Enzyme Technology in Bioethanol Production from Lignocellulosic Biomass: Recent Trends with a Focus on Immobilized Enzymes

Beatriz Meneghetti Costa de Araújo,[#] Isabela Oliveira Costa,[#] Hellockston Gomes de Brito,[#] Nathália Saraiva Rios,* and Everaldo Silvino dos Santos *

Enzyme immobilization is a useful tool to produce biocatalysts with improved performance, such as high activity, high stability at operational conditions, and easy recovery and reuse. In this context, the production of second-generation ethanol using immobilized enzymes was reviewed. Emphasis was placed on the pre-treatment and/or hydrolysis steps to increase efficiency and reduce the cost of the process. In addition, the process design of bioethanol using immobilized enzymes was critically reviewed. In the enzymatic pre-treatment, laccases, manganese peroxidases, lignin peroxidases, and lytic polysaccharide monooxygenases are the main enzymes involved. When considering processes with heterogeneous biocatalysts, laccases are the most explored enzyme. In the hydrolysis step, cellulases and hemicellulases or a mixture of them are the main enzymes explored in the literature frequently using magnetic nanoparticles as support for enzyme immobilization. Although enzyme immobilization is a mature technology, the use of these biocatalysts is frequently limited to only one step of the bioethanol production process, such as pre-treatment or hydrolysis performed using Benchmark Technology. However, some recent papers have explored innovative design processes using a mixture of immobilized enzymes or co-immobilized enzymes to join some process steps, thereby decreasing the cost of enzymes and equipment.

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*Contact information: Departamento de Engenharia Química, Universidade Federal do Rio Grande do Norte, Natal-RN, Brazil; #: The authors equally contributed to this paper; *Corresponding authors: nathalia.rios@ufrn.br; everaldo.santos@ufrn.br*

INTRODUCTION

To reduce the environmental damage generated by the burning of fossil fuels, the production of fuels and chemicals precursors from renewable sources has been well explored in the literature (Kumar *et al.* 2009; Kang *et al*. 2014; Wyman *et al.* 2019). The lignocellulosic biomass, such as green coconut fiber and corncob, among other agro-industrial wastes, appears as a good option to reduce environmental impacts and produce second-generation ethanol (Wu *et al.* 2022).

Lignocellulosic biomass is mainly composed of cellulose, hemicellulose, and lignin. It is lignin that makes the structure recalcitrant. Cellulose, a linear and crystalline polysaccharide, is formed by glucose units linked by β-1,4 glycosidic bonds (Haghighi *et al.* 2013). Hemicellulose is an amorphous polysaccharide, formed by pentoses (xylose, arabinose), hexoses (glucose, rhamnose, mannose), and uronic acids (glucuronic acid, methyl-glucuronic acid). Because of its low molecular weight and amorphous structure, hemicellulose is more susceptible to acid and enzymatic hydrolysis. Lignin is a complex organic polymer that is a three-dimensional, cross-linked macromolecule made up of phenylpropanoid units, primarily p-coumaryl,

coniferyl, and sinapyl alcohols (Zeng *et al.* 2014). Lignin is responsible for providing structural support to the plant and protecting it from external stresses such as pathogens and environmental factors.

Therefore, to produce second-generation ethanol from lignocellulosic biomass, it is necessary to remove the lignin, the non-polysaccharide portion of lignocellulosic biomass, through pre-treatment strategies. This step also exposes the cellulose portion of the material, facilitating the action of enzymes in the next step, saccharification. In this case, enzymes can be used to hydrolyze the cellulose and hemicellulose polymeric structure into reducing sugars that can be metabolized by yeast to produce bioethanol in the fermentation step. Chemical pretreatment (acid, alkaline, ionic liquids, and organosolv) methods, for example, have been widely used to decrease cellulose crystallinity, remove lignin and/or hemicellulose, and reduce the degree of polymerization of carbohydrates, increasing their biodegradability (Akram *et al.* 2023). Therefore, enzymes can be used in the pre-treatment and saccharification steps, being an important tool to innovate in the production of second-generation ethanol (Plácido and Capareda 2015; Ríos-Fránquez *et al.* 2019a).

The use of enzymes for the pre-treatment and hydrolysis of lignocellulosic biomass has many advantages, such as a reduction in the use of toxic reagents due to the enzymatic reactions are conducted at mild conditions (mild temperature, pH, and solvent-free systems). In addition, the use of enzymes reduces the generation of inhibition compounds that can affect the yeast in the fermentation step (Souza and Kawaguti 2021). However, the use of soluble enzymes can increase the cost of the process due to the difficulties of enzyme reuse (Olivieri *et al.* 2021). In this context, enzyme immobilization is a useful tool to innovate in this field, since the immobilized enzyme is easily recovered and reused, decreasing the cost of the process. In addition, some strategies of enzyme immobilization produce biocatalysts that are highly stable, thereby expanding the reaction conditions enabled to the enzymes for catalysis (Rodrigues *et al.* 2021).

Therefore, this review paper explores the main enzymes used in the pre-treatment (namely laccases, manganese peroxidases, and lignin peroxidases) and hydrolysis (namely cellulases and hemicellulases) of lignocellulosic biomass, detailing, firstly, the properties and mechanisms of these soluble enzymes in the process of bioethanol production. Then, the production of bioethanol using immobilized enzymes applied in the pre-treatment or/and hydrolysis of different lignocellulosic biomasses is also explored. Finally, some process designs of bioethanol production using immobilized enzymes are detailed.

Enzymes Used In the Process of Bioethanol Production

Enzymatic pre-treatment of lignocellulosic biomass for bioethanol production

Currently, lignocellulosic ethanol biorefining technology based on the sugar platform is considered to be the most efficient way to produce fuel ethanol from biomass (Gou *et al.* 2020). The components of biomass are entangled together in complexes, which contributes to a recalcitrant nature (Liu *et al.* 2019). Due to their resistance to degradation, a pre-treatment step is needed for their destruction to separate the components. Therefore, the pre-treatment process plays a crucial role by assisting in the removal of lignin and, in some cases, hemicellulose. In addition, the pre-treatment reduces the degree of polymerization and crystallinity of cellulose, while also increasing the porosity of pre-treated materials (De La Torre *et al.* 2017). These properties of lignocellulosic biomass significantly differentiate the production of 2G ethanol from established processes for the production of 1G ethanol from sugar and grain.

Table 1. Main Physical, Chemical, and Physicochemical Pre-treatments Studied in the Literature

Pre-treatment	Main Strategy	Effect on the Lignocellulosic Biomass	References
Physical	Milling and extrusion	Reduction the particle size, increasing the surface area of biomass	(Akram *et al*. 2023; Kumar *et al*. 2009)
	Microwaves	Causing thermal stress and disruption of cell wall structure, increasing the porosity of the material	
	Ultrasonication	Breaking down the biomass cell walls using high-frequency sound waves, increasing the accessibility of enzymes	
Chemical	Acid (typically with sulfuric acid) at high temperature and pressure.	Acid hydrolysis of the hemicellulose portion of biomass, breaking it down into simple sugars, leaving the cellulose and lignin partially modified.	(Mosier et al. 2005)
	Alkaline (typically with sodium hydroxide) at high temperature and pressure	Solubilization of the lignin portion of the biomass	(Silva *et al.* 2022)
	Organosolv with a mixture of organic solvents and a catalyst at high temperature and pressure	Solubilization of hemicellulose and remotion of lignin	(Xu *et al.* 2023)
Physicochemical	Steam explosion with steam at high pressure and temperature	Disruption of the lignocellulosic structure recondenses lignin, which reduces cellulose crystallinity and removes hemicellulose	(Bhatia *et al*. 2020)
	Subcritical water	The acidic nature of the medium performs hydrolytic reactions, such as the degradation of hemicellulose, proteins, lignin, fats, lipids, and polyphenolic compounds	(Sarker *et al.* 2021)
	Liquid hot water (typically at high pressure)	Solubilization of hemicellulose and remotion of lignin	(Beig *et al*. 2021)

The pre-treatment strategies can have a direct impact on the final bioethanol yield and therefore need to be well-planned and effective. Unlike hemicellulose and cellulose, lignin is a non-sugar-based polymer, and therefore its hydrolysis does not provide products that contribute to the production of 2G ethanol (Nguyen *et al.* 2017). Another unfavorable aspect is that the lignin might deposit onto cellulose, thus making its surface inaccessible to the adsorption of cellulases, which negatively affects the enzymatic hydrolysis of cellulose (Li *et al*. 2014; Jönsson and Martín 2016; Su *et al*. 2018).

Several pre-treatment processes aim to disrupt the lignocellulosic matrix, through the cellulose depolymerization and solubilization of lignin and hemicellulose, leaving the structure more susceptible to enzymatic attack in the hydrolysis step (Costa *et al.* 2009). In bioethanol production, some pre-treatments have been extensively used, such as physical, chemical, and physical-chemical pre-treatment (Akram *et al.* 2023). Some of the main strategies are summarized in Table 1. In addition, some authors have studied a combination of different pre-treatments to disrupt the lignocellulosic biomass (Costa *et al.* 2019; Ribeiro *et al.* 2022).

Among the common strategies of pre-treatment approached in Table 1, biological pre-treatment of lignocellulosic biomass involves the use of microorganisms or enzymes, such as fungi or bacteria (single or in co-culture), microbial consortia, or soluble and immobilized enzymes (pure or mixtures) (Deivayanai *et al.* 2022). This strategy of pre-treatment is more attractive due to lower energy consumption and lower additives required (Beig *et al.* 2021). In the specific case of using soluble enzymes to carry out the pre-treatment, the efficiency of the process depends on the type of enzyme and the composition of lignocellulosic biomass used (Hosseini *et al.* 2019). Enzymes activate the biomass surface by breaking down the molecules and providing radicals, generating more reactive sites on the biomass fiber (Sun *et al.* 2022).

