Are Biological Pretreatments of Lignocellulosic Residues a Real Option for Biofuels Production?

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The use of lignocellulosic residues as feedstocks for biofuels production represents an economic and ecofriendly option, since they are generated as byproducts or wastes from different industrial areas. Nevertheless, a pretreatment method aimed at eliminating the lignin content of these residues must be performed. This is required in order to increase cellulose bioavailability, which favors the production of reducing sugars through microbial or enzymatic attack. Some performed pretreatments can be classified as physical, chemical, and physicochemical methods. Although such methods are the most used pretreatments, they are expensive and generate or make use of harmful compounds. Biological methods, by the action of microorganisms or their enzymes for lignin content reduction, may be regarded as an alternative, being cheaper and more friendly to the environment than the aforementioned methods. However, until now, biological pretreatments have not shown the same yield as the previously mentioned methods in both sugar recovery and biofuel production. In that sense, the aim of this work is to review the efficiency of these methods, with the goal of clarifying their advantages and disadvantages for improvement of biofuel production.

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INTRODUCTION

The depletion of fossil fuels reserves has created a real need for new energy sources in order to satisfy growing demands for energy. Lignocellulosic residues represent a sustainable alternative to fossil fuels, since this kind of residue is highly abundant. Biofuels derived from lignocellulosic biomass are considered the biggest energy source, which covers 10% of current demands for energy (Liu *et al*. 2017). Lignocellulosic biomass includes residues and wastes from forestry, wood processing, municipal solid wastes, and paper industry areas (Arora *et al*. 2020). Lignocellulose is composed of cellulose, hemicellulose, and lignin, which form a complex and recalcitrant structure. Although agro-industrial residues are rich in carbohydrates and essential compounds, the presence of

crosslinked lignin with hemicellulose and crystalline cellulose represents a huge barrier for the usage of these residues as feedstock for biofuels production (Zhang *et al.* 2023a).

Pretreatment is considered as the key step for the usage of these residues, and an effective pretreatment includes the breakage of both hydrogen bonds within crystalline cellulose and the crosslinks between lignin and hemicellulose (Bhurat *et al*. 2023). Furthermore, an increase in porosity and surface area must be considered in order to improve enzymatic activity to enable the release of free sugars (Li *et al*. 2010). Several physical (extrusion, milling and microwave), chemical (acids, alkalis, and ionic liquids), and physicochemical methods (steam explosion, humid oxidation, and carbon dioxide explosion) have been applied for pretreatment purposes (Saravanan *et al*. 2022). However, the aforementioned methods can produce inhibitory compounds that are associated with negative effects on microbial activity. Microbial action is often a key to subsequent processing steps during lignocellulosic residue usage (Mood *et al*. 2013).

Biological pretreatments through microbial or enzymatic biomass degradation offer an alternative to physical, chemical, and physicochemical methods. Biological methods, compared to aforementioned methods, require a lower energy input and avoid the usage of harmful chemical compounds as alkalis and acids, making them environmentally friendly (Bhushan *et al*. 2023). However, they have not shown the same yields as the physicochemical methods (Zhang *et al*. 2023a).

Therefore, the main aim of this work to review the different kinds of pretreatments and their yields in fermentable or free sugar production, in order to compare them during biodiesel, bioethanol, and biohydrogen production.

LIGNOCELLULOSIC RESIDUES

Before reviewing different pretreatment methods, an overview will be provided of the main compounds of lignocellulosic residues. One goal of this work is to provide a better understanding of how each of these pretreatment methods modifies the structure and composition of biomass waste for subsequent biofuel production.

Cellulose is the main compound of lignocellulosic biomass, and it constitutes 30 to 50% of its dry weight. Cellulose is a polysaccharide of D-glucose units linked by the β-(1,4) glycosidic bond, thus forming a linear chain. During biosynthesis, the cellulose chains assemble themselves into fibrils that are linked by hydrogen bonds, yielding a fibrillar arrangement called fibers (Ahmad *et al*. 2018).

Hemicellulose is made of substituted pentose and hexose chains contributing 20 to 35% of lignocellulosic biomass. The side groups may be either pentoses or hexoses, in different cases. Hemicellulose is bonded to cellulose fibers by hydrogen bonds and Van der Waals forces. Hemicellulose is very sensitive to temperature and pretreatment time; thus, uncontrolled pretreatment conditions may generate undesirable products such as furfural that inhibit microbial fermentation (Palmqvist and Hahn-Hägerdal 2000).

Lignin, which often contributes 15 to 30% of biomass dry weight, provides rigidity, impermeability, and resistance to the attack of microorganisms in plants. Lignin is the result of three cinnamyl alcohol precursors linked to each other by different kinds of bonds (Shanmugam *et al*. 2020). The degree of lignin removal determines the success of a pretreatment method (Arora *et al*. 2020). Lignocellulosic structure and composition are shown in Fig. 1.

