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Fiber degradation and carbohydrate production by combined biological and chemical/physicochemical pretreatment methods of lignocellulosic biomass - a review

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Abstract

Sustainable biorefinery concepts based on lignocellulosic biomass are gaining worldwide research interest because of their inexpensiveness and abundance. The recalcitrance of lignocellulosic biomass poses a major hindrance to enhance biofuel production. Therefore, a pretreatment step is critical to prepare the substrates for the downstream process. Combining pretreatment steps help to lower the severity of the drawbacks of a single pretreatment step. This paper systematically reviews the combined biological and chemical/physicochemical pretreatment based on fiber degradation and sugar yield. An energy-efficient biological pretreatment method combined with a chemical pretreatment that accelerates the pretreatment times has been seen to be efficient for fiber degradation and sugar yields. However, fungal species, culture conditions, biomass type, the severity of chemical pretreatment and the order of sequential pretreatment influences the relative component contents and sugar yield. Even the same biomass from different sources undergoing similar pretreatment conditions could result in a varying amount of digestibility.

Keywords: Lignocellulosic biomass, combined pretreatment, fiber degradation, sugar yield

1. Introduction

The energy crisis is receiving worldwide attention and the current need is to fulfill the ever-growing energy demand sustainably. It is not only enough to produce energy, heat, and transport fuel but also to increase the security of the energy supply. Improving energy security is essential to transition out of conventional energy (Ošlaj and Muršec, 2010). The move towards renewable energy has to be done without diverting land use or food crops for the production of energy (Tomei and Helliwell, 2016). Lignocellulosic biomass (LCB) resources like energy crops, agriculture, and forest residues are abundant and are good renewable feedstocks for bioenergy production. It is assessed that the annually produced terrestrial biomass stores 3-4 times greater energy than the existing global energy demand (Guo et al., 2015). In the last decade, the debate of food and land use versus fuel has been raised with the assumption that a crop has a single utility. Many feedstocks have multiple uses including human food consumption, animal feed, industrial applications and generation of energy (Tomei and Helliwell, 2016). Generally, crop residues from cereals such as rice and wheat are mainly used as fodder and for manure applications. The surplus unutilized crop residues are openly burned in most of the developing countries which is a major cause of air pollution (Sukumaran et al., 2010). Therefore, the safe disposal of waste is another biggest challenge to humankind. Combining these challenges, it is possible to utilize the full potential of organic waste to produce energy and to reduce the dependence on fossil energy resources (Ošlaj and Muršec, 2010).

Biomass is a theoretically viable and economical source of renewable energy carrier for the production of bio-oil, biogas, biodiesel, and bioethanol using a wide range of technologies. Over the last decade, there have been numerous researches to produce biofuels from lignocellulosic feedstock (Valdivia et al., 2016). However, the physical and chemical structural rigidity and recalcitrance nature of lignocellulose has made it difficult and highly expensive to produce sugars from carbohydrates in lignocellulose (Mosier et al., 2005). The major components of lignocellulosic biomass are lignin, hemicelluloses, and cellulose. The substrates enabling biofuel production are sugars contained in cellulose and hemicelluloses but are protected by the resistant structure of lignin. Therefore, a pretreatment step is required before the downstream process to break the lignin seal and reduce the overall crystallinity of the biomass structure so that the surface area for the enzyme accessibility and microbial attack can be increased. The microbial breakdown of polymer chains of cellulose and hemicellulose will help to increase the rate of biomass degradation and help to convert the fermentable sugars into biofuel (Anwar et al., 2014). The pretreatment process has to be chosen based on the techno-economic feasibility of integrating into the downstream process with considerations of configurations and efficiency of downstream operations (Zheng et al., 2014). A mechanical pretreatment step such as a hammer mill will help to break the tubular structure and reduce the size, which will prevent floatation. It has been observed that mechanical pretreatment provides a minimal improvement in biogas production but the high energy demand of the process makes it an expensive addition to the AD process (Kratky and Jirout, 2011). Chemical pretreatment uses either acid, alkali, oxidants, or organo-solvents and each chemical uses a different mode of action to efficiently remove the lignin or hemicelluloses present in the biomass (Abraham et al., 2020). In comparison to physical and biological pretreatment methods, chemical methods have a better degradation effect and faster rate of degradation of the complex lignocellulosic structure. Although chemical pretreatment is the majorly investigated strategy, the disadvantages of this method are also significant. For example, acid pretreatment using sulfuric acid (H_2SO_4) and nitric acid (HNO_3) can increase the content of H_2S and N_2 in biogas, which results in the need for additional gas cleaning and thereby increasing the investment costs. In a study where several chemical and physicochemical pretreatment methods for biogas production from wheat straw were compared, the authors found none of them to be cost-effective as additional chemicals or high energy were required. Albeit NaOH pretreatment being proven as an efficient and significantly cheaper pretreatment method for biogas yield in AD, the probability of Na^+ ion inhibition for methanogenesis is higher. Besides, disposal of digestate containing Na^+ is difficult as it causes soil salinization (Zheng et al., 2014). A method to solubilize lignocellulosic components efficiently without the formation of inhibitors is the physicochemical method. This includes steam explosion, hydrothermal, and Ammonia Fiber Explosion (AFEX). However, this pretreatment step which exposes the lignocellulosic structure for hydrolysis based on the temperature and moisture content and which produces higher yield in the subsequent bioprocesses is an expensive method because of the high energy needed (Hernández-Beltrán et al., 2019). Biological pretreatment using fungal, microbial consortium and enzymes is an inexpensive and more sustainable strategy. While the advantages of biological pretreatment include substrate and reaction specificity, low energy requirements, and no generation of toxic compounds, the disadvantages are relatively low efficiency, a considerable loss of carbohydrates, and long residence periods (Zheng et al., 2014).

From the highlights and challenges of pretreatment methods discussed, it can be observed that although single pretreatment makes a significant contribution, no single method provides efficient results with its intrinsic limitations. Therefore, combined pretreatment strategies could lower the severity of the disadvantages and provide the desired result (Zheng et al., 2014). For example, the combination of microbial and chemical pretreatments is perceived to shorten the pretreatment times, reduce the strength of chemicals used and thereby the secondary pollution associated with it, and as a cost-effective strategy. While pretreatment by physical, chemical, and biological methods has been studied extensively, the combined pretreatment strategies are gradually being developed in recent years for their synergistic effect. Physical and chemical combined pretreatment is a more commonly used combined pretreatment method but the combination of biological and chemical pretreatment is yet to be well studied (Shirkavand et al., 2016). Therefore, the aim of this work is to present an updated review of combined microbial-chemical/physicochemical pretreatment strategies used for different LCBs based on fiber content degradation (Chapter 2) and sugar yield (Chapter 3), for final biofuels like bioethanol or biogas production.

2. Use of fiber content degradation analysis to evaluate the combined pretreatment effect

The major components of lignocellulosic biomass are cellulose, hemicellulose, and lignin. These form the highly ordered crystal structure of the plant cell wall, which causes heterogeneity, and the recalcitrant nature of the lignocellulosic biomass. The relative quantity of the three major components varies widely among the various biomasses (40–50% cellulose, 25–30% hemicellulose, 15–20% lignin) and amongst the same biomass depending on its cultivation and harvesting conditions. The minor compounds are usually proteins, starches, pectins, tannins, etc., and are called extractives (Adjalle et al., 2017; Chen et al., 2017). The structure of lignocellulose is such that cellulose microfibrils are integrated into the hemicelluloses matrix and covalently cross-linked with the heterogeneous lignin. The main fraction that causes recalcitrance of the lignocellulosic biomass is lignin, which is made up of non-linear phenolic polymer built with chemically diverse and poorly reactive linkages. Lignin is relatively hydrophobic and aromatic (Cesarino et al., 2012; Paudel et al., 2017). Klason lignin, also known as acid insoluble lignin (AIL) is the most abundant lignin content in most lignocellulosic biomass and is the insoluble residue portion after removing the ash by concentrated acid hydrolysis of the biomass. On the other hand, the acid soluble lignin (ASL) fraction that is soluble in 72% sulfuric acid is the reminder fraction. The sum of ASL and AIL is used to determine the total lignin content (Technical Committee ISO/TC, 2020). Hemicellulose is a heterogeneous polysaccharide and is non-covalently bonded (weakest bonded) to the surface of the cellulose fibrils and forms an amorphous matrix. It is thermo-chemically sensitive and includes arabinoxylan, glucomannan, glucuronoxylan, xylan, and xyloglucan. Xylan is the main component of hemicelluloses, which contains C5 sugars, C6 sugars, and sugar acids. Cellulose is the major fraction of lignocellulose and is made up of linear (1-4) β-D-glucan, which is a glucose polysaccharide. Albeit its large size, crystalline cellulose is hydrophilic. The intermolecular and intramolecular hydrogen bonds provide the strength to cellulose by forming a crystalline and amorphous structure (Paudel et al., 2017). In some papers reviewed in this chapter, xylan content is taken as a measurement of hemicellulose fraction while glucan content is considered as a measurement of cellulose fraction of biomass.

During a pretreatment process, changes occur in the microstructure, macrostructure, and chemical composition of lignocellulose. Lignin is broken down and removed, hemicelluloses are degraded and the crystalline structure of cellulose is changed (Paudel et al., 2017). A detailed characterization of the fiber content will help to determine the nature of lignocellulosic biomass, characterize the biodegradability properties of a pretreatment method, and to estimate the biofuel yield. Many such characterization methods for component analysis have been developed with specific applications and industries in mind. The most widely cited method for application in second-generation biofuels and chemicals is "Determination of structural polysaccharides and lignin in biomass" by Sluiter et al. (2008 and 2010) provided by National Renewable Energy Laboratory (NREL) (Karimi and Taherzadeh, 2016). NREL procedure is a two-stage method, where the first stage is a time-consuming process of removal of non-structural components using both water-soluble and ethanol-soluble extraction materials. The second stage involves using strong sulfuric acid to hydrolyze the polymeric carbohydrates to monomers and determination of the monomers using High-Pressure Liquid Chromatography (HPLC) (Mourtzinis et al., 2014). For forage and feed analysis in which digestible fiber is the most desired fraction, the Van Soest method (Soest and Wine, 1967) and the Association of Official and Analytical Chemists International (AOAC) standards are used. The AOAC standards cannot measure all non-digestible carbohydrates and therefore, the Van Soest method is preferred (Agblevor and Pereira, 2013; Theander et al., 1995). The Van Soest method is also known as the Neutral Detergent Fiber (NDF) method as it is based on extracting the soluble fraction quickly using a neutral detergent, followed by the extraction of the insoluble part, i.e., lignin using an acid detergent. The lignin is determined using the Klason method (Mourtzinis et al., 2014). According to Mourtzinis et al. (2014), the Van Soest method is less time-consuming and more cost-effective than the NREL method, whereas the reliability of the NREL procedure was better. This is because the Van Soest method underestimates the lignin content and overestimates cellulose due to the long hydrolysis during the acid detergent lignin (ADL) determination step. Van Soest method also overestimates the hemicelluloses content as compared to the NREL method because extractives not solubilized during the NDF step will be solubilized during the acid detergent fiber (ADF) step (Adjalle et al., 2017). For analysis of woody biomass in which cellulose is the most desired fraction, the Technical Association of Pulp and Paper Institute's (TAPPI) procedure is used (Agblevor and Pereira, 2013). Browning (1967) estimated lignin using nitro-benzene oxidation method and quantifying the yield of vanillin or vanillin plus syringaldehyde (Kirk and Obst, 1988). The other methods used for fiber determination by the articles discussed in this chapter are the Iiyama and Wallis (1988) (perchloric acid method), Kaar and Brink (1991), Updegraff (1969), and Wise et al. (1946). Whichever method is used, care should be taken for sampling, particle size, moisture content, and presence of debris as it can affect the results. Though these different methods of analysis lead to wide compositional variation reported in the literature for the same biomass (Karimi and Taherzadeh, 2016), it is effective to study the fiber degradation of the combined pretreated lignocellulose to that of untreated biomass for the 23 articles discussed in table 1.