The main enzymes used in the pre-treatment of biomass are the manganese peroxidase, laccases, lignin peroxidases, which can be either pure or mixtures (Bian *et al.* 2020), and the lytic polysaccharide monooxygenases (LMPO) (Koskela *et al.* 2019). Ligninases are enzymes that partially remove lignin from biomass and modify its properties, facilitating the hydrolysis process (Dantzger *et al.* 2016). This type of enzyme can cleave phenolic and non-phenolic portions of the lignin structure (Sun *et al.* 2022). Lignin degradation is a complex process due to depending on the synergic action of intracellular and extracellular enzymes to break the polymer's bonds, producing large amounts of side products (Asina *et al.* 2017; Carvalho *et al.* 2011). The main ligninolytic enzymes are produced extracellularly by fungi, such as laccase (Lac), manganese peroxidase (MnP), lignin peroxidase (LiP), and versatile peroxidase (VP), the LiP with the highest oxidative power (Azevedo 2022).

The application of laccases in the field of biomass utilization and sustainable energy generation has gained attention in recent years because laccases also can be used to degrade and modify the lignin and improve the efficiency of following hydrolysis (Fillat *et al.* 2017). Laccases are polyphenol oxidases that catalyze oxidation reactions of inorganic substances and aromatic compounds by reducing the oxygen from water molecules. They generate phenoxy radicals by extracting an electron from phenolic substrates (Azevedo 2022). Masran *et al.* (2020) evaluated the effect of laccase pre-treatment (45 U/g) and saccharification (cellulase loading at 25 FPU/g) of oil palm empty fruit bunch, obtaining 8.73 g/L of reducing sugars and 23.9% of hydrolysis yield. Also, the authors studied the effect of simultaneous pre-treatment and saccharification using a laccase-cellulase cocktail and demonstrated an increase of 27% hydrolysis yield compared to the process in separate steps.

Classified as heme peroxidases like LiP, manganese peroxidases (MnPs) act on substrates *via* multistep electron transfers, generating intermediate radicals. MnPs catalyze Cα-Cβ cleavages, Cα-H oxidation, and alkyl-aryl C-C bonds cleavages of phenolics structures in lignin. Mn^{2+} is a reducing substrate for MnP to be oxidized to Mn3+, which in turn oxidizes the phenolic compounds. After this, Mn^{3+} is liberated from the enzyme and becomes stabilized through a chelating reaction with α-hydroxy acids (Asina *et al.* 2017). Despite that, the contribution of MnP can be expanded to non-phenolic substrates by using veratryl alcohol as a phenolic mediator (Nousiainen *et al.* 2014). This enzyme has been found in *P. chrysosporium, Panus tigrinus, Lenzites betulinus, Agaricus bisporus*, among others (Janusz *et al.* 2017). Min *et al.* (2022) tested the effect of MnP on the decomposition of beechwood and demonstrated that this ligninase shows a cellulose-decomposing activity like endoglucanases, exoglucanases, xylanases, and β-glucosidase. Also, MnP has a significantly delignification activity, improving the cellulase activity in hydrolysis.

Lignin peroxidases (LiP), a mediator-free peroxidase, degrade the non-phenolic lignin substrates, forming radical cations *via* one-electron oxidation (Asina *et al.* 2017). According to Janusz *et al*. (2017), this enzyme was found in *Phanerochaete chrysosporium, Trametes versicolor, Bjerkandera* sp., and *Phlebia tremellosa*. Colonia *et al*. (2019) evaluated a pre-treatment, after an alkaline treatment, using xylanases and LiP produced by *Aspergillus* sp. LPB-5 and the lignin content was 3.37% on solid fraction compared to raw material with 20.0% lignin content, causing 83.9% of lignin loss.

The lytic polysaccharide monooxygenases (LMPOs) perform a specific oxidation reaction on cellulose's C1 or C4 position, producing aldonic acids or gem-diols, respectively, containing an ionizable carboxyl group. In other words, the enzyme breaks the cellulose chain, and by decreasing the crystallinity, it could potentially increase cellulose fibrillation. In general, these accessory enzymes have a metal site with copper ion; this is used to break glycosidic bonds by oxidative mechanism, involving molecular oxygen, or using hydrogen peroxide and an electron donor (Hemsworth *et al.* 2015).

Breaks in the cellulose chain by LMPO mainly occur in the crystalline regions of cellulose and can result in oxidation at carbon C1 or C4 in the sugar ring, generating soluble oligosaccharides with an aldonic acid at their reducing end and a ketose in the non-reducing part. Thus, the action of LMPOs leads to an increase in glyco-hydrolytic enzyme activity by introducing new ends on which they can act (Silva 2017b). In addition to the copper metal cofactor and oxygen, the action of LMPOs also depends on the source of electrons to be activated, which can be provided by fluorescent pigments or enzymes. Cellobiose dehydrogenase (CDH) is an enzyme capable of donating electrons to LMPOs and is usually expressed by lignocellulose-degrading fungi and co-expressed by AA9 family LMPO-producing fungi (Johansen 2016). Koskela *et al*. (2019) used LMPOs from the ascomycetous fungus *N. crassa*, for 2 days, in cellulose fiber, after a pressurized hot water pre-treatment followed by extensive washing in hot water. The cellulose nanofibers were successfully produced from delignified softwood fibers by the method with a reduction of 2% in the cellulose crystallinity and the surface charges obtained from the ζ-potential measurements showed a 1.5-fold increase.

Enzymes Used in the Hydrolysis of Lignocellulosic Biomass

Enzymatic hydrolysis is based on breaking the polymeric structure of cellulose and hemicellulose through the action of cellulases and hemicellulases enzymes,

respectively; releasing monomeric units of fermentable sugars (such as glucose, and xylose), to improve fermentation yield (Nascimento 2016).

Cellulases make up about 20% of the global enzyme market (Balla *et al.* 2022). These enzymes constitute a complex that acts in synergy during the hydrolysis of cellulose (Castro and Pereira 2010). The main groups of this complex are the endoglucanases, exoglucanases, and β-glucosidases. The endoglucanases act randomly in the amorphous region of cellulose (Yennamalli *et al.* 2013). By contrast, the exoglucanases act at the reducing and non-reducing ends (Hamrouni *et al.* 2022). The β-glucosidases hydrolyze cellobiose into glucose (Florencio *et al.* 2017). Thus, when these enzymes are combined in the right proportions, the mixture is more effective, releasing more reducing sugars that can be converted to ethanol, thus potentially increasing the rate and extent of cellulose conversion (Ximenes *et al.* 2013).

Endoglucanases indiscriminately attack the inside regions of the amorphous structure of cellulose fiber, which causes the beginning of hydrolysis. This is because the center region is structurally less organized and has more erratic intermolecular interactions than the crystalline region (Yennamalli *et al.* 2013). As a result, there is greater exposure to glycosidic bonds, making them more easily cleaved by enzymes. This consequently causes the release of oligomers with various degrees of polymerization and therefore new side pathways for exoglucanase action (Castro and Pereira 2010). These new branches result in a reducing terminal when glucose has a free heterosidic hydroxyl group, and a non-reducing one when the hydroxyl group participates in the bond with the adjacent glucose (Silva 2017a). Glucanohydrolases and cellobiohydrolases make up exoglucanases. In the saccharification of cellulose, they stimulate the catalysis of most glycosidic bonds, producing cellobiose from the reducing and non-reducing ends of the molecule, which is then hydrolyzed by β-glucosidase (Sharma *et al.* 2022). β-glucosidases are non-processive enzymes, as they act on soluble substrates, thus catalyzing the hydrolysis of cellobiose into glucose (Borin *et al*. 2015; Silva 2017a).

Hemicellulases are a varied group of enzymes that hydrolyze hemicelluloses, which are made up of numerous polymeric branches of sugars, for example, D-xylan, D-mannan, D-galactan, and L-arabinans (pentoses and hexoses). Therefore, for the efficient degradation of these polymers to occur, the synergistic action of specific enzymes is necessary, such as xylanases, xylosidases, acetyl-xylanesterase, mannanases, glucuronidases, arabinase, arabinofuranosidases, and hemicellulolytic esterases (Souza and Kawaguti 2021). Among the main hemicellulolytic enzymes are the endoxylanases, which hydrolyze the glycosidic bonds of the xylan structure; the β-xylosidases that catalyze the release of xylose from xylobiose and the β-mannanases that hydrolyze hemicelluloses composed mainly of mannans (Souza and Kawaguti 2021).

Endoxylanases (endo β-1,4-xylanase/1,4-β-D-xylan xylanohydrolase, EC 3.2.1.8) hydrolyze the main component of hemicellulose, the xylan. They hydrolyze the β-1,4-type glycosidic bonds into the xylan chain, releasing xylooligosaccharides (XOS) with a low degree of polymerization (Brigham *et al.* 2018). β-xylosidases (1,4-β-D-xylan xylohydrolase, EC 3.2.1.37) play a central role in xylan hydrolysis together with endoxylanases. They are exo-type glycosidases that hydrolyze xylobiose and the short-chain xylooligomers from the non-reducing ends, releasing xylose monomers (Brigham *et al.* 2018). Xylanases are enzymes made up of endoxylanase and β-xylosidase. Together with cellulolytic enzymes, these are the main enzymes used in the production of bioethanol (Gonçalves *et al.* 2015; Zhou *et al*. 2012). β-mannanases (endo 1,4-β-mannanase/1,4-β-D-mannan mannanhydrolase, EC 3.2.1.78) are endohydrolases that cleave random or specific bonds in mannan, the second component of hemicellulose.