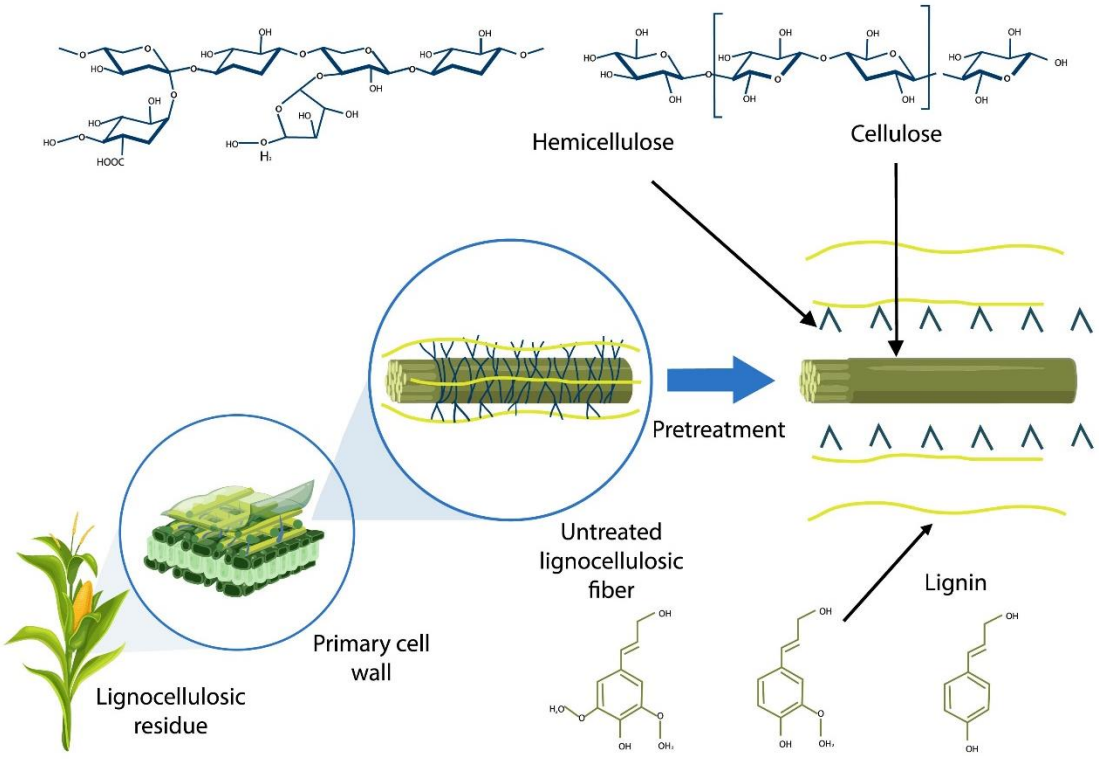

Fig. 1. Structure of lignocellulosic residues. Cellulose fibrils are linked by hydrogen bonds forming a fiber; these fibers are bonded to hemicellulose by hydrogen bonds. The polysaccharides are then linked to lignin by different bonds which increases its resistance to enzymatic or microbial attack.

PRETREATMENTS

The main aim of pretreating the lignocellulose usually is to achieve both the highest amount of removed lignin and the greatest exposure of cellulose to microbial or enzymatic activities. Such pretreatment will ideally result in a minimal loss of cellulose and hemicellulose and will not use or generate harmful compounds (Saravanan *et al*. 2022; Wu *et al*. 2022). Pretreatments can be classified into physical, chemical, physicochemical, and biological methods.

Physical Pretreatments

Within physical pretreatments, milling is considered as one of the first steps for the usage of lignocellulosic biomass. The aim of milling is to reach a particle size between 0.2 and 2 mm that increases the surface area. Despite being an efficient method, it consumes a considerable amount of energy, which reduces its applicability (Mood *et al*. 2013).

Extrusion is a method in which the lignocellulosic residues are mixed, heated up, and exposed to shear stresses. The main advantage of extrusion is that this method does not generate harmful inhibitory compounds such as furfural and hydroxymethyl furfural (Kurunanithy and Muthukumarappan 2011). Just like milling, the cost of energy is an enormous disadvantage in extrusion, which makes its scaling up to industrial levels a complicated task (Zhu and Pan 2010).

Sonication uses ultrasound waves in a range of 10 and 100 kHz. These waves form small cavitation bubbles that break up the cellulose and hemicellulose, increasing the material's accessibility to cellulolytic enzymes (Gogate *et al*. 2011). Freezing is a recently developed approach, that has demonstrated an increase in enzymatic activity over lignocellulosic residues; the approach offers as unique feature a lower environmental impact (Chang *et al*. 2011).

Chemical Pretreatments

The most used chemical pretreatments employ acids and alkalis such as sulfuric acid (H_2SO_4) and sodium and potassium hydroxides (NaOH and KOH). In these methods the hemicellulose polysaccharide is hydrolyzed to monosaccharides, which improves enzymes accessibility to cellulose chains (Taherzadeh and Karimi 2008).

Concentrated H_2SO_4 is the most used acid in this kind of pretreatment. Nevertheless, it has shown huge disadvantages such as equipment corrosion, degradation of fermentable sugars, and production of inhibitory fermentation compounds (Mood *et al*. 2013).

Alkaline pretreatment methods are generally less harmful and toxic than acid pretreatments. The most used alkalis are NaOH and KOH. Alkaline pretreatments decompose ester and glycosidic lateral chains, which alters lignin structure, swells cellulose, and breaks down hemicellulose into monomers (Ibrahim *et al*. 2011).

Ozonolysis is a method that degrades lignin content through the use of ozone as an oxidant agent. This oxidation releases cellulose and hemicellulose, increasing their biodegradability (Balat 2011).

Ionic liquid pretreatments use liquids having low fusion temperatures, low vapor pressure, and high stability and polarity. Ionic liquids dissociate the lignocellulosic complex, breaking down hydrogen bonds. Compared to other conventional methods, ionic liquids have some advantages such as less harmful process conditions and high reusability. However, their main disadvantage is the inhibitory effect that these liquids exert over cellulase activity during the saccharification process (Yang *et al*. 2010).

Physicochemical Pretreatments

Steam explosion pretreatment is a method involving the breakage of structural components throughout steam heating, decompression, and moisture evaporation, yielding an autohydrolysis of glycosidic bonds mediated by organic acids. These events result in the removal of hemicellulose, which increases cellulolytic enzymes accessibility and promotes the saccharification process (Mabee *et al*. 2006; Kabel *et al.* 2007). The severity of this treatment is related to many variables including temperature, retention time, moisture content, biomass type, and the presence or absence of catalytic compounds (Rabemanolontsoa and Saka 2016).

