate	Lactose hydrolysis	*A. oryzae* (Sigma)	The binding between β-galactosidase and alginate resulted from the equilibrium between enthalpy and entropy contributions. The thermal stability was improved compared to FE	Souza et al. 2019
Covalent binding	Chitosan	Lactose hydrolysis	*B.stearothermophilus*	Support activation with GA was performed. Superior activity, reusability, thermostability, and storage stability was achieved compared to FE	Chen et al. 2009. Lima et al. 2013

Covalent binding	1. Polyaniline coated with magnetite 2. Polysiloxane-PVA polymer	GOS synthesis	1. *A. oryzae* (Sigma) 2. *K. lactis*	1. Similar product composition was obtained and similar kinetic behavior was observed compared to the FE 2. Immobilization yield was 99% with 78.5% of enzyme activity recovery. Authors studied experimentally the reaction mechanism to produce GOS and proposed a mathematical model	1. Neri et al. 2011 2. González-Cataño et al. 2017
Covalent binding	Chitosan macroparticles	Lactose hydrolysis and GOS synthesis	Commercial *K. lactis* (Maxilact LX 5000)	pH and temperature operational range was larger with IE in PBR. The combination of continuous flow with a high content of lactose can increase enzyme stability	Klein et al. 2012, 2013
Covalent binding	Silica gel	Whey lactose hydrolysis	*K. lactis*	Pretreatment with lactose prior to immobilization. The pretreated β-galactosidase activity was 2.6 times higher than non-pretreated. Temperature and pH stability and reusability of the IE was improved by the pretreatment.	Seok Song et al. 2010; Seok Song et al. 2013
Covalent binding	Glyoxyl Sepharose	*o*-NPG hydrolysis	Commercial *K. lactis* (Lactozym 3000)	Multi-point attachment enhanced thermal and pH stability, and made the enzyme less susceptible to inactivation in the presence of solvents (dioxane 30%)	Bernal et al. 2013
Covalent binding	Porous acrylic beads with oxirane functionality (Eupergit C)	GOS synthesis	Commercial *B. circulans* (Biolacta N5)	The use of IE in a continuous PBR was compared to FE in a batch reactor. 90 days was the half-life time of the IE. PBR productivity was more than six times higher than FE in a batch reactor	Warmerdam et al. 2014
Covalent binding	chitosan	lactulose synthesis	*K. lactis*	Enzyme stability, conformation and kinetic properties were improved. 17.3 g/L of lactulose, and 86% of lactose conversion was obtained	de Albuquerque et al. 2018
Covalent binding	chitosan-coated magnetic Fe3O4 nanoparticles	GOS synthesis	Commercial *A. aculeatus* (Pectinex Ultra SP-L)	Half-life and thermal and pH enzyme stability was improve. A maximum GOS yield of 17% mol/mol was obtained after 36 h of reaction using 2.34 M of initial lactulose concentration.	Nguyen, Styevkó, et al. 2019
Cross-linking	Gelatin	Lactose hydrolysis	*E. coli*	IE preserved its activity for 3 months, being more stable to pH variations than FE	Sungur and Akbulut 1994
Cross-linking	Graphite electrode surface	Lactose hydrolysis	*K. lactis*	Higher specific activity than free enzyme. Stable and active at 37 °C and 50 °C	Zhou and Dong 2001
Cross-linking	PVDF membrane	GOS synthesis	Commercial *B. circulans* (Biolacta N5)	The units of enzyme immobilized per gr of membrane increased with enzyme concentration. The formation of GOS product increased with the initial lactose concentration. A recirculation loop allowed improving the process. All this was satisfactorily incorporated in a mathematical model	Palai and Bhattacharya 2013; Palai, Singh, and Bhattacharya 2014
Cross-linking and aggregation	—	GOS and f-GOS synthesis	Commercial *A. oryzae* (Enzeco)	Immobilization favored the generation of disaccharides over higher oligosaccharides and allowed enzyme reuse increasing lactulose production per unit mass of biocatalyst and in cumulative productivity	Guerrero et al. 2015; Guerrero et al. 2018, 2019
Cross-linking	Glyoxyl-agarose, amino-glyoxyl, carboxy-	Lactulose, GOS and f-GOS synthesis	Commercial *A. oryzae* (Enzeco)	Glyoxyl-agarose and amino-glyoxyl-agarose derivatives retained the selectivity of the FE for lactulose synthesis while carboxy-	Urrutia et al. 2013, 2018; Guerrero et al. 2017, 2018

(*continued*)

Table 1. Continued.

Immobilization method	Support	Application	Enzyme source	Results/observations	Reference
	glyoxyl and chelate-glyoxyl agarose			glyoxyl-agarose and chelate-glyoxyl-agarose favored the synthesis of transgalactosylated oligosaccharides	
Cross-linking	Glass beads	GOS synthesis	Commercial *A. oryzae* (Megazyme)	IE retained higher enzymatic activity than the FE at higher temperatures. It was studied in a PBR	Eskandarloo and Abbaspourrad 2018
Cross-linking	Electrospun gelatin nanofiber mats	GOS synthesis	*A. oryzae*	Hexamethylenediamine (HMDA) was used as activation agent in order to increase their stability in water. Achieved GOS yield using this catalyst was 31%, higher than that obtained with free enzyme	Sass and Jördening 2020

PVA, polyvinyl alcohol; PVDF, polyvinylidene fluoride; o-NPG, o-nitrophenyl-b-D-galactopyranoside.

claiming that IE reactor operated more efficiently regarding stability and FOS production.

Nishizawa, Nakajima, and Nabetani (2000) tested immobilization of β-fructofuranosidase on ceramic membranes operating in a forced-flow membrane reactor in order to compare physical adsorption and covalent bonding efficiency. They used GA to activate membranes of different pore size, promoting the formation of covalent bonds with aldehyde groups at the surface of porous membrane. In a few seconds, the forced-flow membrane reactor achieved FOS yields similar to those obtained after few hours using batch systems, thanks to the high quantity of IE within the membrane.

Mussatto, Aguilar, et al. (2009) got a deeper insight on the mechanisms of FOS synthesis with IE in vegetal fiber, finding that immobilization allowed for the reduction of the time necessary for repeated batch fermentation from 42 to 14 days (including the microorganism growth and the fermentation process for seven batches). Nevertheless, the main disadvantage of this method was that the hydrolyzing activity of this enzyme increased along cycles, while the transfructosylating activity decreased. Later, this research group (Mussatto et al. 2012) found that among several carriers, synthetic fiber and the polyurethane foam were the best options to operate batch fermentation to obtain FOS.

Although adsorption is a simple method, enabling high enzyme loading, high immobilization yields are usually associated to low enzymatic activities. In general terms, the engineering of this method needs to contemplate a compromise between the enzyme concentrations, the amount of support, and the amount of actually active IE. From literature data, it can be concluded that the immobilization strategy for these two kinds of enzymes involves ionic interaction. In this sense, any additive or change in the nature of the enzyme (i.e., isoelectric point, hydrophilicity) can affect the effectiveness of the immobilization and the enzyme activity yield. According to recent findings about this method, it seems that the innovation focus is more on the type of support than on the immobilization strategy itself.

Entrapment

Ateş and Mehmetoğlu (1997) developed a method for immobilizing β-galactosidase in cobalt alginate beads *via* entrapment, and analyzed the utilization of IE in a plug flow reactor, where there was retention of 83% of the relative activity and increased stability at high temperatures. These results were much better than the β-galactosidase immobilization through entrapment as enzyme fibers composed of alginate and gelatin and hardened with GA (Tanriseven and Doğan 2002), which preserved 56% of activity of FE but also conferred more stability at higher pH and temperature. In both cases, the systems were specially designed for lactose hydrolysis.

Jovanovic-Malinovska et al. (2012), studied the synthesis of GOS with immobilized β-galactosidase in polyvinyl alcohol (PVA) lenses and in sol-gel carriers and compared it to

Table 2. Characteristics of the principal innovative strategies to immobilize fructosyltransferase.