These enzymes release short chains of β-1,4-manno-oligomers, which can subsequently be hydrolyzed to mannose by β-mannosidases (β-Dmannosidase/1,4-β-D-mannoside mannohydrolase, EC 3.2.1.25) (Bonechi *et al.* 2017; Brigham *et al.* 2018).

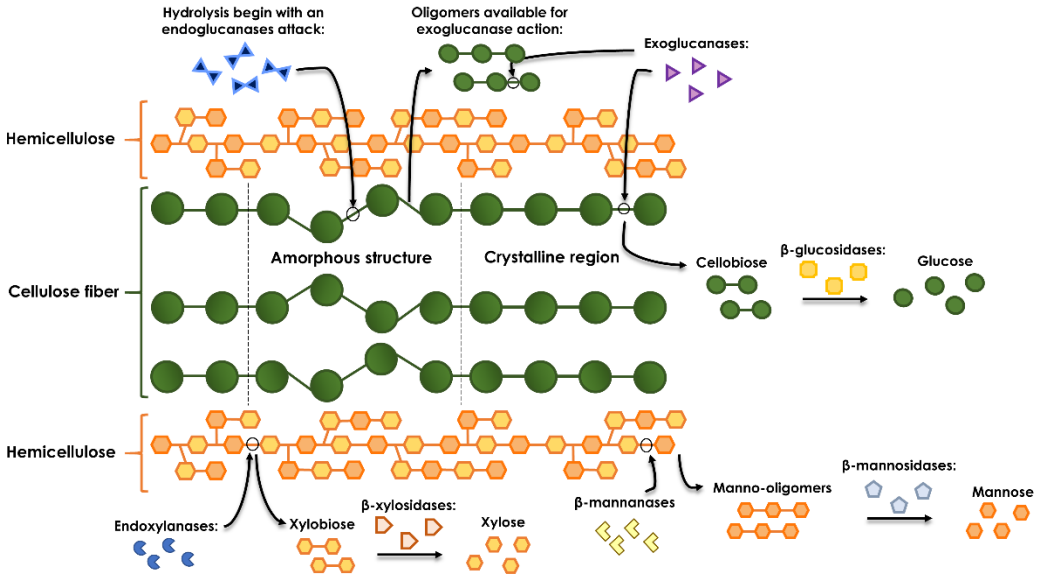

Fig. 1. Schematic illustration of the enzymatic hydrolysis of hemicellulolytic biomass

To perform the hydrolysis of cellulose and hemicellulose, commercial enzymatic cocktails, such as Cellic CTec (cellulase) and Cellic HTec (xylanase) families, have emerged. The Cellic CTec cocktail stands out for having high β-glucosidase activity in its composition, making it the protagonist in the production of reducing sugars in the process of bioethanol production (Lee *et al.* 2021; Triwahyuni *et al.* 2015). In addition, the Cellic HTec cocktail has excellent endoxylanase activity and selectivity for soluble hemicellulose, contributing to the hydrolysis of lignocellulosic materials into fermentable sugars. According to studies in literature, the addition of these commercial cocktails during the hydrolysis step results in greater amounts of bioethanol, producing 89.8%, 60.8%, and 82.7% mass yield of ethanol, respectively, making them widely effective in a variety of lignocellulosic materials (Costa *et al.* 2019; Oliveira *et al.* 2022; Singhania *et al*. 2022).

Strategies to Improve the Enzyme Properties: A Special Emphasis on Enzyme Immobilization Strategy

As mentioned before, some enzymes (MnP, laccases, LMPOs, cellulases, and hemicellulases, among others) are strategic in the bioethanol design process. However, enzymes are designed by nature to enable their physiological requirements. In industrial conditions, natural enzymes can have some limitations (Sharma *et al*. 2019) due to the low stability of enzymes at high temperatures, extreme pHs, and in the presence of organic solvents or metal ions (Choi *et al.* 2015; Giri *et al.* 2021). In addition, it is important to mention that activity, selectivity, and specificity of natural enzymes were developed for their physiological substrates, which, in most cases, are different from the industrial interest substrates (Souza *et al*. 2022). Based on the high industrial interest in enzymes as biocatalysts, it was developed some techniques to improve the enzymatic properties of natural enzymes, namely chemical modification (Giri *et al*. 2021), rational design (Teixeira and Milagre 2020), direct evolution (Romero and Arnold 2009), and immobilization (Mateo *et al.* 2007).

Among these strategies, enzyme immobilization is one of the most studied and efficient strategies to improve enzyme properties (Mateo *et al.* 2007). The enzyme

immobilization technique consists in confine the enzyme in/on a defined region of space, such as on the surface of solid supports, with retention of its catalytic activity, producing a heterogeneous biocatalyst that can be reused in repeated batches (Sato *et al*. 1971; Mohamad *et al*. 2015). To produce an immobilized biocatalyst, the researcher can use some strategies according to the type of interaction between the enzyme and support or matrix. The traditional strategies of immobilization are physical adsorption, covalent attachment, entrapment, and cross-linked enzyme aggregates (CLEAs) (Mohamad *et al*. 2015; Reis *et al*. 2019).

The physical adsorption immobilization strategy promotes mainly hydrophobic or ionic interactions between enzymes and support and these types of interactions classify the method as reversible (Arana-Peña *et al.* 2021). Covalent attachment between enzyme and support can produce biocatalysts highly stable, mainly in the case of multipoint covalent attachments, due to the amount and nature of enzyme-support bonding, thus generally having higher operational and storage stability than physically adsorbed enzymes (Rodrigues *et al.* 2021; Othman *et al.* 2023). In the entrapment strategy, the enzymes are trapped within a matrix during its formation. In this case, there are no covalent bonds between the enzyme and matrix, allowing the enzyme structure to remain intact (Imam *et al.* 2021). The most important parameter is the pore size of the matrix, which if well designed, can avoid diffusional problems and enzyme release (Imam *et al.* 2021). Finally, Cross-linked Enzyme Aggregates (CLEAs) are a carrier-free immobilization method that enables enzyme-enzyme covalent attachments. Furthermore, CLEAs´ strategy combines the purification and immobilization of enzymes into a single operation and through an easy preparation method (Sheldon 2011). The specific application of CLEAs technology in the production of ethanol and biodiesel was recently reviewed (Costa *et al.* 2023).

Enzyme immobilization was first designed to enable the easy recovery and reuse of enzymes (Sato *et al.* 1971). In addition, this technique can improve the enzyme properties, such as activity, stability, and selectivity, among others (Mateo *et al.* 2007; Garcia-Galan *et al.* 2011; Rodrigues *et al.* 2013). This improvement in enzyme properties can be due to conformational changes in enzymes or due to environmental effects (Rodrigues *et al.* 2013). The hyperactivation of lipases when immobilized on hydrophobic support through interfacial activation (Rios *et al.* 2018; Rios *et al*. 2019a; Silva *et al*. 2023) and the improvement of enzyme stability due to enzyme rigidification through an immobilization by multipoint covalent attachment (Arana-Peña *et al*. 2019; Rios *et al*. 2019c) are examples of conformational changes on the enzyme structure due to immobilization. However, it is important to mention that, in general, enzyme rigidification also can lead to a decrease in enzyme activity (Silva *et al*. 2023). Therefore, the researcher can use a compromise solution in this case. On the other hand, there are positive impacts on enzyme properties that it is not related to structural change, such as the protective effect when the enzyme is immobilized inside the porous support or the prevention of enzyme aggregation with the immobilization, maintaining the enzymatic activity at precipitation conditions (Rodrigues *et al*. 2013).

Regardless of the reason, immobilization can alter the enzymatic properties and can facilitate the reuse of the enzyme. These are remarkable advantages since some enzymes still are expensive to be used in each batch allied to the fact that soluble enzymes can be unstable at operational conditions. Therefore, this technique has been used in many works to obtain industrially efficient biocatalysts (Sheldon and Van Pelt 2013). Thus, in the next sections, it will be detailing the use of some immobilized biocatalysts in the enzymatic pre-treatment and the enzymatic hydrolysis of lignocellulosic biomasses. Also, some recent approaches to bioethanol process design using immobilized enzymes will be explored.

Immobilized Enzymes Involved in the Production Process of Bioethanol

Immobilized enzymes for enzymatic pre-treatment of lignocellulosic biomass

Most studies relate that an efficient step of pre-treatment is the basis for developing an improved and efficient bioethanol production process (Bhutto *et al.* 2017; Rezania *et al.* 2020; Jayakumar *et al.* 2023). The most recent strategies of pre-treatment have advanced in the field of genetic modification, such as the engineering of plant cell walls (Huang *et al*. 2019), in the development of green solvents, such as deep eutectic solvents (DES) (Santos *et al.* 2022a; Li *et al.* 2023), and in the use of immobilized enzymes. This last will be the technique critically analyzed in this topic.

As mentioned before, the immobilization of enzymes emerges as a strategy to overcome some drawbacks of using soluble enzymes as biocatalysts in bioprocess (Xia *et al.* 2016). Several authors have applied nanotechnology in the enzymatic field (Chen *et al.* 2020; Gou *et al.* 2020; Muthuvelu *et al.* 2020; Shanmugam *et al.* 2020). Materials with a magnetic core have attracted a lot of attention as a support for enzyme immobilization due to their high surface area, resistance to physical and chemical conditions, low toxicity, and their ease of synthesis (Teng *et al.* 2012; Rios *et al.* 2019a; Zanuso *et al*. 2022; Costa *et al.* 2023). To the best of our knowledge, studies of ligninolytic enzyme immobilization and their application in the delignification of lignocellulosic biomass are very limited in the literature. However, some studies were found in which laccases in particular were immobilized for purposes of delignification. Some of these will be briefly discussed below.