Table 1 (to be inserted here)

2.1 Biological – Alkaline Pretreatment

Biological-alkaline combination has been the most commonly studied combined pretreatment for lignocellulosic biomass so far. Of the seven research articles discussed in table 1, six of them have studied biological pretreatment followed by alkaline pretreatment while only Si et al. (2019) have studied the opposite sequential treatment. Biological pretreatment is preferred as the first step as this helps in the delignification of lignocellulosic fibers, which leads to a reduced concentration of alkali needed for the pretreatment of the substrate. An advantage of alkali pretreatment is that many of the caustic salts used can be regenerated (Zhong et al., 2011). The most common alkali pretreatment process is using NaOH as it causes a delignification reaction and decreases cellulose crystallinity. This results in an increase in surface area and enhanced enzymatic hydrolysis (Zhao et al., 2008). Zhong et al. (2011) state that NaOH pretreatment needs to be carried out at elevated temperatures (around 100°C) to have a satisfactory lignin degradation rate and sugar yield. The combination with biological treatment can enhance delignification and help lower the temperature of alkaline treatment, thereby reducing heating costs. Yu et al. (2010a), Yang et al. (2013), and Alexandropoulou et al. (2017) studied white-rot fungal treatment combined with NaOH treatment at around 75-80°C. Although the fungal pretreatment helped to either lower the temperature or shorten the duration of alkaline pretreatment (Yu et al., 2010a), this combination also resulted in a higher loss of carbohydrates (Alexandropoulou et al., 2017; Yang et al., 2013; Yu et al., 2010a). Wang et al. (2013a) studied white-rot fungus, *Trametes velutina* in combination with chlorite pretreatment at 80°C. Fungal pretreatment degraded lignin and hemicellulose partially while subsequent chlorite pretreatment greatly enhanced delignification compared to hemicellulose degradation. Beyond a threshold level of lignin degradation, Wang et al. (2013a) found out that lignin did not have an effect on cellulose conversion as either lignin was no longer a hindrance to enzyme attack or extensive delignification caused the lignocellulosic pores to collapse and thereby reducing the available surface area for enzyme adsorption (Zhu et al., 2008). On the contrary, Fissore et al. (2010) used a brown-rot fungus, *Gloeophyllum trabeum*, in combination with NaOH treatment at high temperatures of 180°C. The brown-rot fungus, as is well-known, decayed carbohydrates extensively compared to lignin. The alkaline medium at high temperatures also favored peeling and hydrolysis reaction of the carbohydrates and thereby lower lignin degradation (Fissore et al., 2010). While complete delignification is not necessary, a higher amount of delignification is required for carbohydrates conversion than hemicellulose removal (Wang et al., 2013a). Use of bacteria in LCB pretreatment is gaining interest in the recent years as it can be rapidly grown and easier genetic manipulation is feasible as opposed to fungi. Dai et al. (2015) and Si et al. (2019) used bacteria in sequential pretreatment with milder alkaline pretreatment, which helped to shorten the overall pretreatment time of rice straw as compared to fungi. Dai et al. (2015) used a combination of NaOH and urea for chemical pretreatment as NaOH helps to break the inter- and intra- hydrogen bonds between cellulose molecules while urea acts as a hydrogen bond donor and receptor between solvent molecules, thereby preventing the reassociation of cellulose molecules and causing cellulose depolymerization. Though the combined bacterial-alkaline pretreatment did not enhance delignification greatly as compared to sole pretreatment, it significantly enhanced the saccharification of rice straw due to the increased content of cellulose and decreased content of hemicellulose in both Dai et al. (2015) and Si et al. (2019).

2.2 Biological – Acid Pretreatment

Dilute acid pretreatment is known best for solubilization of hemicellulose fraction (Martínez-Patiño et al., 2018) and this can be observed in all the six articles discussed in table 1 on biological-acid pretreatment. To reduce the severity of the acid pretreatment and the consequential inhibitory compounds formation, a combination with biological pretreatment is preferred. Martinez-Patiño et al. (2018) studied fungal pretreatment followed by dilute acid pretreatment and the converse sequential pretreatment for olive tree biomass. Though both the sequential pretreatment resulted in similar fiber degradation, the glucose and ethanol yield from fungal pretreatment followed by dilute acid pretreatment were much more remarkable. Wang et al. (2013a), Ishola et al. (2014), and Martin-Sampedro et al. (2017) also studied fungal pretreatment followed by dilute acid pretreatment. It can be observed in all three cases that while there was almost complete solubilization of hemicellulose, there was not substantial lignin removal in the combined pretreatment, resulting in cellulose being preserved. Similar results were obtained when Si et al. (2019) studied acid pretreatment in combination with bacterial pretreatment. In the study by Ishola et al. (2014) and Martinez-Patiño et al. (2018), it can be seen that percentages of cellulose and lignin are lower in the raw material (irrespective of the order of pretreatment steps) because the pretreatment methods removed at least most of the extractive fraction and thereby increasing the proportion of the rest of the components. The increase in lignin content in the study by Yan et al. (2017) was ascribed to the high temperatures at which the chemical pretreatment was conducted. SEM study showed that high temperatures caused lignin to expand and become mobile while the aqueous environment triggered the lignin molecules to coalesce and form droplets on the surface of rice straw. The bacteria Cupriavidus basilensis B-8 remarkably acted as a scavenger, was bound to active sites on lignin droplets, and used laccase to modify lignin morphology from droplets to creaks with flexible edges. The laccase uses H atom extraction mechanism to form reactive phenoxy radicals, which help to undergo further enzymatic or non-enzymatic reactions. The authors believe that this modification of the lignin and laccase mechanism changes the hydrophobicity and subsequently the polarity on the surface, leading to accessibility to cellulose and reduced nonspecific binding of the cellulase enzyme (Palonen and Viikari, 2004; Yan et al., 2017). By and large, the combination of biological-dilute acid pretreatment did not yield higher saccharification as compared to sole pretreatment.

2.3 Biological - Oxidative Pretreatment

With the understanding of the action of white-rot fungi in the biodegradation process, it helps to overcome the limitations in traditional bio-treatment like long residence time and ineffective delignification. The lignocellulose biodegradation mechanism by white-rot fungi involves a Fenton-based oxidation reaction. Therefore, mimicking the Fenton reaction-induced decay using oxidizing reagent like Hydrogen peroxide (H_2O_2), will help enhance delignification without generating inhibitory by-products and unreacted chemical residues (Eastwood et al., 2011; Paudel et al., 2017). Six research articles have been compared for fiber degradation by biological-oxidative pretreatment of which four studies used fungi and two studies used bacteria for biological pretreatment. In the study by Yu et al. (2009), the oxidative treatment (H_2O_2) followed by fungal pretreatment (*Pleurotus ostreatus*) was very effective as the delignification rate doubled and the carbohydrates losses reduced compared to that of sole biological treatment. The fungal pretreatment time reduced from 60 days for sole pretreatment to 18 days for combined pretreatment as structural changes during the oxidative pretreatment allowed for rapid penetration of fungal hyphae into the feedstock. *T.versicolor* showed the highest lignin-degrading ability amongst *Ganoderma lucidum* and *Echinodontium taxodii* in the sole fungal pretreatment studied by Yu et al. (2010b) but it also resulted in high cellulose loss, which subsequently resulted in low sugar yield. Therefore, the authors preferred *E. taxodii* to be used for combined pretreatment as cellulose loss was significantly lower. Yu et al. (2010b) and Xie et al. (2017) carried out oxidative pretreatment in alkaline conditions because H_2O_2 produces hydroxyl and superoxide radicals which are very reactive at high pH (Paudel et al., 2017). The combined white-rot fungi-alkaline/oxidative pretreatment resulted in significant enhancement of delignification compared to individual pretreatment, which helped to decrease the unproductive adsorption of cellulase in the subsequent enzymatic hydrolysis step. Wang et al. (2013b) studied both a brown-rot fungus (*Fomitopsis palustris*) and a white-rot fungus (*Trametes orientalis*) in combination with $FeCl_3$. It is very evident from the results that compared to the white-rot fungus, brown-rot fungus selectively degrades carbohydrates in lignocellulosic materials without removing the surrounding lignin. This is possible because the lignin is solubilized at temperatures (operating temperature of 180°C) higher than the temperature range of lignin phase transition. This causes the lignin molecules to coalesce into large bodies and migrate out, which then get redeposited on the cell walls causing an increase in the amount of lignin (Donohoe et al., 2008; Liu et al., 2009). The tremendous hemicelluloses degradation was attributed to the ability of Fe^{3+} ion in $FeCl_3$ to act as good electron acceptor capability and synchronize with the oxygen donor atoms of carbohydrates to hydrolyze hemicelluloses (Yu et al., 2011). This masked the synergy of the combined biological-oxidative pretreatment in hemicelluloses degradation (Wang et al., 2013b). Zhang et al. (2018) and Si et al. (2019) conducted oxidative pretreatment followed by bacterial pretreatment on rice straw. Ligninolytic bacteria such as *Cupriavidus basilensis* and *Pandoraea sp.* showed the outstanding potential to selectively remove lignin while the saccharolytic bacteria *Acinetobacter sp.* easily utilized amorphous hemicelluloses (Si et al., 2019; Zhang et al., 2018). In the study by Zhang et al. (2018), the free radicals produced by the Fenton reaction resulted in the oxidation of cellulosic substrates, which lead to rapid degradation of cellulose. This created more sites for the cellulases to bind and increased the hydrolysis rate. The surface oxidation of cellulose by Fenton reaction along with selective removal of lignin and partial hydrolysis of hemicellulose by the ligninolytic bacteria provided new insights into effective pretreatment strategies of lignocellulosic biomass (Zhang et al., 2018).