Immobilization method	Support	Enzyme source	Results/observations	Reference
Adsorption via ion exchange	DEAE-cellulose	*β-fructofuranosidase Aureobasidium sp*	pH stability was improved with immobilization and also the enzyme became less susceptible to metal ions. Long-term continuous operation in a column reactor yielded a total production of FOS of 105–127 mg/mL	Hayashi et al. 1994
Adsorption via ion exchange	Porous styrene-derived resin	*Fructosyltransferase Aureobasidium pullukzns*	High porous support and a column system produced high volumetric activity, more stability against pH changes, and long-term continuous operation	Yun, Kang, and Song 1995; Yun and Song 1996; Yun and Song 1999
Adsorption and covalent binding	Ceramic membrane	*β-fructofuranosidases A. niger*	Smaller pore size membranes showed larger immobilized activity, and covalent bonding presented higher immobilization capacity (units of enzyme activity U per m2 of membrane) than adsorption	Nishizawa, Nakajima, and Nabetani 2000
Adsorption	Synthetic fiber, polyurethane foam, stainless steel sponge, loofah sponge and cork	*Penicillium expansum*	The best carriers were the synthetic fiber and the polyurethane foam. Repeated batch fermentation with high FOS yields were possible thanks to the enzyme activity that remained constant for 6 cycles	Mussatto et al. 2012
Entrapment	Calcium alginate gel	*β-fructofuranosidase A. japonicus*	Great mechanical strength of the support and immobilized *Asp. japonicus* mycelium achieved an increase in enzyme stability (pH and temperature). Sucrose diffusion did not affect the conversion yields of FOS	Cheng et al. 1996
Entrapment	Potato dextrose agar	*A. japonicus*	The method enhanced the hydrolyzing activity of this enzyme but decreased the transfructosylating activity	Mussatto, Aguilar, et al. 2009
Entrapment	Sodium alginate	*Aureobasidium Pullulans*	It was able to immobilize *Aureobasidium pullulans* culture, and intra and extra cellular enzyme were studied for FOS production. Intra cellular enzyme was allowed obtaining higher FOS yield	Ganaie, Pathak, and Gupta 2011
Entrapment	Dried alginate gel beads	Comercial *Fructosyltransferase A. aculeatus (Pectinex Ultra SP-L)*	The IE resulted stable during batch operation and when compared to calcium alginate gel beads, promoted higher volumetric activity and enhanced the space-time-yield of fixed-bed bioreactors for continuous operation	Fernandez-Arrojo et al. 2013; Zambelli et al. 2016
Covalent binding	Shirasu porous glass	*β-fructofuranosidase Aureobasidium sp.*	Immobilization in a strong support allowed producing continuously FOS in a packed glass column. A selective production of 1-kestose was possible operating at fast flow rate of concentrated sucrose	Hayashi et al. 1991
Covalent binding	Oxriane-containing polymer	*β-fructofuranosidases A. niger* and *A. japonicus*	Both IEs showed an increase in Michaelis Menten constants, but regarding pH and temperature stability and product composition, there were no differences between free and IE	Chiang et al. 1997
Covalent binding	Polymethacrylate-based polymer (Sepabeads EC)	Comercial *Fructosyltransferase A. aculeatus (Pectinex Ultra SP-L)* and *A. niger (Rapidase TF)*	Efficient immobilization of commercial enzymes can be achieved without adding external salt or buffer. The reaction course	Ghazi et al. 2005

(*continued*)

Table 2. Continued.

Immobilization method	Support	Enzyme source	Results/observations	Reference
			of FOS formation under batch operation was not affected by enzyme immobilization	
Covalent binding	Porous acrylic beads with oxirane functionality (Eupergit C)	*Fructosyltransferase A. aculeatus Pectinex Ultra SP-L*	High immobilization efficiency and more stability for higher pH and temperatures were achieved. The enzyme retains its activity constant for 20 days	Tanriseven and Aslan 2005
Covalent binding	Acrylic copolymers	*Fructosyltransferase Aureobasidium pullulans*	IE behaves quite similar than free enzyme when analyzing the influence of pH and temperature. IE decreases considerably its activity when operating at sucrose concentration higher than 500 g/dm^3	Onderková, Bryjak, and Polakovič 2007
Covalent binding	GA-activated chitosan	Partialluy purified comercial *B-fructosyltransferase (Viscozyme L)*	Immobilization enhanced the thermal stability comparing to FE and retained its activity after 50 cycles of batch FOS synthesis. The combination of enzyme partial purification and immobilization allowed obtaining high FOS yield	Lorenzoni et al. 2014, 2015
1. Covalent binding 2. Adsorption	1. Polymethacrylate porous beads with epoxy functions 2. Epoxy resin	*A. terreus* expressed in *K. lactis*	Covalent immobilization was promoted because it maximized enzyme activity, stability and enhanced the yield of FOS	Burghardt et al. 2019
Covalent binding	Fe_3O_4 chitosan-magnetic nanoparticles	Comercial *A. aculeatus* (*Pectinex Ultra SP-L*)	Hydrolytic and transfructosylating activities and retention was 70% and 86%, respectively, after 6 cycles of reuse. High thermostability was achieved obtaining a maximum FOS concentration of 101.56 g/L	de Oliveira et al. 2020
Cross-linking	Commercial anion-exchange resins and polymethacrylate carriers	Comercial *Fructosyltransferase Aureobasidium pullulans*	Six different commercial carriers (with different functional groups) were tested for the immobilization. The addition of the cross-linking agent produced a drop of the activity. The most appropriate based on specific activity and storage stability of the IE were immobilized through direct attachment and consisted on styrene with quaternary amine groups and polymethacrylate with epoxide groups	Platková et al. 2006
Cross-linking	—	Mutant-type *Aureobasidium pullulans* NAC8	CLEA reusability was 100% of residual activity after four catalysis cycles, for this reason authors promoted this method for the industrial synthesis of FOS. The produced FOS had prebiotic properties comparable to those obtained from commercially obtained FOS	Ademakinwa et al. 2018
Cross-linking	—	Inulosucrase (R483A-LrInu) of *L. reuteri 121*	When comparing CLEAs with soluble inulosucrase to produce IFOs, there was obtained a product composition with a lower degree of polymerization when using the immobilized form, obtaining a product with higher prebiotic effect	Charoenwongpaiboon et al. 2019

the synthesis with the FE. Authors found that polyvinyl alcohol immobilization was the most appropriate method. It retained 95% of its initial activity after seven repeated uses and retained more of the initial activity after 3 months of storage than sol–gel-immobilized β-galactosidase. Also, polyvinyl alcohol-IE achieved higher lactose conversion rates than sol–gel enzyme. IE was adapted to operate in a PBR to produce GOS from both lactose and whey.

Recently, Yu and O'Sullivan (2018) developed a method to produce GOS with immobilized whole cells of *Lactococcus lactis* containing high levels of a hyper-thermostable β-galactosidase from *Sulfolobus solfataricus*. The approach involved as first step the degradation of the recombinant DNA with UV treatment and then immobilization, comparing two supports: chitosan and alginate beads. Although both supports were able to entrap whole cells, alginate beads swelled during prolonged exposure to high temperatures, so chitosan was the appropriate carrier to perform GOS synthesis.

Another interesting approach is the combination of entrapment and adsorption *via* ionic interaction. Souza, Garcia-Rojas, and Favaro-Trindade (2018), complexed β-galactosidase with different polysaccharides (sodium alginate or λ-carrageenan), mixing them with the enzyme solutions, and varying the pH (from neutral to acid). Authors evaluated lactose hydrolysis at different pH values, until achieving a change in the three-dimensional conformation as result of the interaction of the amino groups of the enzyme with the sulfate and carboxyl groups of the polysaccharides. Although the complex was affected by pH variation, this alteration was observed in a lesser extent when alginate was used as the polymer (when compared to λ-carrageenan). Low pH also reduced the enzyme denaturation rate. More recently, Souza et al. (2019) got an insight on the immobilization through complexation using alginate, stating that when the enzyme-complex was exposed to high temperatures for a long time, thermal stability was improved, compared to FE.

The latest innovations in β-galactosidase immobilization through entrapment indicate that using alginate as a carrier seems to be proper when lactose hydrolysis is the final objective but when immobilization is designed for GOS synthesis, carriers less common, like fibers or chitosan, are more adequate to implement the entrapment.

In early studies, the mycelium from *Aspergillus japonicus* was immobilized using calcium alginate gel Cheng et al. (1996), to improve enzyme (β-fructofuranosidase) stability in terms of pH and thermal changes. When performing FOS synthesis, the obtained yields were very similar to those obtained with FE, and only 17% of enzyme activity was lost over one month of continuous operation. Authors claimed that mass transfer was effective thanks to a high ratio of transfructosylating to hydrolyzing activity. A similar approach was applied by Ganaie, Pathak, and Gupta (2011), by immobilizing whole cells of *Aureobasidium pullulans* with sodium alginate through extruded drops. Authors evaluated separately extracellular and intracellular enzyme performance for FOS production. The last one showed a higher conversion yield (54% wt/wt FOS) than extracellular mass (46% wt/wt FOS).

Also using the entrapment method but with a commercial enzyme preparation, Fernandez-Arrojo et al. (2013) immobilized the enzyme in calcium alginate gel beads and then, dried the gel with the entrapped enzyme. This strategy was successful, and IE resulted stable because it did not swell in the concentrated sucrose solution, thus avoiding enzyme leakage. The IE was tested for FOS synthesis using both batch and continuous fixed bed reactors at lab scale. The continuous operation promoted higher volumetric activity and enhanced the space-time-yield of fixed-bed bioreactors. FOS yield was stable for long term operation and the enzyme system could be stored at room temperature without microbial attack.