Chen's research group proposed a study of the catalytic conversion of some chemical bonds into lignin structure (Chen *et al*. 2020). Three lignin model compounds (2-phenoxy-1-phenylethanol, benzyl phenyl ether, and phethethoxybenzene) were selected to compare the catalytic effects using soluble and immobilized laccase. Analyzing the results, for all components, laccase immobilized on amino-functionalized magnetic nanoparticles improved the degradation efficiency. As an example, for some degradation products of 2-phenoxy-1-phenylethanol a 6-fold increase (2-styrylphenol) and a 4-fold increase (phenol and 2-phenyloxirane) were observed when immobilized laccase was used.

Gou *et al*. (2020) investigated the laccase from *Myrothecium verrucaria* 3H6 immobilization on amino-modified magnetic nanoparticles (Fe_3O_4–NH_2) by metal affinity adsorption to pre-treat corn stover (Gou *et al.* 2020). The corn stover was treated with water, inactivated soluble laccase, soluble and immobilized laccase, and, after this, was subjected to cellulase hydrolysis. The soluble laccase showed a good candidate for delignification, promoting 44.8% of lignin degradation. The degradation rate of lignin was higher than using the immobilized laccase (40.8%). The term "delignification" represents the lignin removal and the promotion of a better carbohydrate's accessibility for the subsequent saccharification step (Malhotra and Suman 2021).

However, when the cellulose conversion rate was analyzed for soluble and inactivated soluble laccase, both showed an inhibitory effect, which resulted in a decreased rate when they were used in saccharification step. On the other hand, the cellulose conversion rate of corn stover using the immobilized laccase was improved by 24.0% compared to the soluble laccase treatment. This indicates that the inhibition was removed *via* recycling the immobilized laccase, resulting in increased conversion.

In a similar study, Shanmugam *et al.* (2020) also used a fungal laccase (from *Trichoderma asperellum*) immobilized on magnetic amine-functionalized nanoparticles. The immobilized enzyme was applied in sweet sorghum stover pre-treatment. Under optimized conditions, the percentage of biomass delignification was 84.3%, which was higher than the soluble laccase (38.4%). This behavior was justified

due to a higher enzyme concentration that can be loaded on the nanoparticle support.

Laccase from *Trametes versicolor* was immobilized onto magnetic nanoparticles coated with chitosan by covalent bonding as a strategy treatment of fibers from *Agave atrovirens* leaves (Sánchez-Ramírez *et al.* 2018). It has been related in the literature that *Agave atrovirens* leaves generated as waste during the production of alcoholic beverage (Mezcal) works as a cellulose source to produce second-generation bioethanol (Sánchez Ramírez *et al.* 2014). The main purpose of coating the nanoparticles with chitosan was to give more stabilization and functionalization for the support. However, this modification influence on interactions between the enzyme and insoluble substrate. In this case, cellulose hydrolysis yield with immobilized biocatalyst decreased by 27.7% in comparison with the pretreatment performed by the soluble enzyme.

The same laccase from *Trametes versicolor* was immobilized on various supports and promoted a considerable reduction in the lignin content of several lignocellulosic wastes (Asgher *et al*. 2018). When entrapped to alginate-chitosan, the immobilized enzyme had the highest delignification (78.3%) of sugarcane bagasse after 15 hours, followed by wheat bran and corn stover (approximately 73% and 68%, respectively). In this immobilization, the chitosan acts reducing the porosity of the alginate gel, decreasing the enzyme leaching, and improving the immobilization efficiency (Won *et al.* 2005).

In theory, materials of various origins could be used as laccase immobilization supports. Among them, graphene oxide (GO) is another promising solid support, providing high chemical stability and specific surface area (Ahmad *et al.* 2018). In the study related to Zhou (2021), the effect of GO-immobilized laccase on lignin degradation was the main subject. Comparing the same reaction conditions, the study revealed that the laccase immobilized on GO showed better efficiency (93.7 % of lignin degradation) than soluble enzyme (56.2% of lignin degradation). The biocatalyst afforded better results than those reported in the literature using other inert supports, in which the degradation rate of lignin model compounds was below 70% in the same context (Chen *et al*. 2020).

Another research field is to improve immobilization by adding agents that contribute to the biomass degradation performed by the main enzyme. Because lignin is a substrate that contains non-phenolic units, the degradation effect performed only by laccase is limited, and, therefore, studies have been carried out to immobilize with a laccase-mediator system proper for lignin degradation. One example of this system was designed by Lin *et al*. (2023), where laccase was immobilized and vanillin was added to the microreactor to enhance lignin degradation and modification. It has been related in the literature that vanillin is a natural mediator that enhances the removal efficiency promoted by laccase in other processes, such as the removal of pesticides (Kupski *et al*. 2019). The immobilized laccase reacted preferentially with vanillin, oxidizing it to phenoxyl radicals to react with the lignin (Jeon *et al.* 2008). The solid–liquid contact between lignin and soluble mediator is more effective than solid–solid contact between lignin and immobilized laccase, leading to a better lignin degradation effect.

For the untreated wheat straw, the cellulose conversion degree and the glucose productivity were 74.6% and 3.50 g L^{-1}, respectively. Wheat straw after being treated with soluble laccase, the conversion degree and the glucose productivity were increased to 77.3% and 3.68 g L^{-1}, respectively. Using the same reaction system with the immobilized laccase, the conversion and the glucose productivity reached 79.6% and 3.82 g L^{-1}, respectively. The highest cellulose conversion (88.1%) and glucose productivity (4.37 g L^{-1}) were obtained in the presence of vanillin (4.37 g L^{-1}), showing that the vanillin can effectively enhance the degradation of non-phenolic units of the

lignin performed by laccase (Lin *et al*. 2023). This causes structural damage and enhances the cellulose accessibility of wheat straw, improving cellulose conversion and glucose productivity. Although laccase-mediator systems are effective for delignifying lignocellulosic biomass, their usage on an industrial scale is constrained by the high cost and potential toxicity of synthetic mediators (Sánchez-Ramírez *et al.* 2018).

Multiple research projects have shown that adding metal ions, such as copper, zinc, calcium, and cobalt can increase the laccase activity (Guo *et al.* 2011; Naresh Kumar *et al*. 2018). Copper, which occurs in lower concentrations than the other metal ions, greatly boosted the laccase activity (Guo *et al.* 2011). In this sense, laccase was immobilized onto amino-functionalized copper ferrite magnetic nanoparticles (CuMNPs) and amino-functionalized ferrite magnetic nanoparticles (MNPs) to compare the lignin degradation efficiency (Muthuvelu *et al.* 2020). CuMNPs and MNPs presented high thermal and pH stability, improved kinetic parameters, and superior storage stability compared to the soluble enzymes. However, the remarkable property was concerning the lignin removal capacity. Laccase immobilized on CuMNPs exhibited the highest enzymatic activity of all biocatalysts, promoting the maximum lignin removal (43.28 ± 1.46%) when applied for *Ipomoea carnea* biomass delignification, compared to MNPs (40.10 ± 1.35%) and soluble laccase (38.16 ± 0.78%).

After the pre-treatment step, it is necessary to separate the immobilized enzyme used (solid biocatalyst) from the pretreated biomass. In this way, the separation is performed depending on the strategy of immobilization. When magnetic nanoparticles are applied in the immobilization their separation is facilitated, since it is performed under a magnetic field. For other strategies of immobilization on solid support, such as those mentioned: entrapment into alginate-chitosan (Asgher *et al.* 2018) or immobilization on graphene oxide (GO) (Zhou 2021), separation is commonly performed by filtration or centrifugation. However, for the specific case of heterogeneous reactions, such as the pre-treatment of lignocellulosic materials, the ideal and recommended method is to use approximation to a magnetic field. The immobilized enzyme on magnetic nanoparticles can be separated from the reaction medium by means of a magnet, and thus the liquor fluid resulting from the pre-treatment can be separated from the solid biomass by classic filtration. In this way, there will be separation of all components of the reaction. Regardless of the technique used for separation, it is common after each cycle to wash the biocatalyst with buffer, and then resuspended in fresh solution containing the material to be pre-treated.

Immobilized enzymes used in the hydrolysis of lignocellulosic biomass

After the pre-treatment, hydrolytic enzymes are used for the biodegradation of lignocellulosic biomass into reducing sugars during the saccharification process. Thus, saccharification is a crucial step, since the final amount of ethanol produced depends on the concentration of sugars acquired during the hydrolysis (Cunha *et al.* 2020). Immobilization is also a promising strategy for developing efficient biocatalysts, thereby improving the lignocellulosic biomass conversion. One of the barriers to producing ethanol from lignocellulosic biomass is to financially support the high enzyme dosage requirement. As mentioned before, immobilization promotes the easy reusability of enzymes, being a powerful way to overcome this obstacle. Nano-carriers have gained space as a promising conventional matrix for enzyme immobilization (Rios *et al.* 2019b; Liu and Dong 2020; Costa *et al.* 2023) and for that reason, it has been the most utilized in this subject of matter.

In this context, immobilized enzymes on nanoparticles have been applied to hydrolyze the pre-treated lignocellulosic biomass (Sánchez-Ramírez *et al.* 2017;

Ariaeenejad *et al*. 2021). This is due to the small size of nanoparticles (NPs), which allows them to penetrate the biomass's cell wall with great ease and efficiency, enabling the conversion of cellulose and/or hemicellulose into fermentable sugars by the immobilized enzymes on NPs (Dey *et al*. 2022). In addition, these nanomaterials have high surface area to volume, enabling higher enzyme loading, high stability, low toxicity, biocompatibility, and reduced mass transfer effects (Husain *et al*. 2011; Rai *et al*. 2019). Some authors have used magnetic nanoparticles as support to immobilize enzymes due to the easy recovery and reuse of biocatalyst after reaction and the possibility of use in a large-scale continuous process (Xu *et al*. 2014; Cipolatti *et al*. 2016; Sánchez-Ramírez *et al*. 2017; Miranda *et al*. 2020).