2.4 Biological – Organosolv Pretreatment

The organosolv process utilizes compounds like methanol, ethanol, butanol, n-butylamine, acetone, ethylene glycol, etc. to break the internal lignin and hemicellulose bonds and separate them. However, all the five articles discussed on biological-organosolv combined pretreatment (Table 1) used ethanol for the pretreatment. Ethanol is preferred in an organosolv process as it is comparatively less toxic and due to its ease of recovery, thereby reducing the recurring costs of chemicals. In addition, all of them studied fungal pretreatment followed by the organosolv process (ethanolysis) as fungal pretreatment helps to improve solvent accessibility, and thereby decreasing the severity of the organosolv process required. The 50-60% by volume ethanol-water solutions under high pressure of about 250 – 350 psi and high temperatures of about 180-200 ^{o}C used in organosolv treatment effectively enhances delignification and produces easily hydrolyzable substrates. However, removal of the solvents is necessary because solvents could be inhibitory to the growth of organisms, enzymatic hydrolysis process, or the fermentation step. It has also been reported that organosolv pretreatment can better hydrolyze biomass with rather low lignin content. Therefore, a fungal pretreatment step before the organosolv process can help with reducing the lignin content and further increase solvent accessibility to biomass (Itoh et al., 2003; Muñoz et al., 2007). Results from Munoz et al. (2007), Kandhola et al. (2017), and Saad et al. (2008) who used white-rot fungi to treat wood showed that the majority of lignin degradation was due to biodegradation, which facilitated the action of chemicals further. Fissore et al. (2010) and Monrroy et al. (2010) both studied the effect of brown-rot fungi *Gloeophyllum trabeum* with organosolv process on *Pinus radiata* wood chips. The severity of the organosolv process based on the time and temperature of the process is described by the H factor. There were only slight severity differences in the organosolv process of these two studies; still, the fiber degradation obtained was quite varied. This shows that the same biomass from different sources undergoing similar pretreatment conditions could result in a varying amount of digestibility. The differences in the relative component contents of pretreated biomass could also arise as a result of different fungal species, culture conditions, culture time, and biomass (Yang et al., 2013). In the five cases discussed biological-organosolv combined pretreatment, it can be seen that fungal pretreatment facilitated the removal of lignin and hemicelluloses. This reduced the severity of the subsequent organosolv process required and produced a greater synergetic effect on cellulose digestibility by improving the solvent accessibility during the organosolv process. Another advantage of the organosolv process is that it presents low residual lignin and high glucan retention as can be observed from the five research articles discussed in table 1. This is because ethanol and the solubilized lignin act as scavengers for the free radicals formed during the organosolv cooking process, thereby reducing the extent of lignin condensation (Fissore et al., 2010).

2.5 Biological – LHW/ HWE/ Autohydrolysis Pretreatment

To avoid the use of chemicals for an environmentally friendly process, liquid hot water (LHW) pretreatment is preferred. This method is conducted at high temperatures of 120-260°C at which water and acetyl groups in hemicelluloses act as acids and catalyze hemicellulose hydrolysis. These severe pretreatment conditions can result in organic acids accumulation and therefore create an acidic condition (Weil et al., 1998). To balance the acidic environment and therefore degradation of fermentable sugars, the severity of LHW pretreatment can be reduced by combining with biological pretreatment (Wan and Li, 2011). Wang et al. (2012) observed that while acid-soluble lignin (ASL) decreased with increasing temperature of LHW conditions, the acid-insoluble lignin (AIL) increased. The increase of AIL is attributed to condensation and precipitation of the lignin due to elevated temperatures whereas reduction in ASL is attributed to hot water liberating acids and thereby breaking ether linkages in biomass. Though LHW is credited for the high hemicelluloses solubilization, initial pretreatment with white-rot fungus is attributed for lowering the temperature needed to conduct LHW (Wang et al., 2012). Wan and Li (2011) observed that there was virtually no lignin removed by liquid hot water (LHW) pretreatment as observed generally in hydrothermal or thermochemical pretreatment. This is attributed to the condensation and precipitation of dissolved lignin and carbohydrate oligomers. However, Wan and Li (2011), suggest that LHW / hot water extraction (HWE) facilitates the fungal pretreatment in the subsequent step by initially reducing the recalcitrant of biomass. The difference between LHW and HWE as used by Wan and Li (2011) is that LHW is conducted at high temperature (170°C) and pressure (110 psi) in a sealed reactor while HWE is a method where the biomass is extracted with hot water at 85°C. The mechanism of LHW differs from HWE, in the sense that HWE helps to extract some hydrophilic compounds and lipophilic extractives that would impose a protective barrier to fungal degradation while LHW facilitates specifically lignin degradation for further fungal degradation. To illustrate the action of HWE, Wan and Li (2011) detected that water extractives in wheat straw partially contributed to recalcitrance to *C. subvermispora*, but an HWE pretreatment prior to fungal pretreatment partially removed water-soluble components of the biomass and significantly improved the sugar yield. It was also observed that different biomass reacts differently to both LHW and HWE depending on the varying amount of chemical bound components that cause recalcitrance in the crop residues (Wan and Li, 2011). Martin-Sampedro et al. (2015) combined endophytic/white-rot fungi before and after a mild autohydrolysis as sequential pretreatment steps. A fungal pretreatment followed by mild alkaline pretreatment was more effective as it resulted in increased digestibility without masking the effect of fungal pretreatment. Irrespective of the order of pretreatment, the combined pretreatment enhanced the decrease in Klason lignin and hemicellulose mainly in the form of xylose. As the first study on endophytic fungi in combined pretreatment, it is interesting to see the results of Martin-Sampedro et al. (2015) where the endophytic fungi performed comparative or enhanced lignin degradation as the white-rot studied for the same case. Even though the endophytic fungi *Pringsheimia smilacis* produced the highest degradation of lignin irrespective of the order of combined pretreatment steps, it did not produce higher saccharification rates. This study proved that endophytic fungi have the potential as primary degraders of lignocellulosic substrates and could be interesting to study further (Martín-Sampedro et al., 2015).

2.6 Biological – Steam Explosion Pretreatment

Steam explosion pairs physical tearing and chemical high-temperature cooking of the biomass, which helps to degrade hemicelluloses and lignin while softening the cellulose (Li and Chen, 2014). To reduce the energy intensity of the steam explosion, it is often combined with biological pretreatment because of its easy integration to existing thermo-chemically treated biomass-to-ethanol processes (Keller et al., 2003). Vaidya and Singh (2012) compared the effect of brown-rot fungi and white-rot fungi on steam-exploded wood (SEW). SEW substrate was observed to be less recalcitrant than the raw biomass as the steam explosion caused some lignin degradation. There was more degradation in cellulose and hemicellulose of brown-rot treated wood samples which caused higher weight losses in them compared to white-rot treated wood samples. However, a steam explosion followed by white-rot fungus pretreatment was observed to work synergistically to enhance enzymatic digestion more than the brown-rot fungus. Sawada et al. (1995) and Asada et al. (2011) conducted white-rot fungal pretreatment followed by steam explosion at around 215°C for 5-6.5 minutes. Sawada observed that *P.chrysosporium* rapidly degraded 42% of lignin and gradually degraded holocellulose up to 17% of beech wood meal during an incubation time of 28 days. Later, the lignin degradation rate slowed down and the holocellulose degradation rapidly increased. Even though there was increase in the area of contact between holocellulose and the enzyme, it was not sufficient for enzymatic saccharification of the wood-meal. Therefore, a consecutive treatment with steam explosion was necessary to enhance saccharification. Above 210°C steam temperature, Sawada et al. (1995) observed that Klason lignin underwent condensation reactions with water-soluble material and methanol soluble lignin. Asada et al. (2011) also noticed that at higher temperatures of 214°C, recondensation of lignin occurs while the cellulose amount remained the same, as thermal degradation of cellulose is about 240°C. Therefore, at around 214°C or 20 atm pressure, there appears to be low Klason lignin, which is desirable for the enzymatic or microbial conversion into sugars as Klason lignin decreases the susceptibility of enzyme and cellulose. On the other hand, Taniguchi et al. (2010) and Li and Chen (2014) conducted white-rot fungal pretreatment on steam-exploded crop residues for 1 minute with lower severity. Taniguchi et al. (2010) confirmed the structural changes in rice straw during the sole steam explosion and steam explosion- *P. ostreatus* combined pretreatment with SEM images. It was observed that the steam explosion solely did not effectively change the cellulose contents of lignocellulose while an increase in Klason lignin was observed due to partial condensation with other components. SEM micrographs helped to understand the impact of steam explosion and biological pretreatment on rice straw. The steam explosion only caused a partial cracking of the surface and slight destruction of the structure, while *P.ostreatus* hyphae growth on the surface and their invasion into the structural networks loosened the fibers and increased the surface area for enzymatic hydrolysis (Taniguchi et al., 2010). Li and Chen (2014) concluded similarly to Taniguchi et al. (2010) from results of pore size distribution, XRD analysis, and chemical composition of corn stalk by studying the effect of steam explosion, fungal treatment, and combined pretreatment strategy, which showed that steam explosion destroyed the rigid structure of the biomass and facilitated fungi penetration. *P. baumii,* being a white-rot fungus selectively degrades lignin and enhances the effect of steam explosion. Therefore, from the five research articles discussed in table 1, it can be concluded that steam explosion is only advantageous because it does not degrade cellulose even at 30 atmospheric pressure and 235°C. Nevertheless, steam explosion needs another treatment in combination, preferably biological treatment with white-rot fungi to offset the energy costs, to efficiently degrade lignin and increase the susceptibility of the biomass to enzymatic hydrolysis.

Therefore, it can be observed from table 1 that biological-alkaline and biological-organosolv combined pretreatment strategies helped to achieve higher fiber degradation. While pretreatment is the most important step in lignocellulosic biomass processing, an efficient enzymatic hydrolysis process is required to obtain an optimum yield of reducing sugar, which can then be used for various applications. The enzymatic hydrolysis process can be used to evaluate the effect of pretreatment more efficiently and therefore, the approach, their effect on different LCBs, and their pros and cons are discussed in Chapter 3.

3. Evaluation of the combined pretreatment based on sugar yield

Biofuels are produced through either chemical reactions, bioconversion, or heat that help to break down the starches, sugars, and other molecules present in lignocellulosic biomass. Due to the association with complex polymers and crystalline state, cellulose is the key carbohydrate that needs to be hydrolyzed to release the hexose and pentose sugars it contains. Generally, enzymes or acids are used to catalyze the hydrolysis reaction. Enzymatic hydrolysis is better preferred due to the high specificity that can be achieved under milder conditions (pH around 4.8 and temperatures around 45 - 50°C). Moreover, enzymatic hydrolysis produces higher yields of glucose without introducing corrosion problems, which is favorable for subsequent processes. Three main steps occur during the enzymatic hydrolysis of cellulose: adsorption of cellulase enzymes to the surface of the cellulose, hydrolysis of cellulose to glucose, and desorption of cellulases. Cellulases consist of endoglucanases, exoglucanases, and β-glucosidase. The β-glucosidase activity is what helps to convert cellobiose and short-chain oligosaccharides into glucose. However, the commercially available cellulase enzymes normally show low β-glucosidase activity, causing incomplete cellobiose hydrolysis. Consequently, extra β-glucosidase enzymes are added to the hydrolysis mixture. Since the cellulase enzymes have to penetrate the cellulose structure for adsorption at the first step, pretreatment is necessary to remove hemicelluloses and lignin barriers and break the crystalline structure of cellulose. This would help to enhance the susceptibility to enzymatic hydrolysis (Chen, 2015; Gupta et al., 2016; Mesa et al., 2010; Soccol et al., 2011). Therefore, the effectiveness of the pretreatment method could be analyzed by estimating the reducing sugar yield. The two widely used methods for determining reducing sugars are namely, the Nelson-Somogyi (NS) (used in 5 articles discussed in Table 2) and 3,5-dinitrosalicylic acid (DNS) assays (used in 13 articles discussed in Table 2). From Breuil and Saddler (1985) and Gusakov et al. (2011), it could be understood that the DNS method, although a very convenient method, overestimates the activity of enzymes and is susceptible to interference by various substances. On the other hand, though the Nelson and Somogyi copper method is more reliable and sensitive, it is not widely used because laboratories are reluctant to use the more toxic NS reagent which is also more sensitive to disturbing factors than DNS (Bailey et al., 1992). Another method (used in 2 articles discussed in Table 2) for the determination of total sugars is the phenol-sulfuric acid method (DuBois et al., 1956), which although easier to use than many other available methods, poses multiple health hazards. The results of this method are presented as glucose-equivalent concentrations and are not accurate for complex carbohydrates (Albalasmeh et al., 2013). These colorimetric methods can only be used for quantifying total reducing sugars (TRS) but not for the pentoses and hexoses separately. Instruments like high-performance anion-exchange chromatography (HPAEC) (used in 4 combined pretreatment studies discussed in Table 2) and high-performance liquid chromatography (HPLC) (used in 16 combined pretreatment studies discussed in Table 2) have been increasingly used for both quantitative and qualitative sugar analysis. Although they require high cost for analysis, they are regarded as the best methods (Chi et al., 2013).