More recently, Zambelli et al. (2016) implemented this immobilization method for whole cells, as most of the cited works do, of *Cladosporium cladosporioides* and performed FOS' synthesis in a continuous flow reactor. The strategy promoted a significant improvement of reactor productivity (1.7 times, compared to batch processes), being stable during 7 days of continuous FOS production without varying significantly the product composition.

In general terms, immobilization *via* entrapment is a good strategy to improve pH and temperature stability for both β-galactosidase and fructosyltransferase. In the particular case of fructosyltransferase, this immobilization method is most employed for immobilizing whole cells rather than isolated enzymes. The main disadvantage of this method is that in general, supports cannot be reusable when the enzyme activity runs out.

Covalent binding

In addition to adsorption methods, Gaur et al. (2006) also immobilized β-galactosidase by covalent coupling to chitosan previously activated with GA. This method led to a high activity yield and the enzyme presented higher temperature stability while GOS yield was comparable with that obtained with FE. A similar immobilization method using an analogous support was implemented by Klein et al. (2012, 2013), claiming that optimal pH was enhanced from 6.5 to a wider range between 6.5 and 8.0. Although the optimal temperature was the same for both FE and IE, this latter immobilization method led to a higher enzyme activity in a wider range of temperatures. Authors also assayed the enzyme thermal stability under different lactose concentrations (50 g/L and 400 g/L) indicating that a higher lactose concentration promotes retaining enzyme activity. These results encourage the production of GOS in a continuous PBR using immobilized β-galactosidase in chitosan macroparticles.

Chen et al. (2009) immobilized a thermostable β-galactosidase from *Bacillus stearothermophilus* using Tris(hydroxymethyl)phosphine (THP) and GA and chitosan as support, but the focus in this work was to enhance lactose hydrolysis in a PBR. In line with this, Lima et al. (2013) investigated the same immobilization method but focusing on the selection of the best strain of *Kluyveromyces* promoting the highest hydrolytic activity. The strategy increased thermal

stability (compared to that of FE) and the enzyme could be reused for 10 cycles, retaining more than 70% of its initial activity.

Again, with GA as a driver for the covalent attachment, Neri et al. (2011) immobilized β-galactosidase using polyaniline coated with magnetite as support. Although highlighted that similar product composition was obtained and similar kinetic behavior was observed, compared to the FE, with the advantage of an easy way to remove the IE from the reaction mixture by a magnetic field, being reusable.

Using the same immobilization technique González-Cataño et al. (2017) employed polysiloxane-polyvinyl alcohol polymer activated with GA, which led to an immobilization yield of 99%, with 78.5% of enzyme activity recovery. Authors studied experimentally the reaction mechanism to produce GOS and proposed a mathematical model estimating rate constants, considering a pseudo steady-state hypothesis for two concomitant reactions. The first one involved lactose hydrolysis forming glucose and galactose, the latter reacting with lactose to form trisaccharides, and with each other (glucose and galactose) to form transgalactosylated disaccharides. In the second one, the galactosyl–enzyme complex reacts with the obtained transgalactosylated disaccharides, and although trisaccharides are still being formed, they are simultaneously hydrolyzed, leading to glucose release. Song et al. (2010) implemented this immobilization method, but using silica gel as the support, inducing a reaction between the protein amine and carboxyl groups and electrophilic moieties previously introduced onto the solid surface treating it with 3-APTES and GA. As a strategy to protect the enzyme active sites, before immobilization β-galactosidase was previously treated with lactose solution. This produced a higher activity yield than non-pretreated enzyme, and a higher thermal stability, as many IE mentioned above. An interesting finding of this work is the fact that not only pH but also buffer molarity affected both FE's and IE's activity, showing that the IE works properly in a more basic medium and tolerates a wider range of buffer molarity. In a more recent work, Song et al. (2013) analyzed the continuous synthesis of lactulose from whey lactose finding that the inhibitory effect of galactose and glucose decreased with the immobilization in a PBR.

Bernal et al. (2013) immobilized β-galactosidase in a glyoxyl Sepharose support comparing both one-point and multi-point attachments. Immobilization was performed at pH 10 to promote the inactivation of the enzyme by displacing cation, which allowed immobilizing in 20 min and retaining 82% of the enzyme activity. Multi-point attachment enhanced thermal and pH stability, increased the rigidity of the three-dimensional structure, and made the enzyme complex less susceptible to inactivation in the presence of solvents (dioxane 30%). Authors also optimized the degree of multi-point attachment, given that they observed that excessive multi-point linkage (longer incubation time during immobilization) caused a decrease in the enzyme stability which was explained by a modification or distortion of the structure.

Güleç (2013) applied covalent binding to immobilize β-galactosidase onto cellulose acetate membranes, modifying membrane's surface with plasma polymerization of ethylenediamine (EDA) and plasma polymerization of 2-mercaptoethanol in order to introduce $-NH_2$ and –SH groups on the membranes. Additionally, plasma polymerization of EDA-modified membrane was coated with layers of IE using polyethyleneimine. Although high enzyme loading (65–83%) was achieved, both methods decreased enzyme activity (11–12%) and GOS yield, probably due to negative effects on active amino groups. The most efficient strategy was to immobilize β-galactosidase onto thiolated membrane surfaces, created by plasma polymerization of 2-mercaptoethanol with high immobilization yield (70%) and especially high enzyme activity (46%).

Warmerdam et al. (2014) used well-known commercial porous acrylic beads (Eupergit C) with oxirane functionality to immobilize covalently β-galactosidase through reaction of its thiol and amino groups with the epoxide groups of the carrier. Although after immobilization the enzyme experienced activity loss, it was stable for 90 days and its productivity during one run in the PBR was more than six-fold higher than the productivity of the FE during one run in a batch reactor.

Using chitosan as support, de Albuquerque et al. (2018) and Nguyen, Styevkó, et al. (2019) have recently immobilized covalently β-galactosidase. The former used GA to activate and to improve catalysts stability for lactulose synthesis using cheese whey and fructose as substrate. The methodology allowed the obtaining of 17.3 g/L of lactulose, and 86% of lactose conversion, suggesting that the immobilization improve not only enzyme stability but also, its conformation and its kinetic properties. On the other hand, Nguyen, Styevkó, et al. (2019) used chitosan-coated magnetic Fe_3O_4 nanoparticles and assayed the immobilized catalyst to produce GOS from lactulose. Also in this case enzyme stability was improved in terms of half-life and thermal and pH tolerance. A maximum GOS yield of 17% mol/mol was obtained after 36 h of reaction using 2.34 M of initial lactulose concentration.

Regarding fructosyltransferase, Hayashi et al. (1991) applied this method with an inorganic support (Shirasu porous glass), previously activated with an aqueous silanization process coupling a monolayer of silane onto the support surface (Weetall 1976) activated with GA. Enzyme activity was tested in a PBR using a fast flow rate of concentrated sucrose solution as substrate, leading to the production of short chain FOS.

Also with an inorganic support, Chiang et al. (1997) used oxirane containing methacrylamide-based polymeric beads to covalently immobilize β-fructofuranosidase from *A. niger* and *A. japonicas*. Although both IE and FE presented their maximum activity at 60 °C, at lower temperatures IE retained a higher percentage of its maximum value than FE. Obtaining FOS with both IEs in a batch reaction presented a similar pattern to that of the FE's reaction.

A similar approach took Ghazi et al. (2005) immobilizing two commercial enzymes using two kinds of polymethacrylate-

based polymers (Sepabeads EC). Authors assayed the influence of pH and ionic strength, finding that pH 5.5 favored the binding of the carboxylic heads of the aspartic and glutamic side chains whereas pH 9, the binding of amino and thiol groups. Additionally, low ionic strength produced more protein bound to the support, which also increased with the porosity of the support. The pattern and the yield of FOS production under batch mode with IE, was similar to the production with FE and other immobilization methods, respectively. After 36 h of reaction they were able to obtain 61.5% of FOS relative solid composition. In line with this, Tanriseven and Aslan (2005) immobilized the same commercial enzyme using also a commercial support (Eupergit C). The covalent attachment was possible through the amino, mercapto, or hydroxyl groups. The maximum efficiency (ratio of activity of IE to the activity of FE) was 96%. In addition, immobilization enhanced the thermal stability of the enzyme comparing with FE. The production of FOS using FE and IE was very similar regarding product composition, and the latter retained its activity for 20 days performing batch reactions of 1 h at 60 °C.

Onderková, Bryjak, and Polakovič (2007) covalently immobilized fructosyltransferase from *Aureobasidium pullulans* using a commercial support composed of butyl acrylate copolymerized with ethylene glycol dimethacrylate. As a first step, the amount of enzyme to be immobilized was optimized. At optimal conditions, the carrier preserved 98% of its activity for one month. While immobilization poorly affected thermal stability (comparing with FE), pH stability was favored, shifting the optimum to the alkaline region. FOS production with FE and IE presented similar apparent initial rate, which increased while sucrose concentration increased. Authors pondered the mechanical resistance of the carrier which was able to perform 11 cycles of FOS synthesis in a stirred vessel with only 8% of activity loss.