As an example, *Trichoderma reesei* cellulase was covalently immobilized on chitosan-coated magnetic nanoparticles using glutaraldehyde as a coupling agent. The immobilized enzyme showed better thermal and storage stability than the soluble enzyme. Moreover, the immobilized cellulase was able to hydrolyze lignocellulosic biomass from *Agave atrovirens* leaves with a yield close to the free enzyme, and it was reused for four cycles, maintaining 50 % of its activity (Sánchez-Ramírez *et al*. 2017). In another study, Ariaeenejad *et al*. (2021) immobilized a bifunctional mannanase/ xylanase on magnetic Fe_3O_4 nanoparticles, and the results demonstrated that the immobilized samples improved the thermal stability, kinetic parameters, and storage stability of the enzyme. In addition, this immobilized enzyme generated 38.7% more reducing sugars than free enzyme, after 168 h hydrolysis of the coffee waste, and it can be reused for up to 8 cycles, maintaining more than 50% of its original activity (Choi *et al*. 2012). Coffee residue waste (CRW) is a non-edible agricultural by-product with a low price (Choi *et al*. 2012) and can be a good source for bioethanol production, having a large number of polysaccharides.

More recently, the same group published a new study to identify a bifunctional enzyme with better β-glucosidase activity (Ariaeenejad *et al*. 2023). This specific enzyme is responsible for hydrolyzing the oligomeric molecules, generating glucose (Allardyce *et al*. 2010). The novel bifunctional enzyme was immobilized on the same nanocellulose carrier obtained from sugar beet pulp (SBP). As in the previous work, better results were achieved with coffee residue waste (Ariaeenejad *et al*. 2021). The amount of reducing sugar produced from enzymatic saccharification enhanced from 15.9 g/L (Ariaeenejad *et al*. 2021) to 19.7 g/L (Ariaeenejad *et al*. 2023) and reached a bioethanol concentration of 51.5 g/L after 72 h fermentation, while 29.3 g/L for the soluble enzyme. The bifunctional biocatalyst reached 53.2% of retained xylanase and 39.3% of its β-glucosidase activity after 8 cycles.

Moreover, Paz-Cedeno *et al*. (2021) immobilized cellulase and xylanase enzymes on magnetic graphene oxide, and the biocatalysts maintained 88 and 66 % of efficiency on the hydrolysis after 10 cycles, respectively. Chitosan has become a frequent agent in nanoparticle preparations, since it is a natural polymer that can form a polymeric shell on the nanoparticle. In addition, chitosan has amino groups that allow the enzyme immobilization by covalent bonding, if a proper cross-linking agent and immobilization conditions are provided. This can produce a more stable biocatalyst compared with immobilization *via* adsorption (Zanuso *et al*. 2022). Zanuso *et al*. (2022) produced 21.8 g/L of reducing sugars from corn cob biomass using chitosan-coated magnetic nanoparticles activated with glutaraldehyde. This result represents a capacity of 8.2-fold higher when compared to a hydrolysis cycle with a non-reusable enzyme, corresponding to a conversion yield of 64.4%.

Halloysite nanotubes also were used as support in the production of a nanobiocatalytic system (Sillu and Agnihotri 2020). Iron oxide nanoparticles were synthesized over the halloysite nanotubes concurrent with their deposition, rendering a

magnet nanobiocatalyst. Then, the nanotubes were functionalized with amino silane and treated with glutaraldehyde to immobilize the cellulase. The biocatalyst effectively catalyzed the cellulose hydrolysis from sugarcane bagasse, obtaining 50.2% of saccharification yield after 48 h of reaction. The practical affordability of this nanobiocatalyst was evaluated through the continuous hydrolysis of carboxymethyl cellulose (CMC), as a model substrate. After the seventh cycle, 68.2% of the initial activity could still be retained.

The traditional synthesis of magnetic nanoparticles is a time-consuming process that uses a high amount of chemicals. To overcome these drawbacks, Yasmin *et al.* (2022) synthesized Magnetized Multiwall Carbon Nanotubes (MMCN) using a water-based system and this support was used for cellulase immobilization. The biocatalysts synthesized with water-based MMCN produced similar results if compared with the cellulases immobilized on magnetic nanoparticles synthesized using chemicals or co-precipitation methods.

However, it is important to mention that the immobilization process on solid supports can also generate diffusional effects, decreasing the efficiency of biocatalysts. This behavior is frequent in biomass hydrolysis because cellulose is a large-polymeric substrate, and some immobilization systems cause drastic mass transfer limitations. In this context, immobilization by encapsulation or entrapment may not be a suitable strategy due to the large substrate can have difficulty passing through the membrane pores to access the enzyme (Zanuso *et al.* 2021) (Fig. 2). Some authors suggested materials with an open pore structure with a high surface area to immobilize enzymes, thereby enabling more sites for enzyme immobilization and facilitating substrate diffusion (Mo *et al.* 2020; Zanuso *et al.* 2021).

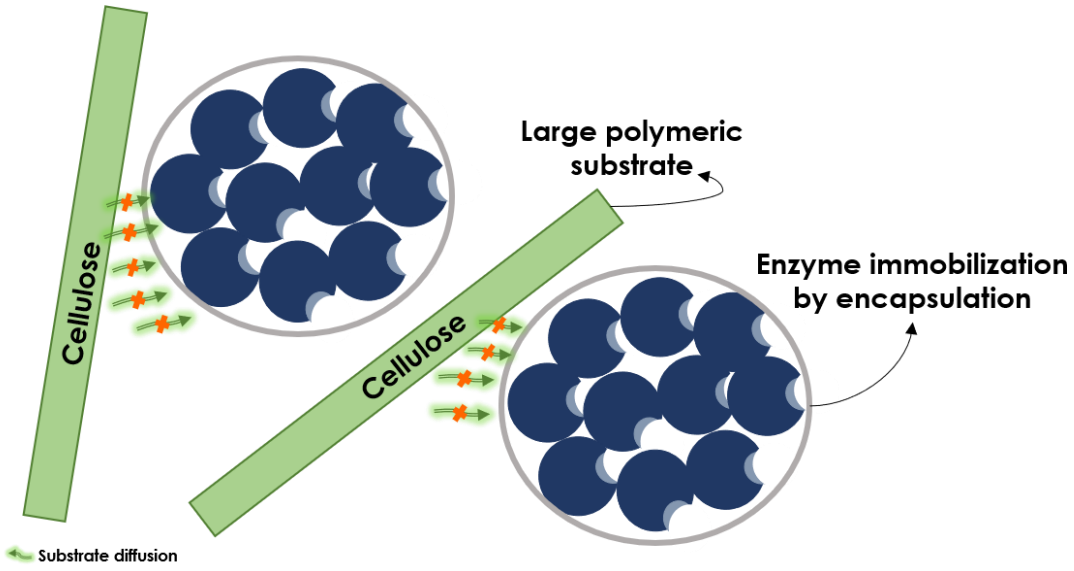

Fig. 2. Diffusional effects on enzymes immobilized by substrate size limitation

To minimize the mass transfer effects, cellulase was immobilized in sodium alginate with polyethylene glycol (Guo *et al.* 2021). The main purpose of using polyethylene glycol (PEG) was to increase the porosity of the carrier to reduce the mass transfer resistance between the cellulase and the substrate. However, it is important to mention that PEG can increase the viscosity of the media. It is noted that solid-solid interactions are one of the challenges of using immobilized cellulases to catalyze the hydrolysis of cellulosic biomass, mainly when the cellulase was immobilized on porous support. In these systems, a good amount of enzyme molecules is immobilized inside the carrier, where their contact with the substrate is limited; thus it can be extremely

important to increase porosity or immobilize on non-porous supports (Bourkaib *et al.* 2021; Santos *et al.* 2022b). Compared with the yield of reducing sugars obtained after the hydrolysis of microcrystalline cellulose, the yield was increased by 133% when utilizing the support with PEG compared to immobilizing cellulase with the one without the treatment. The biocatalysts could be reused for as many as five cycles, but the overall yield of reducing sugars was almost three times higher for immobilized cellulase with PEG.

Sugarcane bagasse was also hydrolyzed using a mixture of immobilized enzymes (Morais Junior *et al*. 2021), generating a glucose-rich hydrolysate with 14.11 ± 0.47 g/L of reducing sugars (94.54% of glucose). The authors immobilized an endocellulase on nanoparticles coated with dextran and immobilized the second enzyme, β-glucosidase, on glyoxyl agarose. This mixture of immobilized was efficiently produced to minimize the mass transfer effects, since endocellulases, which catalyze insoluble substrate, were immobilized on non-porous support, and β-glucosidases, which catalyze soluble substrate (cellobiose), were immobilized on the porous support. Therefore, β-glucosidase was protected from the medium without inducing high mass transfer effects due to the nature of the substrate. However, it is important to mention that the result using immobilized enzymes was approximately 5% lower than the amount of reducing sugars achieved by soluble enzymes. In both tests, cellobiose concentration was less than 1.0 g/L after 48 h, indicating that the load of β-glucosidase used was adequate, stipulating that optimization of the concentrations of endocellulase to be optimized if the desire is to obtain a higher production with this biocatalyst. The possibility of recovery and reuse the same portion of each biocatalyst was studied and immobilized endo-cellulase maintained 61.40 ± 1.17% of its enzymatic activity after seven reuse cycles, and immobilized β-glucosidase maintained up to 58.20 ± 1.55% after nine reuse cycles.