The aim of the articles discussed in table 2 was to maximize the enzymatic digestibility by studying these combined pretreatment strategies. After the hydrolysis of the pretreated substrates, the substrates were either digested to produce biogas (as in the case of Zhao et al. (2017)) or fermented to produce ethanol. The sugar yields obtained from 35 articles using different combined biological and chemical/ physicochemical pretreated biomass are listed in Table 2. The fold increase or decrease in sugar content after combined pretreatment as compared to sole pretreatment is calculated according to the formula given in Eq.1.

$$\text{Fold increase/ decrease} = \frac{\text{Sugar yield from combined pretreatment}}{\text{Sugar yield from sole pretreatment}} \qquad \text{(Eq. 1)}$$

Table 2 (to be inserted here)

Biological- alkaline pretreatment is seen to be the most studied biological – chemical combined pretreatment strategies. In table 2, ten research articles are discussed for biological-alkaline combination, followed by eight and seven scientific publications for biological-acid and biological-oxidative combined pretreatment respectively. Five articles on the biological-organosolv process are also discussed in table 2. For biological-physicochemical combined pretreatment strategies, six papers on biological-steam explosion and three papers on biological-LHW/HWE are discussed.

3.1 Biological – Alkaline Pretreatment

In most cases of biological-alkaline combined pretreatment, fungal pretreatment was conducted before alkali pretreatment because it has been reported to enhance cellulose digestibility and also reduce the production of inhibitors that are toxic for subsequent fermentation (Salvachúa et al., 2011). Of all biological-chemical combined pretreatment studied, many of the biological-alkaline pretreatment studies produced lower reducing sugar yield compared to the single pretreatment step. Even though Hatakka (1983), Fissore et al. (2010) and Salvachúa et al. (2011) studied fungal pretreatment followed by alkali pretreatment similar to that of Zhong et al. (2011), Yang et al. (2013), Wang et al. (2013a), Yu et al. (2010a) and Dai et al. (2015) their results are not in correspondence. Indeed Hatakka (1983), and Fissore et al. (2010) conducted alkaline delignification at much higher temperatures (115°C and 180°C respectively) and yet obtained lower reducing sugar yield from combined pretreatment, unlike the rest who conducted alkaline pretreatment in the range of 25°C - 80°C. This was presumed to be because of a lower degree of polymerization of cellulose chains in fungal treated wood in the case of Fissore et al. (2010) which resulted in higher amounts of carbohydrates solubilized than lignin and therefore, the effect of strong alkali treatment being masked off by the effect of fungi. The other plausible reason for the low reducing sugar yield in the study by Hatakka (1983) could be the low efficiency of the cellulase enzyme used in the hydrolysis step. Therefore, the saccharification efficiency is dependent on both the pretreatment and the enzyme efficiency. Generally, alkali treatment helps in the removal of the hydrophobic restriction of lignin and alters the lignin-hemicellulose network while leaving the high crystalline cellulose unchanged (Si et al., 2019). To enhance the saccharification, Si et al. (2019) combined alkali pretreatment with bacteria. Saccharolytic bacterium *Acinetobacter sp.* B-2 significantly enhanced the sugar yield in the combined pretreatment with alkali. In another study by Zhao et al. (2018) where pretreatment of maize straw was carried out with *T. harzianum,* with the enzyme of *T. harzianum* and pretreatment with NaOH followed by *T. harzianum,* the reducing sugar concentrations were 1.20 mg/ mL, 2.20 mg/ mL and 0.19 mg/ mL. The decrease in the reducing sugar yield in the combined pretreatment method was presumed to be due to the washing away of soluble material dissolved during alkali treatment and the greater ability of the fungi to consume than to produce reducing sugar (Zhao et al., 2018). Hence, pretreatment with enzymes rather than microorganisms themselves was more effective.

3.2 Biological – Acid Pretreatment

The dilute acid pretreatment is primarily a hydrolytic process that causes solubilization of hemicellulosic sugar and therefore, a fungal pretreatment step is required first to degrade the lignin in the biomass and make cellulase enzyme accessibility to cellulose easier (Shirkavand et al., 2016). Many studies on only acid pretreatment have reported an initial increase and later a drop in TRS yield with an increase in acid concentration, reaction time, and temperature. This is presumed to be due to the formation of inhibitory products. Therefore, mild acidic conditions are preferred to obtain a high yield of TRS (Kootstra et al., 2009; Rajan and Carrier, 2014; Timung et al., 2016). To make mild acidic pretreatment more effective, combining with fungal pretreatment is seen to improve the sugar yields from 1.09-1.74 fold (Gui et al., 2013; Ma et al., 2010; Wang et al., 2013a). Martínez-Patiño et al. (2018) compared sequential chemical and biological pretreatment and vice versa for enzymatic hydrolysis of olive tree biomass. The best combination to significantly increase the glucose concentration was biological pretreatment followed by mild acid pretreatment. Moreover, acid treatment as a first step could produce some inhibitors which may hinder the fungal pretreatment efficiency (Martínez-Patiño et al., 2018). Ramirez et al. (2014) performed fungal pretreatment followed by acid hydrolysis and enzymatic hydrolysis of corn leaf and obtained a 9% increase in the reducing sugar yield as compared to sole pretreatment. This increase could be attributed to the multiple-step hydrolysis process, which helped to obtain higher saccharification of the pretreated biomass while making this process more complex to carry out. In the study of Martín-Sampedro et al. (2017), although the endophytic fungi could enhance sugar yield, there was no significant increase in sugar yield obtained during combined pretreatment. This was attributed to the fungal effectiveness hindered by the high water-soluble extractives content of olive tree pruning. The authors concluded that better pretreatment strategies need to be explored to commercially valorize olive tree pruning biomass. Yan et al. (2017) and Si et al. (2019) observed the complementary effect of acid hydrolysis which mainly deconstructs the cellulose and hemicelluloses along with ligninolytic bacterium, improved the digestibility significantly. Their results served new insights into bacteria-acid synergy for the pretreatment of lignocellulosic biomass. Its potential is interesting to explore further as the use of bacteria reduces the pretreatment time and the associated costs (Si et al., 2019; Yan et al., 2017).

3.3 Biological - Oxidative Pretreatment

The combination of powerful oxidant (H_2O_2) followed by white-rot fungi (*Pleurotus ostreatus*) to pretreat rice hull by Yu et al. (2009) showed enhanced net yields of sugar, while effectively reducing the pretreatment time from 60 days for sole fungal pretreatment to 18 days for combined pretreatment. The reduction in the carbohydrates' loss was also minimal as can be seen from table 1. The structural changes observed from SEM results are mainly attributed to easy penetration of fungal hyphae into the rice hull structure chemically degraded by H_2O_2 first and thereby increased production of sugar almost 6 times than that of sole fungal pretreatment (Yu et al., 2009). In the study of white-rot/ brown-rot fungus followed by mild oxidizing agent ($FeCl_3$) treatment of poplar wood (Wang et al., 2013b), an increase in temperature during oxidant treatment led to a further increase in sugar yields. From table 1, it could be observed that there is no delignification due to both fungal or oxidant treatment while a higher hemicelluloses degradation was observed in the combined treatment. Yet, the synergy of combined treatment increased the internal surface area and porosity, leading to a decrease in the unproductive binding of the enzyme with lignin. Wang et al. (2013b) presume that an alteration in the structure of lignin such as a change in the content of hydrophilic phenolic hydroxyl groups could have led to a reduction in the enzyme's irreversible adsorption. These factors thereby increased the enzyme accessibility to cellulose (Wang et al., 2013b). To decrease the power and energy demands during the oxidative process, Yu et al. (2010b) and Xie et al. (2017) used oxidative pretreatment under mild alkaline conditions but this did not enhance the enzymatic hydrolysis process. So, by using white-rot fungal pretreatment along with alkaline-oxidative pretreatment, a higher sugar yield at lower enzyme concentration was achieved, thereby reducing the cost of an enzymatic hydrolysis step (Yu et al., 2010b). From the cellulase adsorption study, Yu et al. (2010b) concluded that the increased yield of reducing sugar was obtained due to a decrease in unproductive adsorption during biological pretreatment. Another important conclusion (based on the results not shown here) by Yu et al. (2010b) was that even though, *T.versicolor* showed the highest lignin-degrading ability, it also produced high cellulose loss which ensued in lower reducing sugar yields. This highlights the need to minimize cellulose loss. An efficient way to specifically target the lignin molecule is by ozonation. Ozone is highly reactive with compounds containing double bonds and high electron densities such as lignin (García-Cubero et al., 2009). These reactions follow the Criegee mechanism and no by-products are formed during the degradation process (Mulakhudair et al., 2017). The study by Mulakhudair et al. (2017) showed that ozonation for 24 hours reduced the biological pretreatment time by 50% but more importantly, a substantial increase in microbial biomass. A drastic increase in glucose concentration of 323% was observed when the ozonation time was increased from 2 h to 24 h. By using microbubble-mediated ozonolysis, there was a significant improvement in dosage efficiency due to the high surface area to volume ratio. Nonetheless, the high cost of the ozonation process makes it an expensive pretreatment method. To lower the cost of the pretreatment process, Zhang et al. (2018) designed a biomimetic system. In nature, fungi and bacteria are in a symbiotic relationship to utilize lignocellulose. The fungi modify the recalcitrant cell wall barrier using an oxidative step and release small molecular compounds which are further degraded by bacteria (Alper and Stephanopoulos, 2009). Therefore, Zhang et al. (2018) utilized low-cost Fenton catalyst (Fe^{3+} and H_2O_2) to stimulate fungal invasion of plant tissue, combined with *Cupriavidus basilensis* B-8 that helped enhance the enzymatic hydrolysis process. On the other hand, the sequential treatment with bacteria followed by the Fenton catalysts had a lower reducing sugar yield (Zhang et al., 2018). Si et al. (2019) also studied the combination of metal salt $FeCl_3$ with various saccharolytic and ligninolytic bacteria. Though there was a significant increase in the sugar yield as compared to that of sole $FeCl_3$ treatment, the reducing sugar yield was still lower due to negligible change in the chemical composition of rice straw (Si et al., 2019).

3.4 Biological – Organosolv Pretreatment

Wood being one of the most recalcitrant biomasses needs efficient pretreatment methods to be developed to improve its saccharification. Combined pretreatment using fungi followed by the organosolv process is an environmentally benign treatment for wood (Baba et al., 2011). Baba et al. (2011), Fissore et al. (2010), Itoh et al. (2003), Kandhola et al. (2017), and Muñoz et al. (2007) have all reported having an increase in the sugar yield by both brown-rot and white-rot fungi when combined with ethanolysis.