Lorenzoni et al. (2014), covalently immobilized partially purified β-fructofuranosidase, from a commercial enzyme preparation (Viscozyme L), on GA-activated chitosan particles. The best biocatalyst activity was obtained with 120 mg/g of enzyme per dry support, achieving an immobilization yield (ratio between immobilization and initial activity) of 90% and immobilization efficiency (ratio between observed and immobilized activity) of 33%. Immobilization enhanced considerably the thermal stability of the biocatalyst comparing to FE at 60 °C, and retained its activity after 50 cycles of batch FOS synthesis. Lorenzoni et al. (2015) evaluated inverted sugar and FOS production using this biocatalyst under two PBR and two fluidized bed reactors (FBR), producing 98 and 94% (grams of invert sugar per grams of initial sucrose), respectively, and 59 and 54% (grams of FOS per grams of initial sucrose), in the PBR and FBR, respectively. In both modes of production, varying the flow rate was possible to modulate the product composition in terms of DP3 and DP4 concentrations.

Burghardt et al. 2019 presented a detailed study of neoFOS production (β-(2,6) glycosidic bonds FOS) using immobilized fructosyltransferase. Authors compared covalently immobilized enzyme using polymethacrylate porous beads with epoxy functions and epoxy resin with ionic immobilization (adsorption) using anion exchange membranes. The former option was preferable because it maximized enzyme activity, stability and enhanced the yield of FOS. Ionic immobilization using membranes seemed to suffer enzyme desorption during the washing step after immobilization.de Oliveira et al. (2020) immobilized fructosyltransferase with a similar approach than applied by Nguyen, Styevkó, et al. (2019) with β-galactosidase. Immobilization carried out using Fe_3O_4-chitosan-magnetic nanoparticles as support and GA as enabler agent. The immobilized biocatalyst, showed both hydrolytic and transfructosylating activities and retained 70 and 86% of them after 6 cycles of reuse. In addition, high thermostability was achieved obtaining a maximum FOS concentration of 101.56 g/L, with predominant presence of 1-kestose in the reaction mixture.

Covalently immobilization of fructosyltransferase and β-galactosidase is one of the most studied techniques for immobilization. During the last years, scientific results show that there are many commercial supports that can be applied for this purpose; nevertheless, most of the protocols of enzymatic covalent immobilization required the treatment with a reactive compound that acts as activator of the support surface, GA being the most widespread.

Cross-linking

Sungur and Akbulut (1994) used a gelatin carrier system and two cross-linking agents (GA and chromium (III) acetate) to immobilize a β-galactosidase. Authors managed to use minimum amounts of crosslinkers, obtaining a stable and hardened gelatin and avoiding enzyme leakage. Activity yield was 25% and 22% for GA and chromium (III) acetate, respectively, and decreased only 9% after 42 days of use. Furthermore, enzyme activity was less susceptible to pH variations during immobilization, accentuated with the crosslinker chromium (III) acetate. From literature search, it seems evident that the enhancement of enzyme activity yield has been prioritized given that GA has been the principal cross-linking agent applied in most studies. In this line, Zhou and Dong (2001) immobilized β-galactosidase using graphite slab and GA as cross-linking agent and found that the average specific activity (ratio of the activity of IE to that of the FE) was between 17% and 25%. Immobilization increased K_m and decreased V_m Michaelis–Menten's constants. The IEs were stable and active in operational conditions for lactose hydrolysis.

The works of Albayrak and Yang (2002), and Matella, Dolan, and Lee et al. (2006) show the use of cross-linking as a final step for coating polyethyleneimine-enzyme aggregates to cotton cloth, with GA as cross-linking agent. Authors stated that this strategy promoted a strong permanently fix bond of enzyme aggregates, avoiding leaching out when exposed to acetate buffer. In addition, low temperatures favored higher enzyme activity. Multilayered polyethyleneimine IE was used in a plug-flow reactor achieving stable and continuous operation with an enhancement in GOS productivity comparing with others previously reported for this type of process configuration.

Gaur et al. (2006) studied a cross-linking method, without any support, to immobilize β-galactosidase along with adsorption and covalent bonding processes. The strategy was to form enzyme aggregates by adding ammonium sulfate and GA under controlled conditions. The activity yield with this method was 13.5% (lower than covalent bonding but higher than adsorption). Also, β-galactosidase immobilized through cross-linking aggregates (as well as covalent immobilization) was thermally more stable and its half-life was enhanced comparing to FE. The disadvantage of this method was that it promoted lactose hydrolysis over GOS synthesis.

These cited works were able to enhance GOS production with this type of immobilization, testing the synthesis under continuous production or repeated-batch operation. For this reason, it is not clear if the disadvantage of the strategy applied by Gaur et al. (2006) rises in the immobilization procedure or if it is a matter of reactor configuration.

In order to design an enzymatic membrane reactor to produce and purify GOS, Palai and Bhattacharya (2013); Palai, Singh, and Bhattacharya (2014) immobilized β-galactosidase in a polyvinylidene fluoride (PVDF) membrane using GA as cross-linking agent. The units of enzyme immobilized per mass of membrane increased with enzyme concentration up to a maximum loading capacity. The formation of GOS increased with the initial lactose concentration. Authors developed a six-step-eleven-parameter model based on Michaelis–Menten kinetics, which was able to reproduce the experimental results. Furthermore, they improved the process' design by incorporating a recirculation loop that allowed producing GOS selectively and, again, mathematically modeled this process. The storage stability of the IE was studied, the enzyme retained 50% of its initial activity after 30 days of storage at 20 °C.

Guerrero et al. (2015; 2018) analyzed diverse strategies to immobilize β-galactosidase by aggregation and cross-linking comparing performances in a repeated-batch operation with a single batch operation with FE, using lactulose as substrate. Immobilization was produced by precipitating β-galactosidase from *A. oryzae* with different concentrations of ammonium sulfate and testing the addition of different concentrations and times of exposure of GA. Authors found that increasing the ratio between GA and protein promoted an increase in specific activity and the immobilization yield up to a certain point, from which consumption of more cross-linking agent did not have any benefit. Thermal stability was not improved under these conditions of immobilization. Regarding lactulose synthesis, immobilization favored the generation of disaccharides over higher oligosaccharides. When comparing with single batch FE operation, immobilization allowed enzyme reuse, increasing lactulose production per unit of mass of biocatalyst. Furthermore, Guerrero et al. 2019 tested this catalyst in continuous packed-bed reactor to produce lactulose from fructose and lactose analyzing the effect of flow rate, substrates ratio and biocatalyst ratio. Under optimal conditions, maximum lactulose yield was 0.6 g/g of total sugars, and lactose conversion was 28%. Authors claimed that operation with recycle had no significant effect on yield.

Guerrero et al. (2017; 2018); Urrutia et al. (2013) also analyzed the use of monofunctional and heterofunctional glyoxyl-agarose supports, as previously reported by Mateo et al. (2010), which consisted on epoxide-agarose with different additional functional groups. This approach involved first the adsorption of the enzyme to the support and then multi-point covalent attachment by means of the amino groups in the enzyme lysine residues and the aldehyde groups of the support. Authors determined the reaction kinetics and the product composition, obtaining a higher immobilization yield (39.4%) with amino-glyoxyl-agarose support. When analyzing enzyme performance during lactulose synthesis, higher yields were obtained with monofunctional glyoxyl-agarose. Moreover, they determined that glyoxyl-agarose and amino-glyoxyl-agarose derivatives retained the selectivity of the FE for lactulose synthesis while carboxy-glyoxyl-agarose and chelate-glyoxyl-agarose favored the synthesis of transgalactosylated oligosaccharides. The restrictions that immobilization produced on the enzyme activity had low effect on transgalactosylation because of the use of high substrate concentrations, concluding that immobilization had a more critical impact on the hydrolysis of lactose. Additionally, Urrutia et al. 2018, using the same experimental design, tested chitosan partially functionalized with aldehyde groups as support. In this case, authors studied two cross-linking agents: GA and epichlorohydrin. The cumulative GOS yield after 10 batches using immobilized enzyme was 4.7 and 4.0 times higher, compared to that obtained with soluble enzyme.

Also, using GA as cross-linking reagent, Eskandarloo and Abbaspourrad (2018) immobilized β-galactosidase on the surface of glass beads, activated with 3-APTES. Different enzyme concentrations were analyzed, founding what many other works reported previously: the amount of IE per mass of support increased when the concentration increased, up to the maximum capacity of the support. However, they showed that increasing enzyme concentration resulted in a considerable decrease in immobilization efficiency (unit of IE per unit of enzyme taken in the solution). Immobilization increased the optimal operational temperature and the highest enzymatic activity was achieved at higher temperatures than FE. Similar behavior was observed with higher pH, related to diffusional constraints, or to secondary interactions between the enzyme molecules and the supports. When studying GOS production in a PBR, it was observed that the GOS yield increased with repeated cycles of operation and demonstrated the high efficiency and reusability of its process configuration with this type of IE.

