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Review

Combined Biological and Chemical/Physicochemical Pretreatment Methods of Lignocellulosic Biomass for Bioethanol and Biomethane Energy Production—A Review

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Abstract: Lignocellulosic biomass is a low-cost and environmentally-friendly resource that can be used to produce biofuels such as bioethanol and biogas, which are the leading candidates for the partial substitution of fossil fuels. However, the main challenge of using lignocellulosic materials for biofuel production is the low accessibility to cellulose for hydrolysis of enzymes and microorganisms, which can be overcome by pretreatment. Biological and chemical pretreatments have their own disadvantages, which could be reduced by combining the two methods. In this article, we review biological–chemical combined pretreatment strategies for biogas and bioethanol production. The synergy of fungal/enzyme–NaOH pretreatment is the only biological–chemical combination studied for biogas production and has proven to be effective. The use of enzyme, which is relatively expensive, has the advantage of hydrolysis efficiency compared to fungi. Nonetheless, there is vast scope for research and development of other chemical–biological combinations for biogas production. With respect to ethanol production, fungal–organosolv combination is widely studied and can achieve a maximum of 82% theoretical yield. Order of pretreatment is also important, as fungi may reduce the accessibility of cellulose made available by prior chemical strategies and suppress lignin degradation. The biofuel yield of similarly pretreated biomass can vary depending on the downstream process. Therefore, new strategies, such as bioaugmentation and genetically engineered strains, could help to further intensify biofuel yields.

Keywords: lignocellulosic biomass; combined pretreatment; biogas; bioethanol; microorganisms

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1. Introduction

With the current rate of global warming and low supplies of crude oil worldwide, replacement of conventional fuels with biofuels is the only viable alternative for the future [1]. According to the World Bioenergy Association, liquid biofuel production has been increasing at a rate of 12%, and biogas production has been increasing at a rate of 9% annually in the last two decades. In 2019, 159 billion liters of liquid biofuels and 62.3 billion m^3 of biogas were produced globally. The average energy content of bioethanol is 21.1 MJ/l, and that of biogas is 23 MJ/m^3 [2]. Currently, bioethanol is the most widely used liquid biofuel for transportation, as it helps to ensure complete combustion and reduce carbon monoxide emissions [3]. Biogas, when upgraded into biomethane, has several uses, namely heat production, electricity production or cogeneration of heat and electricity, and transport fuel production. Because biomethane represents an energy source identical to natural gas, many countries, such as France, Germany, Sweden, Switzerland and the Netherlands are injecting it directly into the existing natural gas network [4]. First-generation biofuels from agricultural crops have been disputed due to the food-versus-fuel debate. Therefore, research activities have been focused on second-generation biofuel from agricultural residues and other cellulosic waste products [5].

Conversion of lignocellulosic biomass (LCB) to biofuels produces net-zero greenhouse gas emissions; therefore, the obtained biofuels are carbon-neutral. Although agricultural biomass is theoretically the most prominently available feedstock for renewable energy, biofuel production at the commercial level has not yet achieved considerable success [6] due to the recalcitrance of the complex lignocellulosic biomass structure, which is mostly caused by lignin. Lignin needs to be disrupted using a pretreatment step in order to increase the accessibility of microbes/enzymes to cellulose and hemicellulose for further conversion of fermentable sugars to biofuels. The available pretreatment strategies, classified as physical, chemical, biological, and physiochemical pretreatments, employ different mechanisms of action and provide various degrees of polymerization. In recent decades, there has been increasing interest in the use of a combination of pretreatments to improve the yield of obtained biofuels while reducing the disadvantages of individual pretreatment methods. Due to its environmentally friendly and low-cost nature, biological pretreatment is a preferred method. Combination with chemicals can help to reduce the residence time and improve the hydrolysis rate, in addition to mutually reducing chemical and energy demands. The biological–chemical combination is also interesting because it can reduce the amount of inhibitors generated, making it easier to integrate into the downstream process. Once the biomass is pretreated, it can be subjected to either microbial saccharification and fermentation to produce ethanol or to anaerobic digestion (AD) to produce biogas [7,8].

1.1. Anaerobic Digestion of Lignocellulosic Biomass

In an anaerobic digestion process, complex organic material is broken down using microorganisms and metabolic pathways under anaerobic conditions. The major products of AD are biogas (made up of 50–65% methane and 35–50% carbon dioxide) and nutrient-rich digestate, which can be utilized as fertilizer or soil improver [9,10]. The biogas can be upgraded using various technologies to produce biomethane (consisting of 95–99% methane), which can be used as a transport fuel, for electricity generation, and as feedstock for chemical industries [11]. The global biomethane market was valued at USD 1.68 billion in 2018 and is expected to reach a valuation of USD 2.61 billion by 2025 [12].

The energy efficiency ratio of AD, calculated as energy gain to energy input, exceeds that of other technologies for energy production from biomass [13]. Biogas production is also advantageous because a wide variety of biomass can be utilized, representing an economical alternative to tackle environmental concerns [14]. Cesaro and Belgiorno (2015) reviewed the composition and methane potential of various crops and reported that maize, sorghum, rice straw, sunflower stalk, and wheat straw had the highest methane potential of the reviewed crops [15]. Nonetheless, biogas production from lignocellulosic biomass has not been scaled-up efficiently despite its high methane potential because AD of lignocellulosic biomass has to overcome various challenges, such as resistance of biomass to microbial and enzymatic degradation, as well as adaptation of existing AD digesters to the high dry solid contents of the particular crops [16].