3.5 Biological – LHW/ HWE/ Autohydrolysis Pretreatment

It has been established by many researchers (Liu, 2010; Mosier et al., 2005; Zeng et al., 2007) that at elevated temperature and pressure, LHW pretreatment is comparatively an environment-friendly pretreatment as it has no sludge generation and limited corrosion problems. At temperatures around 200°C, the water and acetyl groups inside hemicelluloses act as acids that catalyze the hemicellulose hydrolysis to mainly xylose. The synergy of biological-LHW combined pretreatment is promising according to the results of Wang et al. (2012), which helps to lower the severity of LHW pretreatment while enhancing biological pretreatment efficiency. The high glucose yield obtained when LHW was carried out at 200°C was attributed to the hemicellulose loss, which facilitates the enzymatic hydrolysis of poplar wood. However, the researchers saw a decrease in the ratio between glucose yield of combined pretreatment and that of sole LHW as the temperature of LHW was increased from 140 to 200°C. This shows that the LHW treatment has higher efficiency at a higher temperature in both combined or sole pretreatment (i.e. 200°C), but the combination with fungal pretreatment triggered a better improvement of the yield at low temperature (Wang et al., 2012). Besides, the more severe the pretreatment conditions are, the more effective is the LHW pretreatment but it also results in the accumulation of inhibitory compounds like hydroxymethylfurfural (HMF), furfural, formic acid, levulinic acid (Weil et al., 1998). Therefore, it is suggested to carry out LHW at less severe conditions and then follow with other pretreatment methods. Wan and Li (2011) studied LHW/ HWE along with *Ceriporiopsis subvermispora* for different biomasses such as soybean, corn stover, and wheat straw. The synergistic effect of the combined LHW and fungal pretreatment process was significant for soybean straw whereas not so much for corn stover. The glucose yield obtained in the combined study was higher for soybean and not for corn stover when compared to sole fungal pretreatment. In the combined hot water extraction (HWE) and fungal pretreatment, there was an almost 2-fold increase in the glucose yield compared to untreated/ fungal-/ HWE- pretreated wheat straw, while soybean showed no increase in yield in the combined pretreatment compared to individual pretreatment step. On the other hand, combined pretreated corn stover showed a drastic increase in glucose yield as compared to HWE pretreated biomass but a slight decrease compared to fungal pretreated biomass. Thus, their research indicated how different biomasses show different pretreatment efficiency to the same combined pretreatment method (Wan and Li, 2011). Martin-Sampedro et al. (2015) used highly specific endophytic fungi with a mild autohydrolysis process to enhance saccharification of *Eucalyptus globulus* wood. The endophytic fungi produced higher saccharification than white-rot fungi, while the mild autohydrolysis helped to boost the fungal effect. It was also established from the results that the highest lignin-degrading fungi (*P. smilacis*) did not produce the greatest saccharification, indicating that the extent of lignin removal is not always correlated with the enhancement of saccharification yields (Martín-Sampedro et al., 2015).

3.6 Biological – Steam Explosion Pretreatment

All studies done so far on biological-steam explosion combined pretreatment produced a significant increase in the net sugar yields (Asada et al., 2011; Balan et al., 2008; Li and Chen, 2014b; Sawada et al., 1995; Taniguchi et al., 2010b; Vaidya and Singh, 2012). Sawada et al. (1995) observed that a sole fungal treatment was not sufficient to increase the enzymatic saccharification of beech wood even though a large amount of lignin had degraded paving the way for increasing contact between enzyme and holocellulose. Consecutive treatment with steam explosion helped to enhance the saccharification. When either steaming time or steam temperature was increased with the other constant, the saccharification increased up to its maximum (82% at a steam temperature of 215°C and steaming time of 6.5 mins) and then decreased with further increase. High steam temperature or longer steaming time caused depolymerized lignin to combine with the holocellulose which in turn led to holocellulose being unsusceptible to the enzyme (Sawada et al., 1995). Asada et al. (2011) carried out a steam explosion on spent shiitake mushroom media (*Lentinula edodes* mushroom grown on a media containing corn and bran for four months). After the harvest of the fruiting bodies, steam explosion pretreatment proved useful for the effective utilization of the spent medium for biofuel production (Asada et al., 2011). Balan et al. (2008) also studied spent oyster mushroom rice straw media to utilize as a potential substrate for biofuel production. The fungal pretreatment helped to reduce the severity of the subsequent AFEX treatment by improving accessibility to chemicals and enzymes and lead to a 15% increase in glucan (Balan et al., 2008). The opposite sequence of pretreatment (steam explosion followed by fungal pretreatment) was also observed to be effective for the conversion of biomass into sugars. Li and Chen (2014), Taniguchi et al. (2010), and Vaidya and Singh (2012) ascertained the effectiveness to the lignin-carbohydrate complex of biomass being destroyed by the steam explosion which further facilitated the fungal treatment.

From all the combined pretreatment methods discussed in this chapter, we can conclude that the order of biological and chemical methods in successive pretreatments should be chosen based on the mode of action of chemical/physicochemical pretreatment. Biological pretreatment combined with oxidative / ethanolysis seems to be the most effective biological-chemical combined pretreatment while mild autohydrolysis followed by endophytic fungi seems to be the most effective biological-physicochemical combined pretreatment, based on the fold increase of sugar yield compared to a sole pretreatment strategy. The hydrolysis rate depends on the ratio of total enzyme ratio to the amount of substrate added. For the quantification of the pretreatment efficiencies, most of the studies used cellulase loading of more than 15 FPU/g as high enzyme doses are required to release sugars from naturally recalcitrant biomasses (Yang et al., 2011). Hydrolysis of lignocellulosic varies with cellulases absorption and efficacy, hemicelluloses and lignin removal, and accessible surface area (Karimi and Taherzadeh, 2016). Therefore, each research group uses a different concentration of enzymes and different residence times to alter the rate of biomass deconstruction into fermentable sugars. According to Breuil and Saddler (1985), the enzyme concentration does not proportionally affect the reducing sugar values obtained from the hydrolysis step. Nevertheless, pretreatment could alter the structure and composition differently for the same biomass from different regions, which could result in different reducing sugar yields. It is noteworthy that the composition of each lignocellulosic biomass could also vary with geographical location and seasons. These non-standardized conditions make it difficult for direct comparison of the sugar yields from different combined pretreatment strategies. In the future, improved analytical methods to determine the enzyme-substrate interaction could help to better optimize the hydrolysis step for each biomass specifically.

4. Conclusion

The correlation between the biomass properties and its degradability remains unclear, even though many researchers have evaluated the effect of pretreatment. Based on the biomass and the downstream process, the appropriate pretreatment steps need to be chosen. It is also important to establish the order of the pretreatment in the combined studies, especially in biological-chemical/physicochemical methodologies by ascertaining the mechanism of action of each pretreatment method. Nonetheless, it is necessary to determine the environmental impact, cost efficiency, and energy balance of these combined processes to scale the process.

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Table captions

Table 1

Fiber content degradation in different biological-chemical/physicochemical pretreatment strategies

Table 2

Comparison of sugar yield of different microbial-chemical/physicochemical pretreatment strategies

Table 1: Fiber content degradation in different biological-chemical/physicochemical pretreatment strategies

Substrate	1st step	2nd step	Analytical technique	Lignin degradation (%)	Hemicellulose degradation (%)	Cellulose degradation (%)	Reference
Biological – Alkaline Pretreatment							
Corn stalks	*Irpex lacteus* (28°C, 15 d)	0.25 M NaOH solution (75°C, 2 h)	NREL (Sluiter et al., 2006)	80	51.37	6.62*	Yu et al. (2010a)
Pinus Radiata	*Gloeophyllum trabeum* (27 °C, 28 d)	25% w/ w NaOH (180 °C, 5 h)	TAPPI 204 cm-97	37.5*	75.41*	N/A	Fissore et al. (2010)
Populus tomentosa	*Trametes velutina* D10149 (28°C, 56 d)	2.5 g $NaClO_3$ and 2 mL acetic acid (80°C, 1 h)	NREL (Sluiter et al., 2008)	72.75*	7.1*	(-) 11.42*	Wang et al. (2013a)
Populus tomentosa	*Trametes velutina D10149* (28°C, 28 d)	70% (v/v) ethanol aqueous solution containing 1%(w/v) NaOH (75°C, 3 h)	Klason method (KCL 1982; Dence, 1992)	23.08*	22.22*	18.91*	Yang et al. (2013)
Rice Straw	*Sphingobacterium sp. LD-1* (30°C, 4 d)	4% NaOH + 6% Urea (-10°C, 4 h)	NREL (Sluiter et al., 2008)	34.38*	28.20*	(-) 34.81*	Dai et al. (2015)
Willow sawdust	*Leiotrametes menziesii* (27°C, 30 d)	1% (w/v) NaOH (80°C, 24 h)	NREL (Sluiter et al., 2008)	59.8	68.1	51.2	Alexandropoulou et al. (2017)
	Abortiporus biennis (27°C, 30 d)			54.2	51.8	29.1	
Rice Straw	70 mL of ethanol–water solution (65:35, v/v) containing 0.5 wt % NaOH. 400W of microwave irradiation for 10 min	*Acinetobacter sp.* B-2 (30°C, 2 d)	(Teramoto et al., 2008)	51.76*	33.44*	(-) 32.80*	Si et al. (2019)
		Bacillus sp. B-3 (30°C, 2 d)		53.58*	31.53*	(-) 28.39*	
		Pandoraea sp. B-6 (30°C, 2 d)		59.95*	19.65*	(-) 21.06*	
		Comamonas sp. B-9 (30°C, 2 d)		59.04*	22.98*	(-) 25.09*	
Biological – Acid Pretreatment							

Populus tomentosa	*Trametes velutina* D1014 (28°C, 56 d)	1% sulphuric acid (140°C, 1 h)	NREL (Sluiter et al., 2008)	23.82*	75.96*	(-) 18.74*	Wang et al. (2013a)
Oil palm empty fruit bunches	*Pleurotus floridanus* LIPIMC996 (31°C, 28 d)	Ball milled at 29.6/s for 4 mins. Phosphoric acid treatment (50°C, 5 h)	NREL (Sluiter et al., 2011).	(-) 8.29	60.63	(-) 37.52	Ishola et al. (2014)
Olive tree pruning	*Ulocladium sp.* (23°C, 28 d)	0.1% sodium hydroxide (5% w/w) at (50°C, 1 h, 165 rpm); 0.5% (w/w) H_2SO_4 (130°C, 1 h)	NREL (Sluiter et al., 2008)	(-) 61.70*	(-) 28.87*	(-) 36.00*	Martín-Sampedro et al. (2017)
	Hormonema sp. (23°C, 28 d)			(-) 62.23*	(-) 24.65*	(-) 37.78*	
	Trametes sp. (23°C, 28 d)			(-) 57.98*	(-) 33.10*	(-) 50.22*	
Rice straw	0.5% H_2SO_4 (121°C, 40 mins)	*Cupriavidus basilensis B-8* (30°C, 3 d)	(Teramoto et al., 2008)	(-) 91.72*	67.03*	(-) 47.13*	Yan et al. (2017)
Olive tree biomass	2% w/v H_2SO_4 (130°C, 1.5 h)	*Irpex lacteus (Fr.238 617/93)* (30°C, 28 d)	NREL (Sluiter et al., 2010)	(-) 116.02*	73*	(-) 90.48*	Martínez-Patiño et al. (2018)
Olive tree biomass	*Irpex lacteus (Fr.238 617/93)* (30°C, 28 d)	2% w/v H_2SO_4 (130°C, 1.5 h)	NREL (Sluiter et al., 2010)	(-) 105.82*	75.29*	(-) 69.52*	Martínez-Patiño et al. (2018)
Rice Straw	70 mL of ethanol–water solution (65:35, v/v) containing 0.5 wt % HCl. 400W of microwave irradiation for 10 min	*Acinetobacter sp*. B-2 (30°C, 2 d)	(Teramoto et al., 2008)	31.59*	46.64*	(-) 37.49*	Si et al. (2019)
		Bacillus sp. B-3 (30°C, 2 d)		34.32*	38.06*	(-) 30.87*	
		Pandoraea sp. B-6 (30°C, 2 d)		49.83*	28.05*	(-) 29.40*	
		Comamonas sp. B-9 (30°C, 2 d)		34.32*	34.25*	(-) 26.83*	
Biological - Oxidative Pretreatment							
Rice Hull	H_2O_2 (2%, 48 h)	*Pleurotus ostreatus* (28°C, 18 d)	(Goering and Van Soest, 1970)	37.54*	54.42*	11.92*	Yu et al. (2009)
Corn	*Echinodontium*	0.0016% NaOH and	Procedures of	52.00	23.64*	(-) 45.45*	Yu et al.