Recently, Sass and Jördening 2020 promoted an innovative strategy to immobilize β-galactosidase on electrospun gelatin nanofiber mats. The findings involved the determination of optimal conditions for solvent system during electrospinning process and the subsequent cross-linking of gelatin nanofiber mats using hexamethylenediamine (HMDA) in order to increase their stability in water. GOS yield using this catalyst was 31% higher than that obtained with FE (27.7%).

Cross-linking of fructosyltransferase has also been evaluated, although not as extensively as β-galactosidase. Platková et al. (2006) studied six commercial anion-exchange resins and polymethacrylate carriers, both by direct attachment, or by the attachment accompanied by GA cross-linking. Increasing operational pH caused a decrease in enzyme activity, probably due to the presence of hydroxyl groups. For all biocatalysts, the addition of the cross-linking agent produced a drop of the activity. The carriers that promoted higher enzyme activity were styrene with quaternary amine groups and polymethacrylate with epoxide groups. The lower enzyme activity in other supports was attributed to enzyme inactivation during the process and diffusional problems. Ademakinwa et al. (2018) prepared and evaluated cross-linked enzyme aggregates (CLEAs) of fructosyltransferase to produce FOS using GA as cross-linking agent. Authors indicated that the best precipitant for CLEAs production was ammonium sulfate being able to maintain 100% of residual activity over four rounds of catalysis. The secondary structure of CLEAs was determined from FTIR spectra, showing that cross-linking with GA promoted protein aggregation causing the transformation of helical and beta sheets structures into beta turns. When analyzing FOS synthesized with CLEAs, authors stated that they had prebiotic properties comparable to those obtained from commercial FOS. Although authors promoted this method for industrial FOS biocatalysts, no comparison between CLEAs and FE activities (i.e., product yield, enzyme stability) at different process conditions (i.e., pH, stirring, temperature) has been made. Charoenwongpaiboon et al. (2019) also made use of CLEAs. In order to immobilize inulosucrase, they used a fructosyltransferase with higher transglycosylation activity than β-fructofuranosidase, and capable to synthesize both inulin and inulin-type fructo-oligosaccharides from sucrose. In this study the optimum conditions for CLEAs preparation were determined in terms of recovered activity and again ammonium sulfate was promoted as the best precipitant, together with 0.5 mM GA and pH in a range of 5–7. Under these optimum conditions, CLEAs retained 42% of original inulosucrase activity. Comparing with FE, the optimum pH of inulosucrase changed from 5 to 4 after immobilization, while the optimum temperature was the same. Nevertheless, immobilization produced higher pH and thermal stability. There was found that the CLEAs promoted the synthesis of inulin-type FOS with the DP ranging from 3 to 8, while the soluble inulosucrase catalyzed the synthesis of inulin-type FOS with the DP up to 13. Authors concluded that CLEAs were useful to produce insulin-type FOS with higher prebiotic effect than FE and also presented operational stability in the batch synthesis conditions.

In an overall view, cross-linking is a methodology derived from covalent binding, which is among the latest advances in enzyme immobilization. Although it seems to be one of the most delicate methods, given that it can modify the enzyme's configuration leading to inactivation, it presents the advantage of being highly specific. As it consists on the intermolecular linking of different enzyme moieties (primary amino groups, thiols, phenols, imidazoles) with the carrier, immobilization can be modulated to enhance the active sites of the enzyme and to increase the specificity for the substrates avoiding the inhibitors. Although this strategy is still under development, recent advances in nanotechnology will allow going further in the understanding of the intermolecular configurations and interactions, thus optimization of IE selectivity and effective activity yield could be improved.

Support materials and approaches for enzymatic production of GOS and FOS

The selection of an enzyme carrier also depends not only of the material's properties (e.g., surface area, particle size, pore structure, presence or absence of functional groups on its surface) but also on practical issues (e.g., cost, availability, stability, and the type of reactor). For instance, depending on the application, a specific material can successfully immobilize the catalyst but may not survive the industrial processing conditions. On the other hand, if a given material can resist the reactor's conditions but its affinity with the enzyme is insufficient, this can be overcome with the use of surface modifiers, changing the properties of the support (Jesionowski, Zdarta, and Krajewska 2014). Besides its simplicity, the main advantage of this method, is its ability to preserve the native structure and the activity of the enzyme. However, the weak interactions between the protein and the carrier result in leaching of enzyme from the support over time.

In terms of the properties, enzyme supports should grant the process some advantages over the use of the soluble enzyme. The most critical issue is the reduction in the overall cost of the industrial process and this can be achieved using a cost-effective support (not always an easy task), by increasing the reusability of the enzyme (enabling the implementation of continuous processes), by facilitating the recovery of the catalyst and the purification of the final product, and by increasing enzyme stability and durability, while performing the transformation(s) and during storage and transport (Boudrant, Woodley, and Fernandez-Lafuente 2020).

The support should also have thermal, mechanical and physical endurance to withstand the (sometimes) harsh conditions of the industrial process. It ought to grant the enzyme with increased specificity toward the substrate, reduce catalyst inhibition and be inert. It should also present easy regenerability, avoid contamination, namely by bacteria, and be eco-friendly (biocompatible and biodegradable). However, the selection of the support and its properties are closely related with the type of immobilization procedure chosen for the enzyme. Hence, physical and chemical properties (e.g., hydrophilicity/hydrophobicity, pore size, presence of surface functional groups, or resistance to certain pH or temperature) must be also considered taking into account the application foreseen (Aehle 2007; Dwevedi 2016; Mohamad et al. 2015; Sirisha, Jain, and Jain 2016).

Selection of the support material: a key factor for enzyme immobilization

Taking into consideration the different methods for enzyme immobilization to produce GOS and FOS, a suitable support

material should be chosen. The selection of the material depends not only on the immobilization method and the type of the enzyme, but also on the conditions of the catalytic process and the enzyme-support interactions that may occur (Guzik, Hupert-Kocurek, and Wojcieszyńska 2014; Boudrant, Woodley, and Fernandez-Lafuente 2020). These interactions may interfere with the properties of the whole biocatalytic system, so special care must be taken in order to enhance the enzyme specificity (Datta, Christena, and Rajaram 2013; Mohamad et al. 2015). Besides, supports should preferentially be low cost and eco-friendly as well as inert, in order to not interfere and not increase the costs of the overall enzymatic operation. Supports that have high stability, thermal and mechanical resistance, a high rate of regeneration and reusability are preferred (Sirisha, Jain, and Jain 2016; Zdarta et al. 2018). Additionally, the reusability of biomacromolecules such as carbohydrates and proteins-based biopolymers is a promising strategy to obtain biopolymeric nanoparticles that are antibacterial, biocompatible, immunogenicity, and biodegradable (Verma, Dhanya, et al. 2020). It is important to remark that the support should act as a barrier that preserves the enzyme structure against extreme process conditions (pH, temperature, mechanical damage) avoiding denaturation and inactivation. Furthermore, the chosen material should provide an efficient establishment of the enzyme-support complex and there should be a good affinity between the functional groups of the enzyme and the support, so an effective binding can occur. Given that an ideal and universal support would not be feasible to obtain, the choice of the most appropriate material will involve analyzing the pros and cons of its properties and usability (Mohamad et al. 2015; Sirisha, Jain, and Jain 2016).

There is a variety of materials that can and have been used as support to enzyme immobilization. Based on their chemical composition, these supporting matrices can be divided in two main categories: inorganic and organic. The latter can then be subdivided into natural and synthetic organic supports (Hettiarachchy et al. 2018). Silica and other oxides, such as aluminum, titanium or zirconium oxides, are the most commonly inorganic suports, as well as hydroxyapatite, activated carbon, glass and ceramic, as described in Table 3. Usually, inorganic matrices are preferred for their lower reactivity, thermal and mechanical resistance, high stability, rigidity and porosity. Some of them can ensure a fixed volume and shape attributable to the invariance of their pore diameters (Sirisha, Jain, and Jain 2016; Zucca and Sanjust 2014). Since most of these materials are not chemically reactive and the functional groups are mainly hydroxyl groups, a previous treatment to modify and activate the matrix is required. The matrix modification generally occurs prior to the activation and it consists on the addition of amino groups, through a treatment with aminoalkyl triethoxysilanes. For the matrix activation, different methods make use of dialdehydes, such as cross-linking agents, being GA the most common one (Hettiarachchy et al. 2018; Sirisha, Jain, and Jain 2016).