Steam explosion pretreatments using ammonia take advantage of ammonia's low boiling point. The treatment yields a solid residue, rather than a sludge requiring the usage of water. The use of supercritical carbon dioxide explosion instead of ammonia fiber explosion has some advantages, since CO_2 is less harmful and toxic and also requires lower critical conditions than ammonia during the pretreatment process (Mood *et al*. 2013).

Hot liquid water pretreatments, without the need of a rapid decompression or expansion, solubilizes hemicellulose and removes lignin, increasing cellulose accessibility for microbial or enzymatic attack. It has been reported that during sugar cane bagasse

pretreatment using this method combined with sulfonation, enzymatic saccharification reached glucose yields of 97.5% (Kane *et al*. 2003).

The use of organic solvents together with the addition of catalyst agents such as organic or inorganic acids is capable of breaking the internal lignin and hemicellulose bonds. These types of treatments are better known as organosolv and, despite being efficient in the removal of lignin, they present disadvantages with respect to other pretreatments such as high operating pressure and high flammability and volatility (Mood *et al*. 2013).

Biological Pretreatments

Biological pretreatments, in contrast to the previously described methods, do not require elevated investment and operational costs (Brémond *et al*. 2018; Zhang *et al*. 2023b). Most of these methods are based on the activity of degrading fungal microorganisms belonging to the group of *Ascomycetes*, *Deuteromycetes*, and *Basidiomycetes*, which produce the needed enzymes in order to degrade lignocellulosic residues. These microorganisms are normally associated with three different kinds of wood rot: white, soft, and brown rot (Eriksson *et al*. 2012).

White rot fungi use a wide range of cellulolytic enzymes that degrade the whole cellulose but do not possess a considerable ligninolytic activity. Among the most reported white rot fungi, *Aspergillus niger*, *Penicillium chrysogenum* and *Trichoderma reesei* can be cited (Shirkavand *et al*. 2016).

Soft rot fungi are one of the most effective wood degrading fungi. These fungi attack all the compounds from lignocellulosic residues. Some soft rot fungal species discovered are *Phellinus pini*, *Phlebia* spp., *Pleurotus* spp., *Dichomitus squalens*, *Lentinus edodes*, *Phlebia radiata*, *Panus tigris*, *Pycnoporus cinnabarinus*, *Xylaria* spp, and *Ustulina vulgaris*, amongst others (Shin *et al*. 2019; Arora *et al*. 2020).

Brown rot fungi, such as *Fomitopsis palustris*, *Lenzites trabea*, *Gloeophyllum trabeum*, and *Fusarium oxysporum*, degrade in a selective way cellulose and hemicellulose, generating a residue with minimal modification of lignin (Martínez *et al*. 2005). During the first steps of brown rot fungi degrading activity, the degradation process is not carried out by the activity of enzymes; instead, small extracellular oxidants and free radicals participate in an extracellular Fenton system that generates free hydroxyl radicals (Monrroy *et al*. 2011).

All of the biocatalysts present in lignocellulose-degrading microorganisms, and specifically the enzymes that decompose polyphenolic substrates from lignin, can be divided into the categories of lignin-modifying enzymes and auxiliary lignin-degrading enzymes. The first group of enzymes directly degrades lignin and includes laccases and lignin and manganese peroxidases. The second one considers enzymes that do not degrade lignin directly, but are related with a degradation process involving hydrogen peroxide produced from alcohols or glucose; these are used as a substrate by lignin-degrading enzymes (Shin *et al*. 2019; Levasseur *et al*. 2008). Delignification either takes place directly through enzymes or is due to the metabolism of microorganisms, which in turn depends on their enzymes. The models that describe its kinetics generally are those that fit the Michaelis-Menten model; however, there are other models that explain the process in terms of first-order models (Pendse *et al*. 2023).

The main steps for biofuels production using biological pretreatments are presented in Fig. 2.