Straw	*taxodii* (25°C, 15 d)	3% H_2O_2 (25°C, 16 h)	AOAC				(2010b)
Populus tomentosa	*Fomitopsis palustris* (28°C, 28 d)	$FeCl_3$ (180°C, 30 mins)	NREL (Sluiter et al., 2008)	(-) 106.07*	99.7	27.81*	Wang et al. (2013b)
	Trametes orientalis (28°C, 28 d)			(-) 63.60*	98.8	2.88*	
Hemp chips	*Pleurotus eryngii* (28°C, 21 d)	3% NaOH and 3% (v/v) H_2O_2 (40°C, 24 h)	TAPPI (1975)	55.7	23.2	25.1	Xie et al. (2017)
Rice Straw	0.02 M $FeCl_3$, 1.5 M H_2O_2 (25°C, 2 h)	*Cupriavidus basilensis* B-8 (30°C, 2 d)	Holocellulose (Wise et al., 1946); Cellulose (TAPPI 203); Klason lignin (Browning, 1967)	67.05*	21.34*	30.10*	Zhang et al. (2018)
Rice Straw	70 mL of ethanol–water solution (65:35, v/v) containing 0.5 wt % $FeCl_3$. 400W of microwave irradiation for 10 min	*Acinetobacter sp.* B-2 (30°C, 2 d)	(Teramoto et al., 2008)	36.79*	21.52*	(-) 11.50*	Si et al. (2019)
		Bacillus sp. B-3 (30°C, 2 d)		34.04*	11.95*	(-) 5.96*	
		Pandoraea sp. B-6 (30°C, 2 d)		45.04*	(-) 5.27*	0.69*	
		Comamonas sp. B-9 (30°C, 2 d)		30.38*	1.91*	(-) 4.48*	
Biological – Organosolv Pretreatment							
Pinus radiata woods chips	*Ceriporiopsis subvermispora* (27°C, 30 d)	60% ethanol in water solvent (200°C, 1 h) (H-factor: 11,360); cold alkaline wash: 1% NaOH for 10 mins; hot alkaline wash: 1% NaOH (75°C, 1 h)	TAPPI 222 om-88	76.17*	57.89* (Xylan)	(-) 86.06* (Glucan)	Muñoz et al. (2007)
Acacia	*Ganoderma australe*	60% ethanol in water	TAPPI 222 om-88	90.55*	78.76*	(-) 85.74*	Muñoz et al.

dealbata woods chips	(27°C, 30 d)	solvent (200°C, 1 h) (H-factor: 10,920); cold alkaline wash: 1% NaOH for 10 mins; hot alkaline wash: 1% NaOH (75°C, 1 h)			(Xylan)	(Glucan)	(2007)
Sugarcane straw	*Ceriporiopsis subvermispora* (27°C, 15 d)	Acetosolv pulping (Acetic acid with 0.3% w/w HCl) (120°C, 5 h)	Lignin (Rocha et al., 1993); Hemicellulose and cellulose (Rocha et al., 1997)	86.8	93.8	32.1	Saad et al. (2008)
Pinus radiata	*Gloeophyllum trabeum* (27°C, 28 d)	60% ethanol in water solvent (200°C, 1 h)	TAPPI 204 cm-97	74.26*	80.74*	N/A	Fissore et al. (2010)
Pinus radiata wood chips	*Gloephyllum trabeum*(ATCC 11539) (25°C, 21 d)	95% ethanol in water solvent (60:40 v/v ratio) with 0.13% H_2SO_4 (w/v) (185°C, 18 min); 1092 H factor	TAPPI T280 pm 99	40.73*	91.58*	(-) 76.18* (Glucan)	Monrroy et al. (2010)
Pinewood chips	*T. versicolor* (28 °C, 15 d)	65% ethanol in water solvent with 1% H_2SO_4 (v/v) (170°C, 1 h)	NREL (Sluiter et al., 2008)	N/A	N/A	17.1* (Glucan)	Kandhola et al. (2017)
Biological – LHW/ HWE/ Autohydrolysis Pretreatment							
Soybean	Liquid Hot water (170°C, 3 mins, 400 rpm, 110 psi, solid to liquid ratio of 1:10)	*Ceriporiopsis subvermispora* (28°C, 18 d)	NREL (Sluiter et al., 2008)	36.69	41.34	0.84	Wan and Li, (2011)
Corn stover				41.99	42.91	7.09	
Wheat straw	Hot water extraction (HWE) (85°C, 10	*Ceriporiopsis subvermispora* (28°C,	NREL (Sluiter et al., 2008)	24.87	13.19	1.86	Wan and Li, (2011)

Corn stover	mins, solid to liquid ratio of 1:20).	18 d)		30.09	28.14	4.96	
Soybean				0.09	0.09	0.09	
Populus tomentosa	*Lenzites betulina C5617* (28°C, 28 d)	Liquid hot water (200°C, 30 mins, 10% w/v of dry matter mixture)	NREL (Sluiter et al., 2008)	(-) 15.52*	92.33	(-) 30.43*	Wang et al. (2012)
	Trametes ochracea C6888 (28°C, 28 d)			(-) 12.93*	87.86*	(-) 36.94*	
Eucalyptus globulus	Autohydrolysis (135°C, 30 mins, Liquid to solid ratio of 6:1)	*Ulocladium sp.* (23°C, 28 d); 0.1% NaOH (5% w/w) at (50°C, 1 h, 165 rpm)	NREL (Sluiter et al., 2011)	19.09*	15.29*	N/A	Martín-Sampedro et al. (2015)
		Hormonema sp. (23°C, 28 d); 0.1% NaOH (5% w/w) at (50°C, 1 h, 165 rpm)		15*	7.01*	N/A	
		Trametes sp. (23°C, 28 d); 0.1% NaOH (5% w/w) at (50°C, 1 h, 165 rpm)		19.09*	6.37	N/A	
		Pringsheimia smilacis (23°C, 28 d); 0.1% NaOH (5% w/w) at (50°C, 1 h, 165 rpm)		29.54*	12.10*	N/A	
Eucalyptus globulus	*Ulocladium sp.* (23°C, 28 d)	0.1% NaOH (5% w/w) at (50°C, 1 h, 165 rpm); Autohydrolysis (135°C, 30 mins, Liquid to solid ratio of 6:1)	NREL (Sluiter et al., 2011)	21.36*	5.09*	N/A	Martín-Sampedro et al. (2015)
	Hormonema sp. (23°C, 28 d)			16.36*	13.37*	N/A	
	Trametes sp. (23°C, 28 d)			25.91*	1.91*	N/A	

	Pringsheimia smilacis (23°C, 28 d)			33.18*	7.01*	N/A	
Biological – Steam Explosion Pretreatment							
Beech wood-meal	*Phanerochaete chrysosporium* (37°C, 28 d)	Steam explosion (215°C, 6.5 mins)	Perchloric acid method (Iiyama et al., 1988; Wayman and Chua, 1979)	42.00	N/A	N/A	Sawada et al. (1995)
Rice straw	Steam explosion (1.5 MPa, 1 min)	*Pleurotus ostreatus ATCC 66376* (25ºC, 48 d)	Lignin (TAPPI) Cellulose (Updegraff, 1969).	(-) 285.71*	N/A	16	Taniguchi et al. (2010)
Sawtooth oak, corn and bran	*Lentinula edodes* (120 d)	Steam explosion (214°C, 5 mins, 20 atm)	William and Brink (1991)	17.1*	80.43*	(-) 5.19*	Asada et al. (2011)
Pinus radiata	Steam explosion (235°C, 1 min)	*Coniophora puteana (Schumach.) P. Karst* (26°C, 42 d)	Ion chromatography extraction method (Sluiter et al., 2008)	(-) 195.55*	(-) 400*	82.55*	Vaidya and Singh (2012)
		Antrodia xantha (Fr.) Ryvarden (26°C, 42 d)		(-) 233.70*	(-) 290*	(-) 66.73*	
		Oligoporus placenta (Fries) Gilb and Ryvarden (26°C, 42 d)		(-) 177.96*	(-) 285*	(-) 55.10*	
		Trametes versicolor (L.) Lloyd (26°C, 42 d)		(-) 111.11*	(-) 400*	(-) 8.57*	
Corn stalk	Steam explosion (1.7 MPa, 1min)	*Phellinus baumii* (28 °C, 21 d)	NREL (Sluiter et al., 2010)	20.81*	26.14*	(-) 18.27*	Li and Chen (2014)

*as calculated by authors using the data given in the research article

(-): represents the increase in the content of the fiber.