In the bioethanol production process, the heterogeneity of media harms the mass transfer in the reactor, which directly reduces the enzymatic hydrolysis and fermentation efficiency. Therefore, the design of a high-performance reactor is very crucial for an efficient process. In this context, microreactors have a large specific surface area and excellent mass transfer efficiency and these characteristics have made them popular for biocatalysis (Lloret *et al.* 2013). Immobilized cellulase was loaded in a microreactor for continuous enzymatic hydrolysis of carboxymethyl cellulose to obtain glucose (Lin *et al.* 2022). Dopamine (DA) and polyethyleneimine (PEI) were applied to enhance activity retention. The glucose production using the immobilized cellulase-loaded microreactor reached 1.70 g/L after 16 h, producing a glucose concentration 7.7% higher than that of the soluble cellulase. In this case, the excess product concentration inhibited the cellulase activity (soluble form), and the glucose productivity was reduced. The addition of PEI improved the glucose yield by 33.2% compared with no addition.

Another important key factor is the inhibitory effects on enzymes due to multiple compounds formed from the degradation of lignocellulosic biomass during pre-treatment, such as phenolic compounds (due to lignin degradation), organic acids, oligosaccharides, and furans, among others (Zanuso *et al*. 2021). It has been related that immobilized enzymes show better tolerance of inhibitors than free enzymes due to two main reasons: The enzyme immobilization can promote a new nano-environment to the enzyme, promoting a partitioning effect of the inhibitor from the enzyme environment and/ or the enzyme immobilization process may distort or block an inhibition site, reducing the inhibition effects (Fig. 3) (Mateo *et al.* 2007). Therefore, in the presence of inhibitors, the immobilized enzymes can have better efficiency in the biomass hydrolysis than free enzymes. Qi *et al.* (2018) showed this effect when they

immobilized cellulases on a magnetic core-shell metal-organic material and tested the hydrolysis efficiency of the immobilized and free enzyme in the presence of formic acid and vanillin, two typical inhibitors found in lignocellulosic biomass. The authors showed an increase in hydrolysis yield of 18.7 and 19.6% using immobilized preparations (concerning free enzyme) in the presence of formic acid and vanillin, respectively (Qi *et al.* 2018). The hydrolysis yield steadily declined with increasing recycle numbers but maintained over 70% of the initial hydrolysis after five reuses, indicating a great reusability.

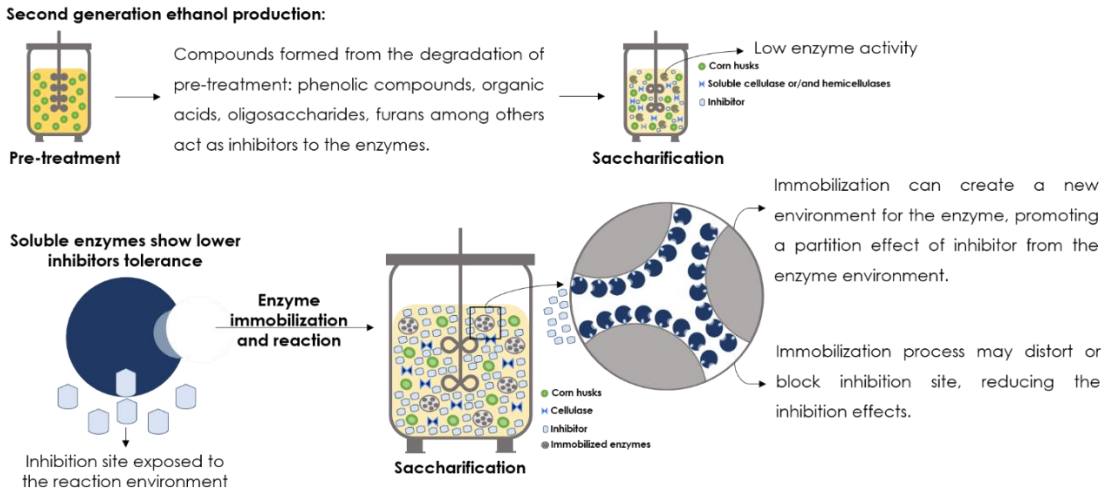

Fig. 3. Inhibitory effects on enzyme due to multiple compounds formed from the pretreatment of lignocellulosic biomass

To reduce inhibition, endo-glucanase and β-glucosidase were co-immobilized on trimethoxysilane-coated superparamagnetic nanoparticles (Gebreyohannes *et al.* 2018). The immobilization in membranes can increase the enzyme stability without affecting the enzyme's catalytic activity, creating a microenvironment with proper substrate diffusion (Giorno and Drioli 2000). In this case, the inhibition effect on the enzymes was avoided due to the co-immobilization and the simultaneous separation of the product, allowing 50% higher efficiency than if the same process was performed in a batch reactor. In addition, the co-immobilization promoted glucose productivity four times higher compared to free enzymes (Gebreyohannes *et al*. 2018). Another study co-immobilized a tri-enzyme mixture of xylanase, cellulase, and amylase-cum-glucanotransferase on magnetic nanoparticles by a covalent attachment with glutaraldehyde for efficient saccharification of corn cob and rice husk (Kumari *et al.* 2018). Amylolytic glucanotransferase degrades starch (Amin *et al.* 2021), providing more comprehensive hydrolysis of biomass waste.

Although many papers explored the enzyme saccharification of lignocellulosic biomass using soluble or/and immobilized enzymes, there are still aspects that need to be overcome. According to studies in the literature (Gebreyohannes *et al*. 2018; Lim and Ghazali 2020), there is a desired minimal concentration of sugars for the fermentation to produce ethanol: between 150 and 250 g/L glucose. However, the glucose concentration obtained in many studies is quite low (0.2 to 20 g/L) (Malmali *et al*. 2015), showing that there are still spaces to improve the saccharification step to effective application in processes on a larger scale. Below is Table 2, which summarizes the application of enzymatic cocktails in different types of biomass.

Table 2. Immobilization of Commercial Enzyme Cocktails and Application on the Hydrolysis of Lignocellulosic Biomass

Support	Biomass	Commercial Cocktail	Main Feature	Reference
Non-porous silica magnetic particles	Wheat straw	Cellic CTec 2	The hydrolysis yield in one cycle was 82.0% and 66.0% with two cycles	Alftrén and Hobley (2014)*
Graphene oxide magnetic particles	Sugarcane bagasse	Cellic CTec 2	The hydrolysis of sugarcane bagasse of 72.0% after the first cycle and which was reduced to approximately 27.0% of conversion after the tenth cycle	Paz-Cedeno *et al*. (2021)*
Silicon dioxide-coated magnetic nanoparticles of Fe_3O_4	Poplar wood	Cellic CTec 2	Regarding hydrolysis, the maximum saccharification rate of 38.4% was reached in 72h. After five cycles the relative saccharification rate was reduced to 16.7%	Huang *et al*. (2022)*
Fe_3O_4 magnetic nanoparticles coated with chitosan	Corn cob	Cellic CTec 2	After 13 cycles, the immobilized cellulase maintained 44.8% of its activity compared to the initial activity	Zanuso *et al*. (2022)*
Supermagnetic iron oxide nanoparticles coated with dimercaptosuccinic acid	Cellulose and Red Wine Grape Pomace	CelluClast 1.5L and Cellic CTec 2	Concerning hydrolysis, when using free enzymes and cellulose as substrate, it was possible to reach a conversion of 81.0%, after 120h, when using the Cellic CTec 2 cocktail, while the mix (50%-50%) of CelluClast 1.5L and Cellic CTec 2 provided a 77.0% conversion which was higher than that shown by CelluClast 1.5L (55.0%)	Murilhas (2021)*
Chitosan gel	Avicel and sugarcane bagasse	Cellic CTec 3	Regarding hydrolysis, the maximum conversion of cellulose to glucose, approximately 37.0%, was achieved when using an enzymatic load of 134 mg of protein/g of support	Pereira *et al*. (2021)*
Iron oxide (II and III) magnetic nanoparticles	Wheat straw	Pectinex 3XL	Regarding ethanol production, the maximum production (0.5 g/L) was observed when using the nano biocatalyst, which was 40% of that presented by the free enzyme. It is noteworthy that at the end of 60 hours, both showed the same result, approximately 0.23 g/L of ethanol	Perwez *et al*. (2017)

* Ethanol production was not evaluated.

With this, it can be seen in Table 2 that Cellic Ctec 2 (Novozymes) is one of the main commercial enzyme cocktails immobilized on different types of supports, with emphasis on: Non-porous silica magnetic particles, Graphene oxide magnetic particles, Silicon dioxide-coated functionalized magnetic nanoparticles of Fe_3O_4 (Fe_3O_4@SiO_2), and Fe_3O_4 magnetic nanoparticles coated with chitosan.

In the case of sugarcane bagasse, iron oxide magnetic particles (< 150.0 μm) were functionalized with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and N-hydroxysuccinimide (NHS), and the immobilization of enzymes from the Cellic CTec 2 cocktail (3-120 μL) occurred during 12 h. Paz-Cedeno *et al*. (2021) recorded that the immobilization yield for total cellulases was 71.1% and reached values greater than 90.0% for β-glucosidase and β-xylosidase. Regarding the hydrolysis and recycling, 72.0% of conversion was obtained after the first cycle. This was reduced to approximately 27.0% after the tenth cycle. In addition, the immobilization of xylanases from Cellic CTec 2 showed better results than those of cellulases. In this case, 96.0% of conversion into xylose was achieved in the first cycle and 61.0% in the last (tenth) cycle.

To perform the poplar wood biomass hydrolysis, the Fe_3O_4@SiO_2 nanoparticles were used to immobilize the Cellic CTec 2 cocktail. Poplar was pretreated with dilute phosphoric acid and using the steam explosion. Huang *et al*. (2022) reported that the biocatalyst showed optimal thermal stability (relative activity for CMCase greater than 95%) at 60 °C, which was 10 °C higher than the free enzyme. Regarding hydrolysis, the maximum saccharification rate of 38.4% was reached in 72 h. After five cycles, the relative saccharification rate was reduced to 16.7%.