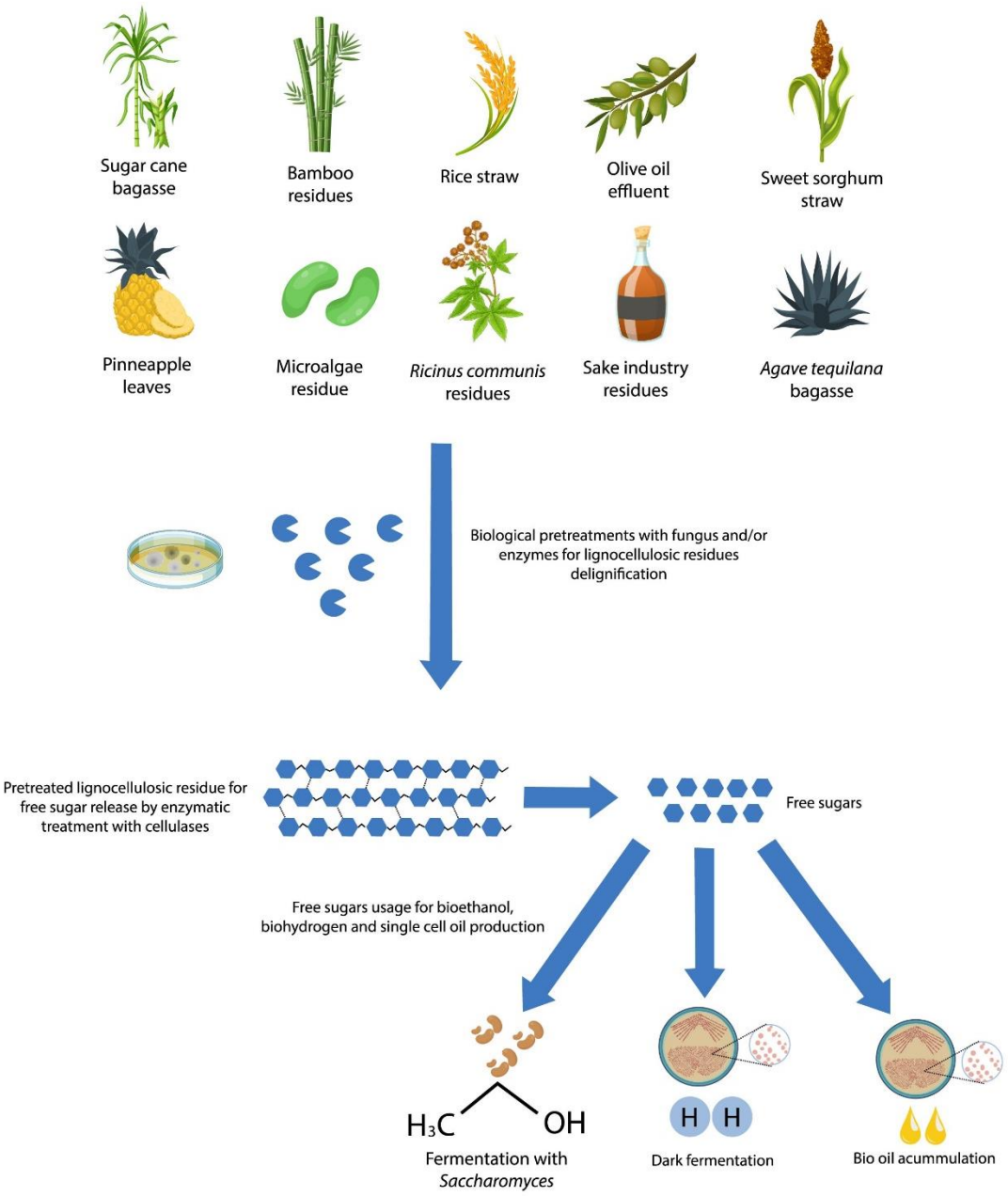

Fig. 2. Lignocellulosic residues used for biofuels production. Lignocellulosic residues are biologically pretreated with fungus or enzymes to reduce lignin content. Pretreated lignocellulosic residues are saccharified with cellulolytic enzymes resulting in free sugars for further biofuels production.

INFLUENCE OF THE KIND OF PRETREATMENT ON SUGAR CONTENT AND FINAL BIOFUEL YIELD

As was mentioned previously, lignocellulosic biomass is a promising alternative as feed stock for biofuel production, since 70% of its dry weight corresponds to polysaccharides. Once all these polysaccharides are hydrolyzed by microbial or enzymatic

processes, they yield pentoses and hexoses, which could be further used for the generation of biohydrogen, bioethanol, and biodiesel biofuels.

Biohydrogen

Biohydrogen is an alternative form of clean energy that seems ideal for the future. It possesses a high energy yield of 122 KJ/g, which makes it 2.75 more energy-rich per unit mass than biofuels derived from petroleum (Chong *et al*. 2009). Bacterial degradation of organic matter under anoxic conditions could produce biohydrogen as byproduct. In that process, pyruvate obtained from glucose degradation within glycolysis pathway is used by anaerobic microorganisms, mainly from the genera *Clostridium*, including the species *C. acetobutylicum*, *C. butyricum*, *C. pasteurianum*, *C. paraputrificum*, *C. lentocellum*, and *C. thermosuccinogenes* (Nagarajan *et al*. 2010).

Although the microorganisms listed above are capable of using simple sugars, there exist different microorganisms such as *Clostridium cellulovorans* and *Rhodopseudomonas palustris*, which possess the ability to use cellulose directly, since they produce cellulolytic enzymes (Lu and Lee 2015). López-Hidalgo *et al.* (2018) carried out a thermochemical pretreatment using 4% w/w of sulfuric acid and 121 °C of temperature on wheat straw. By this means they obtained a glucose yield of 21 g/L. Something to highlight from this work is the fact that, once the pretreatment was carried out over a mixture of agro-industrial residues and cheese whey, the mixture was subjected to anaerobic fermentation using an activated sludge with a previous thermal inactivation of methanogenic microorganisms, reaching a biohydrogen yield of 4132 mL H_2/L (López-Hidalgo *et al.* 2018).

A recent study, through the use of *Agave tequilana* bagasse, using milling as the only pretreatment and further subjecting the material to enzymatic hydrolysis with commercial cocktail, achieved sugar concentrations of 5.3 g of sugar/L. Notably, all these sugars were consumed during dark fermentation, achieving a volumetric production rate of 6.0 L H_2/L.d (Montiel-Corona and Razo-Flores 2018).

Despite there being several physical pretreatments that do not employ polluting compounds, sugar production yields are considerably lower than those yields obtained using acid or alkaline compounds. An example of the latter is the work of Choiron *et al*. (2020), who used lignocellulosic residues from the sake production industry pretreated with only hot compressed water; however, they did not achieve a significant increase in usable sugar concentration (0.12 g of fermentable sugar/g of pretreated residue) for biohydrogen production.

Compared to the aforementioned pretreatments, biological degradation of lignin using ligninolytic microorganisms or its enzymes, offers gentler process conditions, lower consumption of energy, and avoidance of the use of toxic chemicals (Sherpa *et al*. 2018). Laccases are multi-copper oxidase enzymes that catalyze the monoelectronic oxidation of a wide spectrum of phenolic compounds. *Trichoderma asperellum* produces extracellular laccases. Shanmugan *et al*. (2018) have shown that an isolated laccase from *T. asperellum* for sweet sorghum straw delignification was able to eliminate close to 77% of lignin. It is noteworthy that, despite the fact that the removal of lignin was high, the difference between fermentable sugar production during cellulose treatment was only 14% higher than control without laccase treatment. That finding was consistent with the biohydrogen production. The same research team, after working with laccase immobilized in Fe_3O_4@SiO_2-chitosan nanoparticles for sweet sorghum straw pretreatment, reached a delignification percentage of 66.6%. Notably, the delignified straw was further saccharified and fermented for

biohydrogen production, showing a yield of 2.8 molH_2/mol of substrate (Shanmugam *et al*. 2020).