Several reported works merit the use of organic material, since these can be chemically modified and also surpass the limitations of inorganic materials such as reduced biocompatibility, low affinity to biomolecules and the inorganic supports inadaptability to be reshaped and to be used with different methods of immobilization (Jesionowski, Zdarta, and Krajewska 2014; Zdarta et al. 2018). The main disadvantage of organic matrices is the low chemical and mechanical resistance, which impair their usage in systems with aggressive thermal and pH conditions leading to the impossibility of regeneration of the matrix. Among the most used organic materials there are reports of the usage of a broad variety of polymers, natural and synthetic (Jesionowski, Zdarta, and Krajewska 2014). Natural polymers, such as carbohydrate species can form inert and strong aqueous gels (hydrogels) such as alginate, chitosan, starch, cellulose and carrageenan. These mentioned carbohydrates can be easily obtained, with low associated costs, since most of them are by-products of different industries (Homaei et al. 2013; Zdarta et al. 2018). As for the most common synthetic polymers described, there are reports of the application of polyvinyl alcohol, polyvinyl chloride, polyurethane, polyaniline, diethylaminoethyl cellulose (DEAE-cellulose), Eupergit and activated nylon (Datta, Christena, and Rajaram 2013; Hettiarachchy et al. 2018; Sirisha, Jain, and Jain 2016). These synthetic polymers are relatively easy to produce and can be used in different methods of immobilization, where they can be modified to satisfy the desired specific requirements of the enzymes and the reactional conditions of a specific enzymatic process, without interfering with other properties such as thermal and chemical resistance (Datta, Christena, and Rajaram 2013; Jesionowski, Zdarta, and Krajewska 2014).

In order to obtain some products through cascade reactions, some inovative methods, like co-immobilization processes, with multienzyme systems, have gained special attention as well as the materials chosen for such reactions (Ansari and Husain 2012; Mohamad et al. 2015).

In summary, there is wide range of possibilities regarding the type of support, as shown in Table 3. Nevertheless, particularly for β-galctosidase and fructosyltransferase, the most assayed supports include chitosan, alginate-based and sol-gel carriers, resins with different functional groups, acrylic or glass porous beads and membranes (cellulose, polyvinylidene fluoride and ceramic).

Nanotechnology and electrospinning as new approaches to produce support materials for enzyme immobilization

Due to an increased popularity over the years for industrial applications and many other areas such as medicine and pharmaceuticals, agriculture and even biodiesel production, the search for new materials and immobilization in micro and nano-scales allowed the continuous development of different enzyme supports and immobilization methods.

Advances in the nanotechnology field allowed for immobilizing enzymes using different nanostructured forms, such as nanofibers, nanotubes, nanoparticles, nanoporous, nanosheets and nanocomposites. These materials provide large surface area to volume ratio which improves enzyme loading, leading to a more efficient immobilization and

Table 3. Principal materials and applications implemented as supports for enzyme immobilization.

Type of material	Immobilizing matrix	Enzymes	Immobilization method	Reference
Inorganic	Silica	Penicillin acylase	Covalent cross-linking with GA	Kheirolomoom, Khorasheh, and Fazelinia 2002
		Lipase	Cross-linking with GA	Lee et al. 2006
		Trypsin	Adsorption	Gómez et al. 2009
	Glass	Pronase (Protease mixture)	Covalent binding	Royer and Green 1971
		β-fructofuranosidase	Covalent binding	Hayashi et al. 1991
		Lipase	Adsorption	Lee and Swaisgood 1997
	Celite	α-chymotrypsin	—	Adlercreutz 1991
		β-galactosidase	Covalent binding	Fai et al. 2017
		Lipase	Adsorption	Kaja et al. 2018
	Activated Charcoal	Amyloglucosidase	Adsorption	Rani, Das, and Satyanarayana 2000
		Papain	Adsorption	Dutta et al. 2009
		Protease	Adsorption	Khan and Bokhari 2013
	Alumina	Invertase	Adsorption	Hu, Haering, and Geankoplis 1985
		Trypsin	Adsorption	Pugnière et al. 1988
		Penicillin G acylase	Adsorption	Bahulekar et al. 1991
	Hydroxyapatite	Urease	Adsorption	Marzadori et al. 1998
		Levansucrase	Ionic binding	Jang et al. 2000
		Protease	Adsorption	Zdarta et al. 2015
Biopolymers	Alginates	Glucose oxidase	Encapsulation	Blandino, Macías, and Cantero 2001
		β-galactosidase	Covalent binding	Eldin, Hassan, and El-Aassar 2005
		β-glucosidase	Entrapment	Keerti et al. 2014
	Chitosan	Laccase	Adsorption cross-linked GA	D'Annibale et al. 1999
		Inulinase	Covalent binding	Yewale, Singhal, and Vaidya 2013
		Lipase	Adsorption	Kaja et al. 2018
	Cellulose	Peroxidase	Covalent binding	Isobe et al. 2011
		Lipase	Covalent binding	Girelli, Salvagni, and Tarola 2012
		Glucose oxidase	Entrapment	Yabuki et al. 2012
	Agarose	α-chymotrypsin	Covalent binding	Guisán et al. 1991
		α-amylase	Entrapment	Prakash and Jaiswal 2011
		β-galactosidase	—	Satar and Ansari 2017
	Gelatin	Urease	Cross-linking with GA	Srivastava, Kayastha, and Srinivasan 2001
		Tyrosinase	Entrapment	Munjal and Sawhney 2002
		α-amylase	Cross-linking with GA	Jaiswal et al. 2012
Synthetic Polymers	Polyacrylamide	Invertase	Entrapment	Abdellah et al. 1992
		Alkaline phosphatase Tyrosinase	Entrapment	González-Sáiz and Pizarro 2001
			Entrapment	Munjal and Sawhney 2002
	PVA	Laccase	Cross-linking with GA	Bai et al. 2014
		α-amylase	entrapment	Nakagawa and Goto 2015
		Alcohol dehydrogenase	Adsorption/covalent binding	Shinde et al. 2018
	Polyurethane	Lipase	Covalent binding	Awang, Ghazuli, and Basri 2007
		Inulinase	—	Silva et al. 2013
		Papain	Covalent binding	Manohar and Doble 2016
	PEG	Cellulase	Covalent binding	Asli et al. 2016
		α-chymotrypsin	Cross-linking with cystamine	Fraas and Franzreb 2017
	PVDF	β-galactosidase	Covalent binding	Palai, Singh, and Bhattacharya 2014
		Lipase	—	Kayhan, Eyupoglu, and Adem 2016
		Tyrosinase	Covalent binding	Algieri, Donato, and Giorno 2017
Resins	DEAE-cellulose	Invertase	Adsorption	Abdellah et al. 1992
		Epoxide hydrolase	Adsorption	Karboune et al. 2001
		Nuclease p1	—	Shi et al. 2010
	Duolite A-568	β-galactosidase	Adsorption	Botelho-Cunha et al. 2010
		Cellobiose 2-epimerase	Adsorption	Wang et al. 2016
		Invertase	Adsorption	Cabral et al. 2017

(*continued*)

Table 3. Continued.

Type of material	Immobilizing matrix	Enzymes	Immobilization method	Reference
Nanomaterials	Dowex	Invertase	Adsorption	Junko Tomotani and Vitolo 2006
		Phospholipase D	Adsorption	Yon et al. 2008
	Sephadex G-25	Glutaminase	Adsorption	Ahmad Mahmod 2016
		Lipase	Adsorption	Kaja et al. 2018
	Chitosan-coated magnetic nanoparticles	β-galactosidase	Covalent binding	Pan et al. 2009
		β-fructofuranosidase	—	Chen, Sheu, and Duan 2014
	Silica nanoparticles	Glutamate dehydrogenase/lactate dehydrogenase	Covalent binding Entrapment	Qhobosheane et al. 2001 Voss et al. 2007
		Horseradish peroxidase	Adsorption/covalent binding/cross-linking with GA	Karimi et al. 2014
		Endo-inulinase		
	Hydroxyapatite nanoparticles	β-glucosidase	Adsorption	Coutinho et al. 2018
	Polyurethane-gold and polyurethane-silver nanoparticles	Maltogenase	Adsorption	Kochane et al. 2017
	Electrospun cellulose nanofiber membrane	Lipase	Covalent binding	Huang et al. 2011
	Electrospun PVA fibers	Lipase	Entrapment	Sóti et al. 2016
	Electrospun polyethersulfone nanofibers	α-amylase	Covalent binding (through EDC)	Ghollasi 2018

stabilization of the enzymes facilitating reaction kinetics (Verma, Puri, and Barrow 2016). They also have the ability to control particle and pore size, tailoring the thickness of nanofibers and nanotubes. Additionally, the need for the use of surfactants and toxic reagents, such as cross-linking agents, is reduced and in some cases specific particles with conductive or magnetic properties can be used in order to control the immobilized system (Homaei et al. 2013; Mohamad et al. 2015; Wang et al. 2009; Zdarta et al. 2018). Nanoscale particles, in general, can be designed and redesigned according to the required necessities of the enzyme system (Cipolatti et al. 2016). This way, enzymes have been successfully immobilized into many nanoparticles and nanomaterials, like those described in Table 3, with positive results verified toward the improvement of enzymatic performance. Different nanomaterials have been used as supports, such as polymers, silica, graphene, gold and magnetic particles (Chen et al. 2017; Cipolatti et al. 2016).