To perform the corn cob biomass hydrolysis, the biocatalyst was prepared using Fe_3O_4 magnetic nanoparticles coated with chitosan and activated with glutaraldehyde (2.5% v/v) as support. This material was used to immobilize Cellic CTec 2 enzymes cocktail. The hydrolysis of corn cob (5.0 w/v) hydrothermally pre-treated (equivalent to a severity of $\log R_0$ = ~ 3.95) was evaluated using the enzymes of the Cellic CTec 2 cocktail in free and immobilized forms and occurred for 120 hours (50 °C and 180 rpm). According to Zanuso *et al*. (2022), the immobilization efficiency was 70.0%. Concerning the hydrolysis, the free enzyme showed higher conversion for all evaluated times. When using free enzyme, the maximum glucose concentration was 25.27 g/L (71.0% conversion), while the maximum glucose concentration when using the immobilized enzyme was 21.8 g/L (64.4% conversion). However, the immobilized cellulase maintained 44.8% of its initial activity after 13 cycles.

As shown in Table 2, Cellic CTec 3 (Novozymes) is also used as a cocktail in enzyme immobilization. This complex is a more recent version of Cellic CTec 2, which comes with a promise to generate better yields than its predecessor. Pereira *et al*. (2021) use this cocktail in the preparation of the biocatalyst using chitosan powder activated with glutaraldehyde (1.0% v/v) as support. The immobilization of enzymes from the Cellic CTec 3 cocktail was performed using citrate buffer (0.1 M, pH 7.0) for 2 hours. In this way, the maximum immobilization yield (80.0%) was reached when using 40.2 g of protein/g of support. Sugarcane bagasse was pretreated with ethanol/water (50.0% w/v). A cellulose slurry was prepared from microcrystalline cellulose (MCC) and water (solid-liquid ratio 1:10, 1200 rpm, 1h). The hydrolysis was performed by adding the biocatalyst (1.0% m/v) to pre-treated sugarcane bagasse (2.5% m/v) in citrate buffer (pH 50), for 48 h (50 °C, 200 rpm). The maximum conversion of cellulose to glucose, approximately 37.0%, was achieved when using an enzymatic load of 134 mg of protein/g of support. Regarding reuse, when using sugarcane bagasse, there was a loss of 75% of the biocatalyst activity after 6 cycles (each with 48h). When using microfibrillated MCC, the loss was 60.0% of its initial activity after 8 cycles.

Finally, Pectinex 3XL (commercial cocktail containing pectinases, xylanases, and cellulases (Novozymes)) was immobilized and applied to wheat straw hydrolysis. The immobilization of the Pectinex 3XL cocktail was performed on the APTS-magnetic nanoparticles activated with glutaraldehyde using an enzymatic load of 25 to 75U of pectinase. Hydrolysis of wheat straw was evaluated, after pre-treatment of this biomass (1.0% w/v) with NaOH (1.0 N) in an autoclave (121°C, 310 min), using the cocktail Pectinex 3XL in the form free and immobilized (100.0 U of pectinase) for 72 hours (50 °C, 200 rpm). To the hydrolyzate, 10.0 g/L of yeast extract and 20.0 g/L of peptone were added and autoclaved (121°C, 30 min). Fermentation was carried out by adding 1.0 g/L of baker's yeast (30 °C, 150 rpm). Like this, Perwez *et al*. (2017) reported that alkaline pre-treated wheat straw (2.0 g/L) provided a maximum concentration of reducing sugars of 1.39 g/L when the cocktail (free enzyme) was used, whereas when it was used the nano biocatalyst the concentration of reducing sugars was 1.59 g/L after 60 h of hydrolysis.

Therefore, most of the compounds discussed in Table 2 are magnetic nanoparticles because the separation is favored by the use of the magnetic field, and the contamination problems are minimized. It is also noteworthy that there are still few studies that used these biocatalysts with these immobilized commercial enzymes and that managed to produce ethanol. Thus, it is clear that there is much to be explored in this theme.

Process Design for Bioethanol Production using Immobilized Enzymes

Process design is an engineering activity applied to the identification of new process configurations able to form products, considering economic, environmental, and social aspects (Scott *et al.* 2013). In the context of bioethanol production from lignocellulosic biomass, there are three main steps, as mentioned before, namely pre-treatment, saccharification, and fermentation. In addition, after bioethanol production, there are two steps before its commercialization, namely bioethanol recovery and wastewater treatment, aimed at biofuel production and effluent treatments according to the regulations (Scott *et al.* 2013). The Benchmark Technology of bioethanol production consists in converting the lignocellulosic materials into bioethanol in three separate steps (pre-treatment, saccharification, and fermentation), using a short sequence of unit operations performed in different equipment with external supplementation of cellulases (Olivieri *et al*. 2021).

Although many bioethanol plants used this process design, the external supplementation of cellulases and the use of many sets of equipment increases the cost of bioethanol production (Klein-Marcuschamer *et al*. 2012; Olivieri *et al.* 2021). To overcome these drawbacks, many researchers have explored new process configurations, such as *in-situ* cellulases production (ISCP), in which cellulases are produced using fungal fermentation; simultaneous saccharification-fermentation (SSF) with external supplementation of cellulases, in which the pre-treated biomass is hydrolyzed to produce reducing sugars and these last are converted into bioethanol by fermentation in only one vessel; and the consolidated bioprocess strategy (CBS), whereby the two processes are used the combination for cellulase production, cellulose hydrolysis, and sugar fermentation in a one-pot process (Liu *et al.* 2020; Olivieri *et al.* 2021).

Among these strategies, ISCP and CBS processes are performed using soluble enzymes, since the cellulases production is coupled. On the other hand, the Benchmark Technology and SSF strategies can be performed using soluble or immobilized enzymes and/or cells (Alvarado-Morales *et al.* 2009).

In the specific case that immobilized biocatalysts are used, the cost of cellulases in the hydrolysis step can be overcome, since the immobilized enzyme could be reused in many batches. In addition, the immobilized enzyme can be more resistant at non-optimal conditions, mainly in the case of the SSF strategy. Figure 4 shows a schematic representation of some process designs of bioethanol production using immobilized enzymes.

In a literature review, most papers that use immobilized enzymes in the hydrolysis step produce bioethanol using Benchmark Technology (Fig. 4). In this context, Ashwini *et al*. (2023) immobilized cellulases from *Aspergillus niger* on Fe_3O_4/GO/GS nanocomposite and applied the immobilized enzyme in the Benchmark Technology of bioethanol production. In this case, the pre-treated acid-alkali biomasses (wheat bran and rice bran) were used as cellulose sources, and bioethanol productivities of 7.32 and 7.05 g/L.h were obtained at the end of the process, respectively. Using soluble enzymes in the same process, bioethanol productivities of 6.50 and 6.52 g/L.h were achieved using wheat bran and rice bran, respectively. Therefore, the immobilized enzyme was more efficient than the soluble enzyme, independent of the cellulose source used. In addition, the immobilized enzyme has the potential to be recycled, since the biocatalyst was reused by 4 cycles without loss of activity, using cellulose 2.5% as substrate. Baskar *et al*. (2016) also immobilized cellulases on magnetic nanoparticles and applied the biocatalyst in the benchmark technology to produce bioethanol, obtaining 5.39 g/L of ethanol at optimal conditions.

Some results in the literature relate that not all reducing sugar produced in the hydrolysis step is consumed in the fermentation step. This depends on the metabolism of the yeast. As an example, Mahapatra and Manian (2022) show that only 38.3% of reducing sugars produced by β-glucosidase immobilized on calcium alginate (2%) was metabolized by the yeast *Saccharomyces cerevisiae*, producing 5.46 g/L of ethanol after 144 h of fermentation. This behavior may be because *S. cerevisiae* is incapable of utilizing pentoses to produce ethanol. In an attempt to use both the cellulose and hemicellulose fractions of the biomass to produce ethanol, Dhiman *et al*. (2017) co-immobilized a mixture of xylanases with a commercial cellulase cocktail (Cellic C-Tec2) on functionalized hematite (Fe_2O_3) nanoparticles. This co-immobilized biocatalyst was used in the Benchmark Technology with simultaneous recovery of ethanol, using the yeast *Kluyveromyces marxianus* in the fermentation step. This yeast was selected for its ability to metabolize the hemicellulose hydrolysates, thermotolerance, and weak glucose repression. Therefore, a high concentration of ethanol (84.6 g/L) was obtained due to the improvement of sugars uptake and simultaneous recovery of ethanol.

The performance of immobilized enzymes in the SSF process for bioethanol production is also highlighted in the literature (Lupoi and Smith 2011; Cherian *et al.* 2015; Vaz *et al*. 2016). Cherian *et al*. (2015) immobilized cellulases on magnetic nanoparticles and applied the biocatalyst in the SSF process (Fig. 4) for bioethanol production, obtaining 22.0 g/L of ethanol. The biofuel concentration produced was higher than when the process was conducted using soluble enzymes (18 g/L of ethanol). The same behavior was found by Lupoi *et al*. (2011). It is important to mention that immobilization produces stabilized biocatalysts, enabling their use at non-optimal conditions of temperature, pH, and in the presence of ethanol normally found in the SSF process (Lupoi and Smith 2011). Therefore, this can explain the higher conversions of ethanol produced by immobilized enzymes, as mentioned before.