Even though several studies only evaluated the saccharification of lignocellulosic residue and not the production of biofuel, the pretreatments used have shown enormous potential to be used for biohydrogen production. Lignin peroxidase produced by *Aspergillus oryzae* has been shown to degrade corn straw lignin, reaching sugar yields of more than 46.3%, indicating a huge potential for biohydrogen production (Fan *et al*. 2019). Despite the fact that corn lignocellulosic waste can be used for fermentable sugars production and further biofuels generation, this residue can be converted directly to hydrocarbon through hydrothermal carbonization, which is an appropriate process for low quality biomass (Raheem *et al*. 2022).

Despite the fact that most of previously cited works have shown the successful performance of biological pretreatments and further saccharification for biofuels production (De Souza *et al*. 2020; Shanmugam *et al*. 2020; Pereira *et al*. 2021), there also exist some research works such as that of Navas *et al*. (2019), who used a recombinant laccase of *Thermus sp*., which did not find a positive effect. The sugar production amounts resulting from enzymatic saccharification were the same as the controls. Such findings suggest that this pretreatment did not yield forms of cellulose and hemicellulose that were more accessible to enzymatic attack. An explanation of the latter behavior could be an inhibition of hydrolytic enzymes due the presence of phenol oligomers produced by the action of laccases. Sometimes, although biological pretreatments have shown better results than physical and chemical pretreatments, one must consider both the time of process and the economic resources that need to be invested in order to obtain higher yields. Bhurat *et al*. (2023), compared chemical (alkali and acid), physical (autoclaving and aeriation), and biological (the white root fungi *Pleurotus djamor*) methods for the pretreatment of food wastes consisting of vegetable peels and leftovers. Interestingly, during dark fermentation, the biological method showed an increase in biohydrogen yield of 3.8-fold compared to the control (same residue without any pretreatment); however, aeriation and acid treatments showed similar results than biological method and, as a remarkably fact, acid pretreatment reached in a lower time almost the same result as the usage of *P. djamor* (Bhurat *et al*. 2023).

To increase the efficiency of laccases for lignocellulosic residue delignification, some authors have reported the use of mediators such as hydroxybenzotriazole (HOBT). This compound is oxidized by laccase, generating a nitroxide radical that increases the oxidative action over lignin (Srebotnik and Hammel 2000). In a recent work, an enzymatic extract from *Trametes maxima* and HOBT as mediator was used for the delignification of jute sticks. The study showed that the presence of this mediator increased porosity and surface area, favoring the attack of cellulolytic enzymes, obtaining a sugar yield of more than 20% higher compared with a control pretreated with only laccase (Suman *et al*. 2022).

A different study using HOBT, linolenic acid, reduced glutathione, and polysorbate 80 (Tween 80) as mediators, mixed with manganese peroxidase isolated from *Phanerochaete sordida*, found that the presence of these mediators increased lignin removal (Mori *et al*. 2021). Recently, it has been reported that the use of activated sludges for biohydrogen generation can produce this biofuel using lignocellulosic residue from palm oil industry without previous delignification. In this work, the saccharification was done with the use of commercial cellulases, and the hydrolyzed material was inoculated with activated sludge obtained from an acetogenic reactor, achieving a cumulative biohydrogen production of 200 mL (Izzi *et al*. 2023).

Table 1. Yields of Delignification, Free Sugar and Biohydrogen Related to Biomass Residue and Pretreatment

Author and Year	Pretreatment and Residue	Delignification Percentage	Free Sugar Yield (g of free sugar/g of lignocellulosic residue	Biohydrogen Yield (mL of biohydrogen/g of free sugar)	Total Yield (mL of biohydrogen/g of lignocellulosic residue)
(López-Hidalgo *et al.* 2018)	Thermochemical treatment with H_2SO_4 in wheat straw	Not reported	0.912	138	126
(Montiel-Corona y Razo-Flores 2018)	Milling and further saccharification with cellulases in *Agave tequilana* bagasse	Not reported	0.174	604	105
(Shanmugam *et al*. 2018)	Milling, delignification with *Trichoderma asperellum* laccase and cellulases saccharification in sweet sorghum straw	66	0.66	60.9	40.2
(Shanmugam *et al*. 2020)	Delignification with *T. asperellum* laccase in sweet sorghum straw	84.4	0.844	346	292
(Izzi *et al*. 2023)	Saccharification with cellulases in palm oil residues	Not reported	Not reported	52.6	Not reported
(Choiron *et al*. 2020)	Hot compressed water pretreatment in lignocellulosic residues from sake industry	Not reported	Not reported	9.07	Not reported

As has been shown previously, lignin content represents the main impediment for cellulose and hemicellulose usage in lignocellulosic residues. Therefore, recent research studies have proposed the use of biomass derived from algae and microalgae, including cyanobacteria, as a new option for biofuel production, since this kind of biomass lacks lignin (Bushan *et al*. 2023). In that sense, El-Sheekh *et al*. (2023), using biomass from *Oscillatoria acuminata* for biohydrogen production, compared thermochemical pretreatments with different concentrations of H_2SO_4, against biological pretreatments consisting of an enzymatic cellulolytic extract from *Trichoderma harzianum* combined or not with Mg-Zn Fe_2O_4 nanoparticles. It is notable that once fermentable sugars obtained during all pretreatments were inoculated with *Klebsiella pneumoniae* and *Enterobacter cloacae* for biohydrogen production, the biological pretreatment using nanoparticles showed a biohydrogen production that was 74% higher than thermochemical pretreatments (El-Sheekh *et al*. 2023). A summary of aforementioned research works is shown in Table 1.