Immobilization in nanomaterials can be adapted accordingly to desired conditions, however it still depends on factors, such as the type of enzyme, the support itself and the immobilization conditions, which will condition aspects such as immobilization yield and specific activity.

Electrospinning is one of the simplest techniques used to produce nanofibers characterized by their exceptional length, possibility to have a diversified composition and the uniformity of the fiber's diameters. Electrospun nanofibers have been appointed as immobilization supports with a great potential to overcome the problems presented by the other materials. The obtained fibers generally have high porosity and interconnectivity that allow the system to benefit from low diffusion resistance leading to an efficient mass transfer process, high reaction rate and conversion.

The surfaces of the fibers can be modified in order to benefit the specific enzyme activity, loading onto the fiber a huge quantity of enzyme (Fang et al. 2011; Wang et al. 2009). Spun membranes can also be produced and used as filters, allowing the enzyme-membrane system to act simultaneously as a biocatalyst and a separation material having huge interests for the enzymatic membrane-bioreactors field (Wang et al. 2009).

The chosen polymer to spin should not only be able to form fibers or membranes by electrospinning, but shoul also be able to interact with the enzyme. The selection of materials for electrospinning comes with a specific range of solvents associated that should not interfere with the activity or conformation of the enzyme (Tran and Balkus 2012).

Polyvinylidene fluoride is a common electrospinning material, inert due to the absence of reactive groups, but different procedures have been developed toward modifications of the surface to make it more reactive for biomolecules immobilization (e.g., enzymes) (Algieri, Donato, and Giorno 2017).

A good immobilization material, depends not only on the enzyme but also the method of immobilization and the processing conditions. The same material does not behave equally with different enzymes, due to differences in the

binding, leakage, matrix effect, and diffusional barriers, among others (Calabrò 2013).

Main supports for β-galactosidase and fructosyltransferase immobilization

β-galactosidase can be immobilized in different types of materials. Bearing in mind that β-galactosidases have both hydrolytic and transgalactosylase activities, the conditions for these two types of reactions are different and thus, different materials should be considered depend on the exact goal activity: transgalactosylation reactions require higher substrate concentrations, higher temperatures and lower water activity than hydrolysis. Hence, such specific characteristics must be considered when selecting the most appropriate immobilization support (Panesar, Kumari, and Panesar 2010).

β-galactosidase immobilization has been thoroughly studied with different reports since the early 1970's. Woychik and Wondolowski (1972, 1973) have studied the immobilization of this enzyme in porous glass beads with and without GA as cross-linker. The 1972 report followed a method previously described by Weetall (1969) (used for trypsin and papain), which consisted in the covalent attachment of the enzyme into a porous glass through a diazotization process (diazo-linkage). About 75% of the enzymatic activity was retained using such method and it did not affect any of the enzyme's properties, such as optimum pH and temperature. The method used by Woychik and Wondolowski (1973) also enabled the retaining of 75% of the enzyme's initial activity and had a better activity at lower pH (80% of the optimum activity at pH 4.5). Moreover, the immobilized system allowed for a greater efficiency of lactose hydrolysis in column when compared with the stirred batch reactors (Woychik and Wondolowski 1972, 1973). Some of the inorganic materials used as supports include silica, glass, activated charcoal, celite and alumina (aluminium oxide) (Husain 2010; Panesar, Kumari, and Panesar 2010).

Finocchiaro, Richardson, and Olson (1980) described a method of β-galactosidase adsorbed into alumina previously activated with tolylene-2,4-diisocyanate. This method led to a minimal enzyme leakage, an increment of 16-fold the catalytic activity when compared to untreated alumina, a broader pH profile, and a slightly decrease of the optimum temperature.

Following this chronological main contributions for β-galactosidase immobilization supports, Verma et al. (2012) promoted the use of silicon dioxide nanoparticles activated with GA. This methodology involved multipoint covalent attachment, which improved the enzyme thermal stability. Additionally, when performing lactose hydrolysis, the enzyme complex retained more than 50% of the enzyme activity up to the eleventh cycle.

In 2017, Fai and his team (Fai et al. 2017) obtained GOS through a fixed-bed reactor with enzyme covalently bound to celite. When compared to the FE, the optimum pH slightly decreased and the optimum temperature was 10 °C higher when celite was used as a carrier. Moreover, the immobilized system had higher storage stability, maintaining its functionality for 270 days when kept at 4 °C, and when used repeatedly for 10 times.

Eskandarloo and Abbaspourrad (2018) developed a covalent immobilization into modified glass by cross-linking with 3-aminopropyl triethoxysilane (3-APTES). The obtained enzymatic system revealed increased pH and temperature stabilities, an increased reusability of the enzyme for packed-bed reactions and allowed for its usage in cycle reactions with the lactose conversion for GOS formation increasing with multiple cycles.

Natural and synthetic polymers such as chitosan, alginate, gelatin, agarose (some of them in the form of Sepharose), polyvinyl alcohol, polyethyleneimine, polyester, polyacrylamide, and some resins, such as DEAE-cellulose and Duolite have also been studied as supporting material for the immobilization of β-galactosidase (Cao 2005; Verma, Kumar, et al. 2020).

Li et al. (2008) reported the production of GOS with β-galactosidase immobilized in calcium alginate. The resulting beads had a wide pH range (from 3.6 to 8.2) with yields around 23% and optimum temperature at 55 °C. The enzyme immobilized in these beads could be reused up to seven times without any prominent reduction of GOS production.

A GA-activated chitosan support system increased the enzyme operational stability alongside its pH range and thermal stability (Klein et al. 2013). The immobilization system obtained was used in a PBR operating for both lactose hydrolysis and GOS production in a stable operation for 15 days.

Botelho-Cunha et al. (2010) assayed adsorption into a commercial porous anion exchange resin (Duolite A-568) and reported no increase in enzyme activity due to diffusional problems.

Carević et al. (2018) adsorbed β-galactosidase from *L. acidophilus via* ionic interaction, comparing different resins with different functional groups, focused on lactose hydrolysis application. Carriers with epoxy groups showed the highest yields, but not the highest activities. Hence, there is a compromise between the amount of enzyme immobilized and the amount of IE actually active, mainly because of the probability of unfavorable conformation of the active site during the immobilization process. Carriers with amino groups leverage the activity yield; carriers with larger pore sites also promote higher enzyme activity since the free space facilitates enzyme mobility and substrate diffusion through the active sites.

Jovanovic-Malinovska et al. (2012) worked with a synthetic polymer, polyvinyl alcohol, and observed that, with the enzyme entrapped into to this polymer, 95% of the initial activity was retained decreasing to 49% after 3 months. Polyvinyl alcohol had an immobilization efficiency of 88.5% and a reusability rate close to 100% even after 7 cycles of reuse. Furthermore, the authors verified a higher lactose conversion for GOS production with polyvinyl alcohol in batch system (31% maximum GOS production) when compared with polyvinyl alcohol in packed bed column (23–30%).

Palai, Singh, and Bhattacharya (2014) immobilized β-galactosidase onto compacted microporous polyvinylidene fluoride membranes via cross-linking with GA. Increasing the initial substrate concentration led to an increased selectivity for GOS formation, despite a GOS yield reduction. Polyvinylidene fluoride membranes were used in a batch mode feed recirculation system, resulting in a maximum yield of 30% for GOS formation with initial lactose concentration of 50 g/L. This immobilization system allowed for a larger storage time without a significant loss of the enzymatic activity (approximated 50% after 30 days), when compared with the loss of the corresponding native enzyme (100% activity loss after 21 days).

Regarding the group of fructosyltransferases used for the synthesis of FOS (β-fructofuranosidase or invertase), the first report of immobilization strategy was described in 1916, and was attempted by adsorption of invertase onto charcoal and aluminum hydroxide (Nelson and Griffin 1916).

β-fructofuranosidase was immobilized in Shirasu porous glass *via* adsorption and used for continuous production of FOS in a packed column (Hayashi et al. 1991). The Shirasu porous glass was modified by silanization and activation with GA. When used in a batch system, the IE catalyzed the production of a wider range of FOS, in contrast with the continuous system, in which the fast flow rates of sucrose as substrate just led to the production of 1-kestose.