Table 2: Comparison of sugar yield of different microbial-chemical/physicochemical pretreatment strategies

Substrate	1st step	2^{nd} step	Hydrolysis method	Sugar yield	Fold increase	Reference
Biological – Alkaline Pretreatment						
Wheat straw	*lschnoderma benzoinum* 108 (28°C, 14 d)	2% (w/v) NaOH (115°C, 10 mins)	Cellulase (10 FPU/g) (40°C, 72 h, magnetically stirred) [Glucose by glucose oxidase method and RS by DNS]	388 mg/g (RS); 190 mg/g (G)	0.95 (RS) and 0.93 (G) fold less than sole alkali treatment*	Hatakka, A.I. (1983)
	Pleurotus ostreatus (28°C, 14 d)			308 mg/g (RS); 90 mg/g (G)	0.76 (RS) and 0.44 (G) fold less than sole alkali treatment*	
	Pycnoporus cinnabarinus (28°C, 14 d)			343 mg/g (RS); 183 mg/g (G)	0.84 (RS) and 0.89 (G) fold less than sole alkali treatment*	
Pinus radiata	*Gloeophyllum trabeum* (27°C, 28 d)	25% (w/w) NaOH (180°C, 5 h)	Celluclast (20 FPU/g) and β-glucosidase (40 UI/g) (50°C, 24 h, 150 rpm) [HPLC]	77 ± 2% (G)	0.93 fold less than sole alkaline pretreatment*	Fissore et al. (2010)
Cornstalks	*Irpex lacteus* CD2 (28°C, 15 d)	0.25 M NaOH (60°C, 2 h)	Cellulase (30 FPU/g) at (50°C, 48 h) [HPLC]	93.86% (glucan digestibility)	14% increase compared to sole alkaline pretreatment	Yu et al. (2010a)
Wheat straw (*Triticum aestivum*)	*P. subvermispora* (28°C, 21 d)	0.1% NaOH (5% w/v) (50°C, 1 h, 165 rpm)	Cellulase (15 FPU/g) and xylanase (30 U/g) (50°C, 60 h, 165 rpm) [TRS by Somogyi, 1945; Glucose by Glucose TR kit]	357 ± 1 mg/g (G)	0.87 fold decrease than sole alkali pretreatment*	Salvachúa et al. (2011)
	I. lacteus (28°C, 21 d)			340 ± 2 mg/g (G)	0.83 fold decrease than sole alkali pretreatment*	
Cornstalks	*Irpex lacteus* (28°C, 15 d)	0.25 M NaOH (30°C, 2 h)	Cellulase (30 FPU/g of substrate) (50°C, 48 h) [DNS]	400.1 mg/g	1.31 fold increase than sole alkali pretreatment*	Zhong et al. (2011)
	Echinodontium taxodii (28°C, 15 d)			319.5 mg/g	1.05 fold increase than sole alkali pretreatment*	

Populus tomentosa	*Trametes velutina* D1014 (28°C, 56 d)	2.5 g $NaClO_3$ and 2 mL acetic acid (80°C, 1 h)	Cellulase (35 FPU/g) and β – glucosidase (37.5 CBU/g) (50°C, 72 h, 150 rpm) [HPAEC].	412.7 mg/g (84.77% cellulose conversion)	2.19 fold increase than sole fungal pretreatment*	Wang et al. (2013a)
Populus tomentosa	*Trametes velutina D10149* (28°C, 28 d)	70% (v/v) ethanol aqueous solution containing 1% (w/v) NaOH (75°C, 3 h)	Cellulase (20 FPU/g) and β-glucosidase (30 CBU/g) (50°C, 144 h, 150 rpm) [HPAEC]	38.8% of cellulose conversion	4.8 fold increase than sole fungal pretreatment*	Yang et al. (2013)
Rice Straw	*Sphingobacterium sp. LD-1* (30°C, 4 d)	4% NaOH + 6% Urea (-10°C, 4 h)	Cellulase (300 U/g) (47.5°C, 72 h, 100 rpm) [DNS]	RS: 9.25 mg/mL; G: 5.97 mg/mL	1.396 and 1.372 fold increase of RS and G respectively than sole alkaline pretreatment	Dai et al. (2015)
Maize Straw	1% w/v NaOH (room temperature, 48 h)	*T. harzianum* (30°C, 6 d)	No separate hydrolysis step [DNS]	0.52 mg/mL	0.96 fold decrease than sole alkali pretreatment*	Zhao et al. (2018)
		Aspergillus sp. (30°C, 6 d)		0.41 mg/mL	0.77 fold decrease than sole alkali pretreatment*	
		T. harzianum + *Aspergillus sp.* (30°C, 6 d)		0.66 mg/mL	1.23 fold increase than sole alkali pretreatment*	
		Enzyme T (*T. harzianum)* (50°C, 6 d)		0.63 mg/mL	1.18 fold increase than sole alkali pretreatment*	
		Enzyme A (*Aspergillus sp.)* (50°C, 6 d)		0.74 mg/mL	1.38 fold increase than sole alkali pretreatment*	
		Enzyme T+A (*T. harzianum* + *Aspergillus sp.)* (50°C, 6 d)		0.74 mg/mL	1.38 fold increase than sole alkali pretreatment*	

Rice straw	70 mL of ethanol-water solution (65:35, v/v) containing 0.5 wt % NaOH. 400W of microwave irradiation for 10 min	*Acinetobacter sp*. B-2 (30°C, 2 d)	Cellulase (12 FPU/g) (50°C, 48 h, 110 rpm)	818.05 mg/g	1.32 fold increase than sole alkali pretreatment*	Si et al. (2019)
		Bacillus sp. B-3 (30°C, 2 d)		719.15 mg/g	1.16 fold increase than sole alkali pretreatment*	
		Pandoraea sp. B-6 (30°C, 2 d)		696.55 mg/g	1.12 fold increase than sole alkali pretreatment*	
		Comamonas sp. B-9 (30°C, 2 d)		611.77 mg/g	0.99 fold decrease than sole alkali pretreatment*	
Biological – Acid Pretreatment						
Water Hyacinth (*Eichhornia crassipes*)	*Echinodontium Taxodii* (28°C, 10 d)	0.25% H_2SO_4 (100°C, 1 h)	Cellulase (30 FPU/g) (50°C, 48 h) [DNS]	366.0 mg/g dry matter	1.13 fold increase than acid treatment alone*	Ma et al. (2010)
Glycyrrhiza uralensis Fisch. Ex DC	2.5% H_2SO_4 (100ºC, 2.5 h)	*Phanerochaete chrysosporium* (28ºC, 21 d)	Cellulase (30 FPU/g) (50°C, 48 h) [DNS]	192.07 mg/g	1.09 fold increase than acid pretreatment alone*	Gui et al. (2013)
Populus tomentosa	*Trametes velutina* D1014 (28°C, 56 d)	1% H_2SO_4 (140°C, 2 h)	Cellulase (35 FPU/g) and β–glucosidase (37.5 CBU/g) (50°C, 72 h, 150 rpm) [HPAEC].	326.73 mg/g (65.58% cellulose conversion)	1.74 fold increase than fungal pretreatment alone*	Wang et al. (2013a)
Corn Leaf	*Trametes sp. 44 (PS 8, 200mL of air/ min, 13 d)*	4% v/v H_2SO_4 (121°C, 24 h, 1.5 atm)	Xylanase (63 AU) and Cellulase (5.44 AU) (50°C, 48 h) [RS by DNS; simple sugars by HPLC]	12.2 g/L	9% increase	Ramirez et al. (2014)
Olive tree pruning	*Ulocladium sp.* (23°C, 28 d)	0.1% NaOH (5% w/w) at (50°C, 1 h, 165 rpm); 0.5% (w/w) H_2SO_4	Cellulolytic complex (15 FPU/g) and β- glucosidase (15 IU/g) (50°C, 168 h, 120 rpm) [HPLC]	136 mg/g	0.99 fold decrease compared to fungi pretreatment with alkali extraction*	Martín-Sampedro et al. (2017)
	Hormonema sp.			130 mg/g	0.94 fold decrease	

	(23°C, 28 d)	(130°C, 1 h)			compared to fungi pretreatment with alkali extraction*	
	Trametes sp. (23°C, 28 d)			131 mg/g	0.95 fold decrease compared to fungi pretreatment with alkali extraction*	
Rice straw	0.5% H_2SO_4 (121°C, 40 mins)	*Cupriavidus basilensis B-8* (30°C, 3 d)	Cellulase (50 °C, 72 h, 120 rpm) [DNS]	442 mg/g	1.7 fold increase than sole acid pretreatment*	Yan et al. (2017)
Olive tree biomass	2% (w/v) H_2SO_4 (130°C, 1.5 h)	*Irpex lacteus (Fr.238 617/93)* (30°C, 28 d)	Cellulase (15 FPU/ g), β-glucosidase (15 IU/ g) and xylanase (30 U/ g) (50 °C, 72 h, 150 rpm) [HPLC]	5.1 ± 0.5 g/L (G)	0.69 fold decrease than acid pretreatment alone*	Martínez-Patiño et al. (2018)
Olive tree biomass	*Irpex lacteus (Fr.238 617/93)* (30°C, 28 d)	2% (w/v) H_2SO_4 (130°C, 1.5 h)	Cellulase (15 FPU/g), β-glucosidase (15 IU/g) and xylanase (30 U/g) (50 °C, 72 h, 150 rpm) [HPLC]	9.9 ± 0.1 g/L (G)	1.34 fold increase than acid pretreatment alone*	Martínez-Patiño et al. (2018)
Rice straw	70 mL of ethanol-water solution (65:35, v/v) containing 0.5 wt % HCl. 400W of microwave irradiation for 10 min	*Acinetobacter sp*. B-2 (30°C, 2 d)	Cellulase (12 FPU/g) (50°C,48h, 110 rpm)	662.64 mg/g	1.21 fold increase than acid pretreatment alone*	Si et al. (2019)
		Bacillus sp. B-3 (30°C, 2 d)		703.61 mg/g	1.29 fold increase than acid pretreatment alone*	
		Pandoraea sp. B-6 (30°C, 2 d)		770.02 mg/g	1.41 fold increase than acid pretreatment alone*	
		Comamonas sp. B-9 (30°C, 2 d)		599.06 mg/ g	1.10 fold increase than acid pretreatment alone*	
Biological - Oxidative Pretreatment						
Rice Hull	H_2O_2 (2%, 48 h)	*Pleurotus ostreatus* (28°C,	Cellulase (15 FPU/mg) (45°C, 48 h, 150 rpm) [TS by	39.8% (TS) 49.6% (G)	5.8 times (TS) and 6.5 times (G) more than sole	Yu et al. (2009)

		18 d)	phenol–sulfuric acid (Dubois et al., 1956) and G by glucose oxidase-peroxidase]		fungal pretreatment for 18 d	
Corn Straw	*Echinodontium taxodii* (25°C, 30 d)	0.0016% NaOH and 3% H_2O_2 (room temperature, 16 h)	2 mg/ml Cellulase (100 FPU/g) (50 °C, 48 h) [DNS]	57.52%	1.43 fold increase than sole A/O pretreatment at 8 mg/ mL cellulase concentration*	Yu et al. (2010b)
Populus tomentosa	*Trametes orientalis* (28°C, 28 d)	$FeCl_3$ (180°C, 30 mins)	Cellulase (30 FPU/g) and β - glucosidase (37.5 IU/g) (50°C, 96 h, 150 rpm) [DNS]	84.5%	1.4 fold increase than sole $FeCl_3$ treatment	Wang et al. (2013b)
	Fomitopsis palustris (28°C, 28 d)			95.4%	1.6 fold increase than sole $FeCl_3$ treatment	
Wheat straw	microbubble mediated ozonolysis (pH 3, 8.87 mg/L, 24 h)	*Pseudomonas putida* KT2440 (30°C, 48 h)	[DNS]	1.1 ± 0.09 mg/mL	1.83 fold increase than sole ozonation pretreatment*	Mulakhudair et al. (2017)
Hemp chips	*Pleurotus eryngii* (28°C, 21 d)	3% NaOH and 3% (v/v) H_2O_2 (40°C, 24 h)	Cellulase (30 FPU/g) (50°C, 48h) [DNS]	372 mg/g	1.29 fold increase than sole chemical treatment*	Xie et al. (2017)
Rice Straw	0.02 M $FeCl_3$, 1.5 M H_2O_2 (25°C, 2 h)	*Cupriavidus basilensis* B-8 (30°C, 2 d)	Cellulase (12 FPU/g) (50°C, 72 h, 120 rpm) [DNS]	462.74 mg/g	1.54 fold increase than sole Fenton treatment*	Zhang et al. (2018)
	Cupriavidus basilensis B-8 (30°C, 2 d)	0.02 M $FeCl_3$, 1.5 M H_2O_2 (25°C, 2 h)		326.80 mg/g	1.09 fold increase than sole Fenton treatment*	
Rice straw	70 mL of ethanol–water solution	*Acinetobacter sp*. B-2 (30°C, 2 d)	Cellulase (12 FPU/g) (50°C, 48h, 110 rpm)	361.13 mg/g	2.02 fold increase than sole $FeCl_3$ treatment*	Si et al. (2019)