Bioethanol

Bioethanol is an alternative and sustainable biofuel that can reduce green gas emission if is used in place of gasoline. The production of first-generation bioethanol has been questioned, since its production uses human food sources as feed stocks, competing with food supply chains (Nishimura *et al*. 2017; Alfonsín *et al*. 2019). Therefore, bioethanol production using lignocellulosic biomass has gained worldwide attention. In that sense, pineapple leaves are promising candidates for bioethanol production owing to their elevated cellulose concentration of 63% on a dry basis (Immam *et al*. 2021). In this work, by means of a pretreatment consisting of hot acid water, demonstrated achieved a maximum glucose yield of 91% and a theoretical bioethanol yield of 94.7%. Something to highlight from this work is the fact that elevated solid charges increased productivity to 0.63 g/L.h (Immam *et al*. 2021).

Ionic liquids are a group of low melting point organic salts that in the last decade have been used for the delignification of biomass, since they have advantages over other pretreatment methods such as high reusability, low toxicity, low vapor pressure, high thermochemical stability, non-flammability, as well as high conductivity and polarity (Da Costa-Lopes *et al*. 2013; Socha *et al*. 2014). The main obstacle for great industrial scale application of ionic liquids is the elevated costs associated to its use, because of expensive synthesis process, excessive use of water or anti-solvent during washing of pretreated biomass, and high price of recovery (Zhao *et al*. 2022). However, it has been reported that by use of compounds such as triethylamine hydrogen sulfate (TEA-HSO_4), it is possible to achieve a 100-fold reduction in the production costs of ionic liquids (Gschwend *et al*. 2018). Ziaei-Rad *et al*. (2021), through the use of ionic liquids, obtained glucose saccharification levels of 87.2%, and a maximum theoretical bioethanol yield of 84.3%. Furthermore, the global mass balance resulted in 198 g of bioethanol over 1 kg of pretreated wheat straw, which was almost 80 times higher than what was obtained from wheat straw without pretreatment (Ziaei-Rad *et al*. 2021). Wu *et al*. (2021), carrying out an alkaline pretreatment followed by eutectic solvent soaking in sorghum straw, achieved delignification percentages and glucose and bioethanol yields of 78%, 21%, and 9.45% respectively.

Although physicochemical pretreatments have shown high saccharification yields, they have been questioned because of the use of toxic and harmful compounds. Therefore, biological pretreatments for bioethanol production have gained interest recently. In that sense, Avanthi and Banerjee (2016), using an extracellular laccase from *Pleurotus djamor*, carried out delignification in *R. communis, L. camara, S. officinarum, S. spontaneum, A. comosus,* and *B. bamboos*, which correspond to grass-derived and non-grass-derived lignocellulosic residues, finding delignification percentages between 78% and 85%. Noteworthy, once the delignification process was carried out, the saccharification with cellulolytic enzymes showed a yield of 0.58 g of sugar/g of dry pretreated substrate, representing a potential bioethanol yield of 0.30 g of bioethanol/g of dry pretreated substrate.

Sugar cane production generates an enormous quantity of lignocellulosic residues, which contain around 40% dry weight of cellulose. White rot fungi have already demonstrated an elevated lignin remotion during biological pretreatments of sugar cane bagasse, since *Phanerochaete chrysosporium, Lentinula edodes,* and *Pleurotus ostreatus* reached a lignin degradation percentage between 85 and 93%, with *P. chrysosporium* achieving the best performance (Dong *et al*. 2013). Rice straw represents a good source of lignocelluloses; therefore, it has a considerable potential for bioethanol production. The

alkalophilic fungus MVI.2011 can simultaneously degrade all lignocellulosic residues compounds. Sreemahadevan *et al*. (2018) carried out simultaneous delignification and saccharification with this fungus in rice husk, obtaining a saccharification percentage of 54% higher than not pretreated control.

Despite the fact that there are many accounts of biological pretreatments showing high delignification percentages over lignocellulosic residues for bioethanol production, there are other works that have not shown elevated results or, at least, the results are confusing. An example of latter is the work of M´Barek *et al*. (2020), who used native fungal strains such as *Fusarium oxysporum* and *Fusarium solani* from the central region of Morocco for delignification, saccharification, and bioethanol production using lignocellulosic residues of olive oil industry. They reported a yield of 0.84 g of ethanol/g of residue, even presenting yields over the theoretical yield because, in this particular research, the lignocellulosic residue was enriched with glucose during fermentation. Such enrichment makes it possible to achieve results above the usual theoretical limit. In order to reach higher yields of lignin degradation, cellulose saccharification, and bioethanol production, the use of coupled physical and chemical pretreatments could be considered. Jin *et al.* (2020), who used rice husk for fermentable sugar production through the enzymatic activity from *Aspergillus fumigatus* and the metabolism of *Saccharomyces tanninophilus*, found that biological delignification combined with H_2SO_4 acid treatment almost inhibited the activity of lignin degrading enzymes. Contrasting results were found following the use of sodium hydroxide, which increased the activity of lignin peroxidase and laccase from *A. fumigatus*, showing a final yield of 189 g of bioethanol/kg of natural rice husk.

Other research studies, which combined chemical and biological pretreatments focused on bagasse and sugar cane straw usage, have shown that laccase from the actinomycete *Pycnoporus cinnabarinus* combined with HOBT and an alkaline extraction using peroxide resulted in a delignification percentage of 27% and 31% and a saccharification yield of 39% and 46% for bagasse and husk, respectively, while pretreatment with just laccase only showed a percentage of 4% and 9% for the same residues. Such results show how the coupling of different pretreatments can increase the delignification and saccharification efficiencies (Rencoret *et al*. 2017). Such coupled pretreatments have been carried out in different studies using substrates as wheat straw, where the use of only laccase as pretreatment showed a delignification percentage of only 5%, while enzymatic pretreatment together with HOBT and alkaline oxidant extraction, resulted in a lignin degradation percentage of 48% (Rencoret *et al*. 2016).