Nishizawa, Nakajima, and Nabetani (2000) studied the immobilization activity in ceramic membranes with different pore sizes and two immobilization methods: adsorption and covalent binding. For physical adsorption, the membranes did not suffer any alterations. For covalent binding, the membranes suffered a pretreatment and activation with GA, and the immobilization occurred chemically in the innermost surface of the membranes. The authors reported the possibility of long-term operations in a sustainable and viable manner, with the system having a half-life of IE of 35 days, due to enzyme denaturation, since no leakage was verified during the process. Immobilization ratios were higher in covalently IEs, with a maximum of 64%, while for adsorption only 6% immobilization ratio was obtained. Covalent bonded membranes represent a better option to apply a forced flow of substrate for FOS production.

Mussatto, Rodrigues, et al. (2009) studied different porous carriers (polyurethane foam, stainless steel sponge, vegetal fiber, pumice stones, zeolite molecular sieves and foam glass) to immobilize cells of *Aspergillus japonicus* and found that vegetal fibers were the best materials for this purpose. FOS production with these immobilized systems was similar to the one obtained with free cells. On the contrary, porous glass was not suitable for FOS production, mainly because of its instability during agitation, and pumice stones and zeolites did not immobilize large amounts of cells.

Despite that inorganic materials are used for this class of enzymes, most works dealing with their immobilization fall upon organic supports, such as alginates, chitosan, resins and polymers.

Alginates have been those most largely reported. Cheng et al. (1996) immobilized β-fructofuranosidase in calcium alginate beads, *via* entrapment, and verified an increment in mechanical strength and enzymatic stability, with a wider resistance to lower pH and greater resistance to higher temperatures.

Tanriseven and Doğan (2001) encapsulated the invertase in calcium alginate capsules treated with GA. Despite that no alterations regarding the optimum pH and temperatures were noted, a higher stability at higher pH and temperatures, together with a long-term were reported.

Fernandez-Arrojo et al. (2013) and Zambelli et al. (2016) confirmed this stability in batch operation with the enzyme entrapped in dried alginate beads. Even though different research groups make different approaches in their alginate preparations, either with sodium or calcium, in capsules or beads, alginates confer an increase in mechanical strength and stability to the enzymatic system.

In a comparative study between immobilization with alginate and chitosan, both activated with GA, the optimum pH and temperature were slightly lower for alginate (Mouelhi, Abidi, and Marzouki 2016) but not significant, with both immobilized systems showing pH stability in a wider range (4–7) than FE and relative activities between 80% and 100%. After 50 cycles of use, both immobilized systems maintained more than 80% its activity. Similar results were obtained by Lorenzoni et al. (2014, 2015), with chitosan particles activated with GA, that lead to not only higher thermal stability but also a retention of high enzymatic activity after 50 cycles of use in a batch production of FOS. For immobilization in chitosan, Nam et al. (2017) proposed that the occurrence of optimum pH and temperature shifts, when compared with the FE may occur due to alterations of the physical and chemical properties of the enzyme during the immobilization process.

Other materials with gelation capacity are agar/agarose, gelatin and some anion-exchange resin, such DEAE-cellulose, referring an improvement in pH stability, a reduction of susceptibility to metal ions for the enzyme alongside to a use of the said system in a long-term continuous operation with a high rate of FOS production (Hayashi et al. 1994).

Chen, Sheu, and Duan (2014) studied the immobilization of β-fructofuranosidase on chitosan-coated magnetic Fe_3O_4 nanoparticles to produce FOS with sucrose as substrate. The enzyme was immobilized on the surface of the nanoparticles without the addition of cross-linking agents. Both immobilized and FE showed maximum activity at the same pH (5.5) and optimum temperature (60 °C), as well as similar FOS yields, both around 50%. However, the immobilized system showed higher activities at wider range of temperatures and pH than the FE, and retained 55% of the initial activity after FOS production in 10 batches. The nanoparticles can be easily recovered from the obtained FOS solution through application of a magnetic field.

Ganaie, Pathak, and Gupta (2011), and Mussatto et al. (2012) immobilized whole cells of *Penicillium expansum* to evaluate the production of FOS and β-fructofuranosidase at lab scale. Cells were immobilized by natural adsorption through their direct contact with the different carriers

studied (synthetic fiber, polyurethane foam, stainless steel sponge -inorganic materials, loofah sponge and cork- lignocellulosic materials) at the beginning of fermentation. The best carriers were the synthetic fiber and the polyurethane foam, based on their immobilization yield and the enzyme activity. When analyzing repeated batch operations, FOS yields of 87, 72, and 44%, in the 3 initial cycles (60 h) were obtained and the enzyme activity remained constant during 6 cycles (96 h). A similar approach was taken by Castro et al. (2017), who tested 16 different carriers including synthetic, agro-industrial and mineral materials for immobilization of *Aureobasidium pullulans* cells. They suggested that the best carriers to enhance the production of FOS were those with high porosity and water absorption capacity, and low critical humidity point. Reticulated polyurethane foam was one with the highest immobilization yield (over 75% w/w of the total cells were immobilized) and achieved a high FOS yield compared to free cells.

Finally, applying electrospining methodology, Gabrielczyk et al. (2018) encapsulated fructosyltransferase by emulsion, suspension, and coaxial electrospinning. Additionally, they compared the electrospun fiber enzyme load performance with a commercial epoxyactivated resin support (covalent immobilization). Analyzing the hydrophilic properties, they found that bioactivity of electrospun support in aqueous medium increased in order of the matrix hydrophilicity. Moreover, enzyme loading and specific enzyme activity was higher in fibers than in the resins. From the three electrospinning methods, coaxial fibers showed the higher specific activity. Operational stability of fiber supports was examined in a plug-flow reactor being the core-shell immobilizates more efficient than one-dimensional fibers both in batch and continuous reaction.

Conclusions

Industrial chemical reactions involving enzymes as biocatalysts often occur under extreme conditions, in terms of temperature, pH and presence of salts, surfactants, and organic solvents, thus affecting enzyme stability. Enzyme immobilization had emerged as a suitable methodology that not only improves enzyme stability, but also guarantees reusability of the catalyst, simplifying its removal from the reaction medium. As evidenced from the discussion provided by this review, enzyme immobilization involves interdisciplinary knowledge including not only enzymology but also nanotechnology, molecular dynamics, cellular physiology and process design. Particularly, for industrial syntheses of GOS and FOS enzyme cost is one of the most critical issues. For this reason, enzyme immobilization deserves special consideration in their design process. This review was focused on different immobilization strategies and support materials to enhance the activity and re-usability of fructosyltransferases and β-galactosidases.

The examples provided, as well as the discussion of their main findings and methods' effectiveness lead to accurate benchmarks. In this line, the type of enzyme, its origin, its purity, together with the type of immobilization method selected and the support will affect the performance during the enzymatic synthesis. There is another factor that comes into play: process design. The same enzyme, immobilized under the same method with the same support, may not have the same yield when operating at batch or continuous process, under (or not) stirring or forced flow. For this reason, the best method will be the one that better adapts to the process design specifications of each case of study.

Despite this general marks, from the consulted bibliography it was shown that the latest advances in β-galactosidase and fructosyltransferase immobilization involve developing efficient material supports taking into account enzyme-support interactions, in this sense, resources from nanotechnology and electrospinning field are the most promising ones to achieve this goal. Nanostructured supports offer the main advantage of increasing surface area, thus the enzyme loading, while electrospinig offers the versatility of a simple method to obtain submicron-sized fibers, thus improving mass transfer limitations. Additionally, the implementation of combined immobilization methods, most of them including cross-linking seems to be the most appropriate to obtain an immobilized catalyst that can be adapted to the a variety of process conditions. The increasing availability of technology facilities has opened a large variety of possibilities to define smart strategies to optimize the activity and re-usability of these enzymes. This indicates that there is still a large gap with great place for innovative developments.

Authors' contributions

M.M.U., G.N.M., P.F.P. and O.F. did the literature search and wrote the first draft P.C.C. and A.G.-Z. proposed the structure and revised the manuscript.

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Disclosure statement

The authors declare that they have no competing interests.

Abbrevations

3-APTES	(3-aminopropyl)triethoxysilane
CLEA	cross-linked enzyme aggregate
DP	degree of polymerization
EDC	1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride
FBR	fluidized bed reactor
FE	free enzyme
FOS	fructo-oligosaccharides
GA	glutaraldehyde
GH-A superfamily	glucosyl hydrolases family (CAZy classification)
GOS	galacto-oligosaccharides
IE	immobilized enzyme
PBR	packed-bed reactor

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ORCID

Maria Micaela Ureta http://orcid.org/0000-0003-1390-6429
Gonçalo Nuno Martins http://orcid.org/0000-0002-1869-6208
Onofre Figueira http://orcid.org/0000-0001-5732-0994
Pedro Filipe Pires http://orcid.org/0000-0001-8583-7990
Paula Cristina Castilho http://orcid.org/0000-0002-8303-4286
Andrea Gomez-Zavaglia http://orcid.org/0000-0002-8705-0160

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