	(65:35, v/v) containing 0.5 wt % $FeCl_3$. 400W of microwave irradiation for 10 min	*Bacillus sp.* B-3 (30°C, 2 d)		297.65 mg/g	1.66 fold increase than sole $FeCl_3$ treatment*	
		Pandoraea sp. B-6 (30°C, 2 d)		287.77 mg/g	1.61 fold increase than sole $FeCl_3$ treatment*	
		Comamonas sp. B-9 (30°C, 2 d)		259.56 mg/g	1.45 fold increase than sole $FeCl_3$ treatment*	
Biological – Organosolv Pretreatment						
Sapwood of beech (*Fagus crenata*)	*C. subvermispora* FP90031 (28 °C, 28 d)	60% (v/v) ethanol solution (180°C, 2 h)	Meicellase (224 FPU/g) and β-glucosidase activity (264 IU/g) (45°C, 96 h, 170 rpm) [Somogyi-Nelson]	0.454 g/g (G)	1.03 fold increase than sole organosolv pretreatment*	Itoh et al. (2003)
Pinus radiata woods chips	*Ceriporiopsis subvermispora* (27°C, 30 d)	60% ethanol in water solvent (200°C, 1 h) (H-factor: 11,360); cold alkaline wash: 1% NaOH for 10 mins; hot alkaline wash: 1% NaOH (75°C, 1 h)	Cellulase (20 FPU/g glucan) and β-glucosidase (40 CBU/g glucan) (50°C, 72 h, 150 rpm) [HPLC]	92.1 ± 0.5% Glucan; 100% glucan-to-glucose conversion	1.13 fold increase than sole organosolv pretreatment*	Muñoz et al. (2007)
Acacia dealbata woods chips	*Ganoderma australe* (27°C, 30 d)	60% ethanol in water solvent (200°C, 1 h) (H-factor: 10,920); cold alkaline wash: 1% NaOH for 10 mins; hot alkaline wash:	Cellulase (20 FPU/g glucan) and β-glucosidase (40 CBU/g glucan) (50°C, 12 h, 150 rpm) [HPLC]	93.8 ± 0.4% glucan; 100% glucan-to-glucose conversion	1.03 fold increase than sole organosolv pretreatment*	Muñoz et al. (2007)

		1% NaOH (75°C, 1 h)				
Pinus radiata	*Gloeophyllum trabeum* (27 °C, 28 d)	60% ethanol in water solvent (200°C, 1 h)	Celluclast (20 FPU/ g pulp) and β-glucosidase (40 UI/ g pulp) (50°C, 24 h, 150 rpm) [HPLC]	82.4% (G)	1.10 fold increase than sole organosolv pretreatment*	Fissore et al. (2010)
Japanese cedar *(Cryptomeria japonica)*	*Phellinus sp.* SKM2102 (28 °C, 56 d)	Ethanol/ lactic acid/ water (40:10:50, w/w) (190°C, 30 mins)	Meicellase (224 FPU/g) and β-glucosidase (264 IU/g) (45°C, 96 h, 170 rpm) [Somogyi-Nelson method]	442 mg/g (63.5%)	7 times increased	Baba et al. (2011)
	C. subvermispora FP-90031-sp (28 °C, 56 d)	Ethanol/ lactic acid/ water (40:10:50, w/w) (200°C, 1 h)		52%	7.32 fold increase	
Pinewood chips	*T. versicolor* (28 °C, 15 d)	65% ethanol in water solvent with 1% H_2SO_4 (v/v) (170°C, 1 h)	Accellerase® 1500 enzyme (15 FPU/g glucan) (50°C, 72 h) [HPLC]	32.0 ±0.9% Glucan content with 100% glucan conversion	1.09 fold increase than sole organosolv pretreatment*	Kandhola et al. (2017)
Biological – LHW/ HWE/ Autohydrolysis Pretreatment						
Soybean	Liquid Hot water (170°C, 3 mins, 400 rpm, 110 psi, solid to liquid ratio of 1:10)	*Ceriporiopsis subvermispora* (28°C, 18 d)	Spezyme CP (10 FPU/g) (50°C, 72 h, 130 rpm) [HPLC]	64.29% (G)	1.51 fold increase than sole LHW pretreatment*	Wan and Li (2011)
Corn stover				53% (G)	1.54 fold increase than sole LHW pretreatment*	
Wheat straw	Hot water extraction (HWE) (85°C, 10 mins, solid to liquid	*Ceriporiopsis subvermispora* (28°C, 18 d)	Spezyme CP (10 FPU/g) (50°C, 72 h, 130 rpm) [HPLC]	43.69% (G)	2.26 fold increase than sole HWE pretreatment*	Wan and Li (2011)
Corn stover				55.19% (G)	2.44 fold increase than sole HWE pretreatment*	

Soybean	ratio of 1:20).			35.24% (G)	1.05 fold increase than sole HWE pretreatment*	
Populus tomentosa	*Lenzites betulina C5617* (28°C, 28 d)	Liquid hot water (LHW) (200°C, 30 mins, 10% w/v of dry matter mixture)	Cellulase (35 FPU/g) (50°C, 96 h, 150 rpm) [HPAEC]	60.29% (G)	2.66 fold increase than sole LHW pretreatment	Wang et al. (2012)
	Trametes ochracea C6888 (28°C, 28 d)			58.79% (G)	1.12 fold increase than sole LHW pretreatment*	
Eucalyptus globulus	Autohydrolysis (135°C, 30 mins, Liquid to solid ratio of 6:1)	*Ulocladium sp.* (23°C, 28 d); 0.1% NaOH (5% w/w) at (50°C, 1 h, 165 rpm)	Celluclast (15 FPU/g) and β-glucosidase (15 U/g) (50°C, 72 h, 120 rpm) [HPLC]	9.03 g/L (G) and 11.22 g/L (TS)	3.29 (G) and 3.34 (TS) fold increase than sole autohydrolysis process*	Martín-Sampedro et al. (2015)
		Hormonema sp.(23°C, 28 d); 0.1% NaOH (5% w/w) at (50°C, 1 h, 165 rpm)		8.17 g/L (G) and 10.19 g/L (TS)	2.98 (G) and 3.03 (TS) fold increase than sole autohydrolysis process*	
		Trametes sp.(23°C, 28 d); 0.1% NaOH (5% w/w) at (50°C, 1 h, 165 rpm)		6.28 g/L (G) and 8.04 g/L (TS)	2.29 (G) and 2.39 (TS) fold increase than sole autohydrolysis process*	
		Pringsheimia smilacis (23°C, 28 d); 0.1% NaOH (5%		5.55 g/L (G) and 7.01 g/L (TS)	2.02 (G) and 2.08 (TS) fold increase than sole autohydrolysis process*	

		w/w) at (50°C, 1 h, 165 rpm)				
Eucalyptus globulus	*Ulocladium sp.* (23°C, 28 d)	0.1% NaOH (5% w/w) at (50°C, 1 h, 165 rpm); Autohydrolysis (135°C, 30 mins, Liquid to solid ratio of 6:1)	Celluclast (15 FPU/g) and β-glucosidase (15 U/g) (50°C, 72 h, 120 rpm) [HPLC]	8.73 g/L (G) and 11.26 g/L (TS)	3.18 (G) and 3.35 (TS) fold increase than sole autohydrolysis process*	Martín-Sampedro et al. (2015)
	Hormonema sp. (23°C, 28 d)			8.35 g/L (G) and 10.65 g/L (TS)	3.04 (G) and 3.17 (TS) fold increase than sole autohydrolysis process*	
	Trametes sp. (23°C, 28 d)			4.93 g/L (G) and 6.02 g/L (TS)	1.80 (G) and 1.79 (TS) fold increase than sole autohydrolysis process*	
	Pringsheimia smilacis (23°C, 28 d)			5.58 g/L (G) and 7.01 g/L (TS)	2.03 (G) and 2.08 (TS) fold increase than sole autohydrolysis process*	
Biological – Steam Explosion Pretreatment						
Beech wood	*Phanerochaete chrysosporium* (37 °C, 28 d)	Steam explosion (210 °C, 10 mins)	Cellulase (37°C, 100 h) [Somogyi-Nelson method]	76% (saccharification)	1.13 fold increase than sole steam explosion pretreatment*	Sawada et al. (1995)
Rice straw	*Pleurotus ostreatus* (25°C, 23 d)	AFEX (Liquid ammonia loading 1:1, 80% moisture) (100°C, 5 mins)	Cellulase (15 FPU/g glucan), β glucosidase (64 *p*NPGU/g glucan) and xylanase (10% of cellulose protein) (50°C, 168 h, 90 rpm) [HPLC]	92% glucan and 55% xylan conversion	1.12 fold increase in glucan conversion than untreated biomass*	Balan et al. (2008)
Rice straw	Steam explosion (1.5 MPa, 1 min)	*Pleurotus ostreatus ATCC 66376* (25°C, 36 d)	Cellulase (40°C, 48 h) [glucose oxidase–peroxidase kit]	33% (G)	1.8 fold more conversion than sole fungal pretreatment*	Taniguchi et al. (2010)
Sawtooth oak (90%), corn and	*Lentinula edodes* (120 d)	Steam explosion (214°C, 5 mins,	Enzyme (45°C, 48 h, 140 strokes/min) [TRS by Somogyi–Nelson method;	62 mg/g (G)	1.94 fold increase than fungal pretreatment alone*	Asada et al. (2011)

bran (10%)		20 atm/ 2.03 MPa)	Glucose by mutarotase GOD]			
Pinus radiata	Steam explosion (235°C, 1 min)	*Coniophora puteana (Schumach.) P. Karst* (26°C, 42 d)	Celluclast (20 FPU/g) β-glucosidase (25 IU/g) (50°C, 24 h, 180 rpm) [YSI-2700D glucose analyzer]	5.86 g/L (G)	1.13 fold increase than sole steam-exploded wood*	Vaidya and Singh (2012)
		Antrodia xantha (Fr.) Ryvarden (26°C, 42 d)		6.38 g/L (G)	1.23 fold increase than sole steam-exploded wood*	
		Oligoporus placenta (Fries) Gilb and Ryvarden (26°C, 42 d)		6.28 g/L (G)	1.21 fold increase than sole steam-exploded wood*	
		Trametes versicolor (L.) Lloyd (26°C, 42 d)		6.40 g/L (G)	1.23 fold increase than sole steam-exploded wood*	
Corn stalk	Steam explosion (1.7 MPa, 1min)	*Phellinus baumii* (28°C, 21 d)	Cellulase (20 FPU/g) (50°C, 48 h, 130 rpm) [HPLC]	313.31 mg/g (G)	1.32 times increase than sole steam explosion	Li and Chen (2014)

*Calculated by the authors according to equation 1 with data in the literature

(G): Glucose; (RS): Reducing sugars; (TS): Total Sugars