Although recent advances in chemical, physical, and biological pretreatments have shown promissory results for the feasibility of cellulosic ethanol production, until now, cellulosic ethanol, as a single product, is not cost-competitive for industrial production (Liu *et al*. 2019).

Table 2. Yields of Delignification, Free Sugar, and Bioethanol Production Related to Biomass Residue and Pretreatment

Author and Year	Pretreatment and Residue	Delignification Percentage	Free Sugar Yield (g of free sugar/g of lignocellulosic residue	Bioethanol Yield (mL of bioethanol/g of free sugar)	Total Yield (mL of bioethanol/ g of lignocellulosic residue)
(Imman *et al*. 2021)	Milling, hot acid water bath and saccharification with cellulases in pineapple leaves	62.3	0.40	0.588	0.235
(Ziaei *et al*. 2021)	Milling, ionic liquid usage and saccharification with cellulases in wheat straw	45	0.542	0.46	0.25
(Avanthi and Banerjee, 2016)	Milling, *Pleurotus djamor* laccase treatment and saccharification with cellulases in grass- and non-grass-derived lignocellulosic residues	83	0.32	0.646	0.207
(Jin *et al*. 2020)	Pulverization, alkaline treatment, simultaneous delignification and saccharification with *Aspergillus fumigatus* in rice straw	Not reported	0.443	0.54	0.23
(M´Barek *et al*. 2020)	Pulverization and simultaneous delignification and saccharification with *Fusarium oxysporum* in olive oil mill effluent	Not reported	0.077	0.44	1.10

In order to benefit the economics of the process, a biorefinery approach must be considered. In that sense, Pereira *et al*. (2021), using an integrated biorefining process for bioethanol production using sugar cane bagasse (SCB), reached a bioethanol final yield of 93.7 g/ Kg of SCB residue and a conversion yield of 0.48 g of ethanol/g of reducing sugar. Interestingly, during the biorefining process, xylooligosaccharides, cellulose nano fibers and lignin nanoparticles were obtained as byproducts. Considering the cellulosic ethanol and all the byproducts, the biorefining process recovered 65% of the SCB as bioproducts (Pereira *et al*. 2021). All the aforementioned research is summarized in Table 2.

Biodiesel

Biodiesel can be defined as a mixture of alkyl esters from fatty acids that are obtained through transesterification of vegetable oils or/and animal fats with short chain alcohols such as methanol or ethanol (Marin-Suárez *et al*. 2019). Such products are considered a renewable and environmentally friendly energy source. Oils produced by single-celled oleaginous microorganisms, including bacteria, yeasts, and fungi are nowadays considered promising feedstocks for third-generation biodiesel production, owing to their composition in fatty acids being similar to that of vegetable oils (Saenge *et al*. 2011).

Sugarcane bagasse and rice husk, due to their elevated cellulase content, can be used for fermentable sugar production, which could be further utilized by microorganisms capable of producing single-cell oils. Ananthi *et al*. (2019) carried out a steam explosion pretreatment in rice husk and sugar cane bagasse, reaching a reducing sugars concentration of 63 and 61.3 g/L, respectively. After the pretreatment, the produced reducing sugars were used by *Meyerozyma guilliermondii*, *Candida albicans*, and *Pichia manshurica*, showing bio-oil yields of 2.21 g of oil/g of glucose. Recent research, also using sugar cane bagasse, have tested alkaline and acid treatments combined with sonication, obtaining a glucose concentration of 1380 mg/L. Something to highlight from this work is the fact that *Yarrowia lipolytica*, an oleaginous yeast, produced 0.587 g of lipids/g of dry biomass when it was cultivated in medium containing the hydrolysate from sugar cane bagasse. Once the lipids were trans-esterified, the final biodiesel yield was 80% (Vasaki *et al*. 2022).

One of the main limitations of acid and alkaline pretreatments is the production of compounds that inhibit the microorganism's growth and oil accumulation. However, recent research studies have reported the use of *Rhodotorula mucilaginosa*, an oleaginous yeast, for biodiesel production using hydrolysate from *Durio zibethinus* Murr peel. This yeast was able to accumulate 16% lipids even in the presence of furfural and hydroxy methyl furfural. Notably, the final mass balance showed a total yield of 15.8 g of biodiesel/kg of peel (Siwina and Leesing 2021). Miao *et al*. (2020) using a strain of *Rhodotorula taiwanensis* for the usage of corn cob hydrolysate, reached a yield of 60.3% of oil over dry weight yeast and, notably, the final mass balance showed a total yield of 55.8 g of oil/kg of untreated corn cob. *Mortierella isabellina*, an oleaginous yeast, has been shown to give robust lipid production. Santek *et al*. (2021), using a corn cob hydrolysate pretreated with alkalis and acids, obtained a final yield of 3.3 g of biodiesel/100 g of corn cob. Moreover, they observed that common inhibitors such as acetic, formic, and levulinic acids, furan aldehydes, furfural, hydroxy methyl furfural, and the phenolic compounds vanillin and syringaldehyde were formed during pretreatment. These compounds inhibited in a synergic way the cellular growth and the accumulation of lipids, demonstrating that, despite physicochemical pretreatments increase sugar production yields, the inhibitors production is still a huge drawback in this kind of pretreatments (Santek *et al.* 2021).