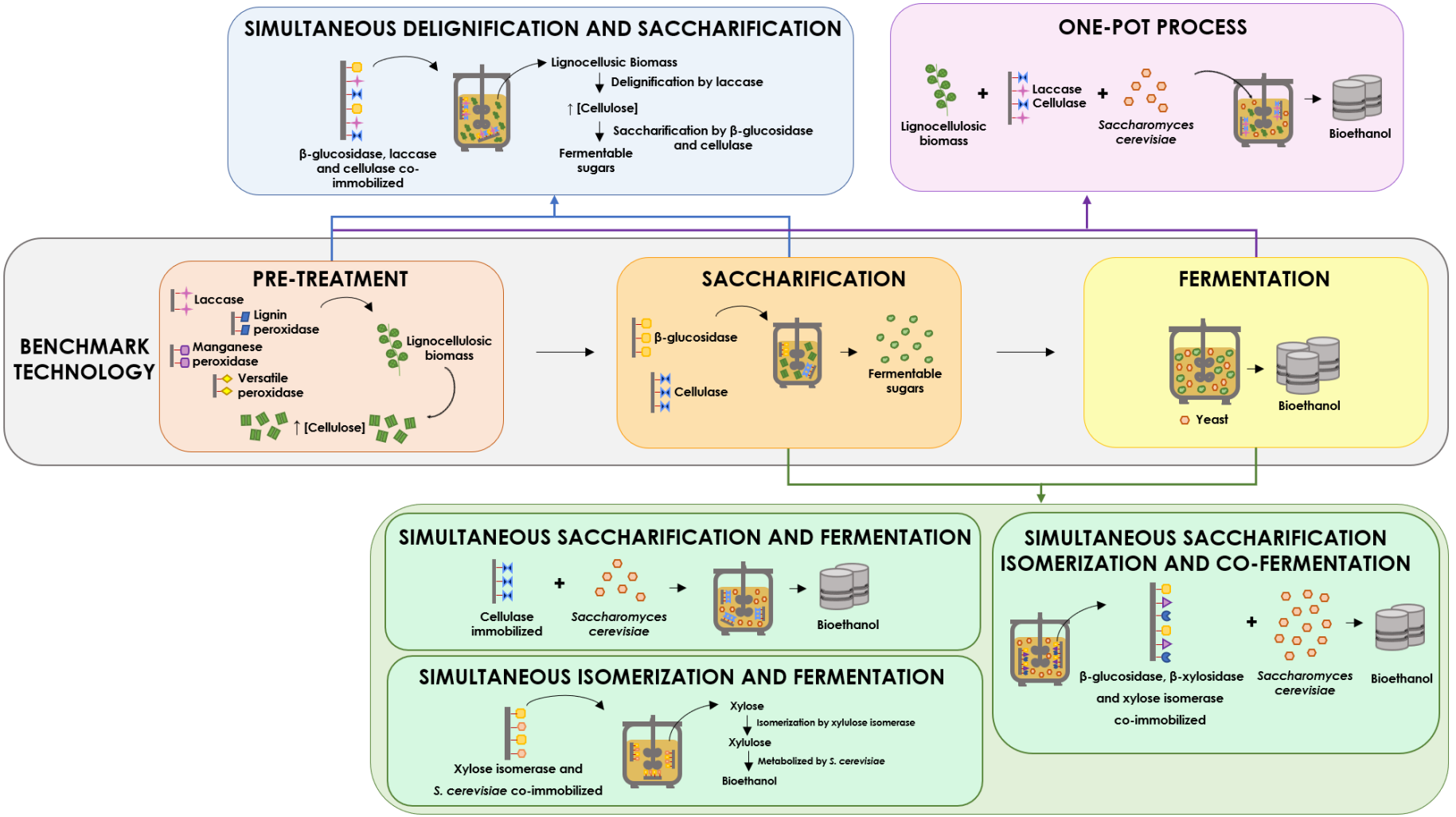

Fig. 4. Main processes design explored in literature for bioethanol production using immobilized enzymes

To improve the metabolic capacity of yeast used to produce bioethanol in the SSF process, a new configuration of the production process of bioethanol was designed, the simultaneous isomerization and fermentation (SIF) process (Silva *et al*. 2012; Milessi *et al*. 2018) (Fig. 4). This new design process was created because the wild type of *S. cerevisiae*, the common yeast used to produce ethanol, is not able to metabolize the xylose (reducing sugar from hemicellulose fraction), but it was able to metabolize the xylulose, an isomer of xylose. Therefore, the isomerization reaction can be performed using a xylose isomerase (D-xylose cetol isomerase; EC 5.3.1.5), in which the equilibrium constant of transformation of xylulose/xylose is unfavorable (1:5). To solve this problem, a SIF process can be performed, enabling the consumption of xylulose and the consequent shift of the equilibrium towards xylulose formation. Therefore, Milessi *et al*. (2018) immobilized xylose isomerase on chitosan support and, subsequently, co-immobilized the yeast using calcium alginate gel to produce ethanol from the xylose source by the SIF process. The results showed an ethanol productivity of 2 g/L.h, with a theoretical yield of 70%.

An evolution of the SIF process, in which only a xylose source is offered in the fermentation, is the simultaneous saccharification isomerization and co-fermentation (SSICF) process (Guilherme *et al.* 2022) (Fig. 4). In this case, β-glucosidase, β-xylosidase, and xylose isomerase were co-immobilized on feroxyhyte magnetic nanoparticles to convert a synthetic medium and pre-treated sugarcane bagasse to bioethanol, using SSICF process. This integrated bioprocess may facilitate ethanol production from both pentoses and hexoses fractions of lignocellulosic feedstocks through simultaneous saccharification with xylose isomerization and the co-fermentation of C6 and C5 sugars using the wild type of *S. cerevisiae*. Therefore, Guilherme *et al*. (2022) produced around 30 g/L of ethanol using this strategy. In addition, the immobilized enzymes were efficient in tolerating ethanol fermentation using the synthetic medium and pre-treated sugarcane bagasse, which makes their use feasible for recycling in an SSICF process.

Another new design strategy to produce bioethanol was explored by Sankar *et al*. (2018). The authors co-immobilized laccase, cellulase, and β-glucosidase by entrapment on sodium alginate to perform a one-pot delignification and saccharification using four different perennial lignocellulosic biomass, as cellulose sources. Using co-immobilized enzymes for pre-treatment of *Ipomoea carnea* biomass, the cellulose content was increased by about 17% and the lignin content decreased by about 11%, compared with no pre-treated biomass. Regarding saccharification, the best results obtained were with around 190 to 200 mg/g of reducing sugars from *Ipomoea carnea* and *Saccharum arundinaceum*, respectively. The biomasses *Ipomoea carnea* and *Saccharum arundinaceum* produced about 4.2 and 4.0 g/L of ethanol, resulting in yields of about 63.4 and 60.8% after 72 h, respectively (Sankar *et al*. 2018).

Hafeez *et al*. (2023) proposed an approach that involved the quick and chemical-free pre-treatment, saccharification, and fermentation of sugarcane bagasse in a single pot. They hypothesized that the combination of these three processes can be accomplished by the simultaneous addition of laccase (a pre-treatment agent), cellulase (a saccharification agent), and the desired microorganism. Together, the two immobilized enzymes (cellulase and laccase) were predicted to effectively pre-treat and hydrolyze the lignocellulose material. The results showed that the immobilized enzymes in polyacrylamide gel yielded 3.01 g/L of ethanol without any supplementation of carbon source and released more sugar (15.2 mg/g) compared with the isolated production of each. Immobilized cellulase released 8.45 mg/g of reducing sugars and immobilized laccase released 2.25 mg/g. It is important to point out that when analyzing the joint capacity, half of the enzyme load is used in the individual analysis.

CONCLUSION AND FUTURE PROSPECTS

In this paper, the main enzymes involved in the production of the second-generation of ethanol were presented and the main strategies of enzyme immobilization using these enzymes for bioethanol production were reviewed in detail. Regarding the enzymes that can perform the pre-treatment of lignocellulosic biomass, studies approaching the immobilization of LPMOs, manganese peroxidase, lignin peroxidase, and versatile peroxidase are still scarce. On the other hand, the immobilization of laccase has been well studied in the literature with applications in many areas. However, studies approaching the effective application of these immobilized enzymes in the pre-treatment of lignocellulosic biomass are limited.

To the enzymes involved in the hydrolysis of lignocellulosic biomass, the immobilization of cellulases and hemicellulases has been well explored in the literature, mainly using magnetic nanoparticles as support. This is a proper approach, since cellulose and hemicellulose are insoluble substrates that could be effectively catalyzed using immobilized enzymes on non-porous support (such as magnetic nanoparticles) to reduce the mass transfer effects. In addition, the magnetic separation of immobilized enzymes in these systems is advised due to the heterogeneity of the reaction. Most papers in the literature that immobilize cellulases or hemicellulases approach only the hydrolysis step, not reaching effectively in bioethanol production. As an example, the immobilization of commercial enzymatic cocktails (Cellic CTec, CelluClast, or Pectinex) reached the high performance of hydrolysis (Table 2), but the ethanol production was not evaluated in most of the papers. In addition, studies involving the mixtures of separately immobilized cellulases and hemicellulases or co-immobilized cellulases and hemicellulases are scarce in the literature.

The process design of second-generation ethanol using immobilized enzymes was also explored in this review. The most studied design is benchmark technology, in which the pre-treatment, hydrolysis, and fermentation are performed in a sequence of separated unit operations. However, some recent papers initiated the exploration of other innovative designs using not only a mixture of different immobilized enzymes but also using the technology of enzyme co-immobilization to join some unit operations.

This work shows a special and detailed revision regarding immobilized enzymes in the production of second-generation ethanol, going deeper into aspects of enzymatic pre-treatment and hydrolysis, commercial enzymes immobilization and bioethanol process design. Therefore, some gaps in the literature were highlighted and some new works exploring these lacks are expected to emerge.

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