Although pretreatments based in the use of alkalis and acids have shown positive results, there exist several research works that demonstrate the opposite. Vignesh *et al*. (2020) carried out an acid pretreatment over *Moringa* leaves, obtaining only a reducing sugars concentration of 0.47 g/L, with low glucose and xylose concentrations of 0.022 and 0.014 g/L respectively. This limited sugar concentration did not generate any positive effect over the accumulation of single cell oil in the microalgae *Coelastrella* sp. M-60 and *Dictyococcus* sp. VSKA18. Owing to the aforementioned work, the use of biological pretreatments represents a real option for lignocellulosic residue usage.

Table 3. Yields of Delignification, Free Sugar, and Biodiesel Production Related to Biomass Residue and Pretreatment

Author and Year	Pretreatment and Residue	Delignification Percentage	Free Sugar Yield (g of free sugar/g of lignocellulosic residue	Bioethanol Yield (g of biodiesel/g of free sugar)	Total Yield (g of biodiesel/ g of lignocellulosic residue)
(Ananthi *et al*. 2019)	Steam explosion in sugar cane bagasse and rice husk residues	Not reported	0.62	0.1	0.062
(Vasaki *et al*. 2022)	Milling, acid treatment and sonication in sugar cane bagasse	Not reported	0.13	1.47	0.192
(Siwina and Leesing 2021)	Milling and acid treatment in *Durio zibethinus* peel	Not reported	0.176	0.09	0.0159
(Santek *et al*. 2021)	Milling, alkaline treatment and saccharification with cellulases in corn cob	Not reported	0.347	0.095	0.033
(Vignesh *et al*. 2020)	Milling and acid treatment in microalgae residue	Not reported	0.363	0.0114	0.004
(Gujjala *et al*. 2019)	Milling and enzymatic treatment with laccase in *Ricinus communis*	85.7	0.288	0.120	0.034
(Miao *et al*. 2020)	Milling and acid treatment in corn cob	Not reported	0.198	0.229	0.044

The fungus *Aspergillus* sp. has been frequently chosen for lipid production due to its capacity to use a wide range of industrial substrates (Muniraj *et al*. 2013). In that sense, a study using *Aspergillus awamori* for bio-oil accumulation evaluated the potential of a lignocellulosic residue from *Ricinus communis* pretreated with laccase from *Pleurotus djamor*. Once the residue was pretreated, the reducing sugar concentration was 3.77 g/L and the yield of bio-oil accumulation was 0.134 g of bio-oil/g of reducing sugar. Notably, the biodiesel obtained during transesterification of the accumulated bio-oil showed the quality required to meet international standards (Gujjala *et al*. 2019). Another fungus, *Pleurotus ostreatus*, has been also used for the degradation of sugar cane bagasse, rice husk, corn cob, wheat fiber, and newspaper residue, showing a delignification percentage of 65% and a reducing sugar concentration of 6 mg/mL. This work demonstrated how the nature of the lignocellulosic residue plays a key role, since each of the residues used showed a different behavior (De Souza *et al*. 2020).

The actinobacteria *Mycobacterium smegmatis* has ligninolytic and bio-oil accumulation abilities. Zhang *et al*. (2019) evaluated these abilities using corn straw as substrate, showing an enormous potential of *M. smegmatis* for biodiesel production. The delignification, bio-oil production, and accumulation all can be done by the same microorganism, thus reducing costs and processing times. In that work, the bio-oil accumulation and delignification percentages were notably higher than the percentages reported in *Rhodococcus opacus* and *R. jostii.* An explanation for these higher percentages is the arsenal of enzymes found in *M. smegmatis*, including laccase, lignin peroxidase, manganese peroxidase, catalase peroxidase, oxide reductase, and super oxide dismutase. Something to highlight from this work is the low percentage of consumed cellulose, which makes *M. smegmatis* and ideal microorganism for lignocellulosic residues delignification and further biofuels production. Table 3 resumes previously cited research works.

Recently, algal biomass has increased its popularity owed to a higher growth rate, lower percentage of lignin, lipid content up to 80% of its dry weight, no use of land resources for cultivation, and the use of wastewater for algal production (Zhang *et al.* 2023a). High yields of 0.43 and 0.39 g of biooil/g of algae has been reported, making this feedstock a real option for biodiesel production (Kumar *et al*. 2013; Malik *et al*. 2022).

CONCLUSIONS

As has been shown in this review, the use of physical, chemical, and physicochemical pretreatments of lignocellulosic biomass, as a means to enhance biomass saccharification, presents drawbacks related with costs and environmental impacts. For example, novel physical methods such as ozonolysis, steam explosion, or the use of supercritical fluids, although they do not generate a great impact on the environment, this kind of processes require expensive equipment, which makes them unfeasible on a large scale. On the other hand, chemical methods such as the use of alkalis and acids, which are simple and cheap, have good yields but have large adverse impacts on the environment. Although biological methods are environmentally friendly and, in theory, require lower energy inputs, they can be strongly influenced by the kind of lignocellulosic biomass, enzyme or microorganism specificity, the presence or absence of mediators, and in addition, they generally consume longer process times in controlled conditions of temperature, pH, nutrients, and aeration. These requirements are related to complicated kinetics and sensitivity to environmental changes. Only by means of sophisticated and instrumented devices can the needed conditions be maintained, which at the end makes the application of biological methods a difficult task.

In that sense, in order to increase lignin degradation yields, which also permits a better activity of cellulolytic enzyme and, furthermore, a higher fermentable sugar production, hybrid pretreatment methods must be considered, combining biological, chemical, physical, and physicochemical treatments that yield higher fermentable sugars production, sugars that can be further used as economic substrates or carbon sources for the growth of microorganisms with the ability to produce biohydrogen, bioethanol, and bio-oil. Such approaches have the potential to both diminish production costs of biofuels and to make them a viable option in order to satisfy nowadays energetic demands.

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