A problem with using biomass, such as straw, is that it is a light and dry material, which would increase the solid content in the digester and float. For such light biomass, special mixers would be required for AD [17]. Ma et al. (2019) [18] studied the AD characteristics of individual purified components of lignocellulosic biomass, namely lignin, cellulose, and hemicellulose. It was reported that lignin and highly crystalline cellulose were negatively correlated with biogas production potential, whereas co-fermentation of hemicellulose and cellulose reduced the rancidity caused by the rapid hydrolysis rate of hemicellulose alone and was positively correlated with biogas production [18]. Therefore, the ultimate aim of pretreatment processes for improved biogas yield should be to enhance the degradation of lignin content, increase the accessible surface area, and decrease the crystallinity of cellulose while avoiding the degradation or loss of carbohydrates. Consequently, the employment of appropriate pretreatment is the best option for improving the digestion rate and methane production from biomass with varying properties and digestibility [13]. AD microbes are highly tolerant to inhibitory compounds that may be generated in the

process of pretreatment; therefore, detoxification is not required, which makes AD an easier process to utilize lignocellulosic biomass [19].

Apart from its structural recalcitrance, the carbon/nitrogen (C/N) ratio of lignocellulosic biomass is higher than the optimum C/N ratio (20–30), which limits the efficiency of methane production in AD [20]. Various techniques are used to counteract this problem, such as pretreatment [21], co-digestion of nitrogen-rich animal manure with carbon-rich biomass [22], high-solid AD [23], bioaugmentation with microorganisms [24], and nutrient supplementation [25]. Song et al. (2014) [21] observed that total carbon (TC) content decreased when corn straw was chemically pretreated. Although it was higher than the optimum C/N ratio, this helped to decrease the high C/N ratio of untreated lignocellulosic biomass. The methane yield of the chemically pretreated biomass thereby significantly increased compared to untreated samples [21]. Therefore, a successful pretreatment method should improve the digestibility of the biomass for the AD microbes, minimize the formation of inhibitors, and be environmentally friendly to avoid the need for waste disposal. In summary, additional research is required to explore other pretreatment methods, as it is the key cost element in the anaerobic digestion of lignocellulosic biomass [26].

1.2. Bioethanol Production from Lignocellulosic Biomass

Bioethanol is the most widely used biofuel to substitute crude oil. Ethanol is produced from lignocellulosic biomass using three major steps, namely pretreatment, hydrolysis, and fermentation. Pretreatment increases the porosity of the fiber matrices, liberates the cellulose from the lignin and hemicellulose complex, and improves the accessibility of enzymes. The pretreated biomass is then hydrolyzed either directly by microbes or with enzymes. Enzymatic hydrolysis offers the advantages of a shorter time period, better yields, and a lower risk of contamination. Commercial cellulase (extracted from microorganisms) is the most commonly used enzyme for hydrolysis. The hydrolysis process converts polysaccharides into monomer sugars, such as glucose and xylose. Subsequently, sugars are fermented to ethanol (as expressed in Reaction (1) below) through the use of various microorganisms. Due to the thermotolerant and highly pH-tolerant nature of *Saccharomyces cerevisiae*, it is the preferred organism for ethanol fermentation. Hydrolysis and fermentation can be conducted separately or simultaneously via processes called separate hydrolysis and fermentation (SHF) and simultaneous saccharification and fermentation (SSF), respectively. During the SHF process, each step occurs sequentially under optimized conditions, and the generated sugars are ready when the fermentation step is carried out. The SSF process occurs in the same reaction vessel, and both reactions occur at the same time. The advantages of SSF include minimized risk of contamination, a shorter time, and lower material requirements, whereas the inherent disadvantages are the generation of intermediate products that can inhibit the fermentation of microorganisms [27,28].

$$\begin{gathered}(C_6H_{10}O_5)_n + n\,H_2O \rightarrow n\,C_6H_{12}O_6 \rightarrow 2n\,C_2H_5OH + 2n\,CO \\ \text{Ligocelluose} \rightarrow \text{glucose} \rightarrow \text{ethanol} + \text{carbon dioxide}\end{gathered} \quad \text{(R1)}$$

Acid pretreatment is considered an efficient pretreatment step, although it generates various intermediaries, such as acetic acid, furfural, and 5-hydroxymethylfurfural, which inhibit the microorganisms during the fermentation step. Therefore, to reduce the intensity of acid required, combination with biological pretreatment can be more effective. Zhang et al. (2018) [29] observed an 8.6% increase in ethanol yield with combined pretreatment using white-rot fungi and dilute acid on water hyacinth as compared to dilute acid treatment alone. The increased yield of combined pretreatment was obtained even without the addition of a hydrolysis agent; therefore biological–chemical pretreatment could also help to reduce the cost of ethanol production [29].

The lack of systematic reviews of microbial and chemical/physicochemical combined pretreatments and the knowledge of the advantages of these methods as established in our previous review [8] make it interesting to review the research on pretreatments for biofuel production. Therefore, in this work, we aim to evaluate combined microbial-

chemical/physicochemical pretreatment strategies used for different LCBs based on biogas yield (Chapter 2) and bioethanol yield (Chapter 3).

2. Comparison of Combined Pretreatment Based on Biogas Yields

Although a number of reviews have been conducted in the last two decades studying the various pretreatment strategies of lignocellulosic biomass for biogas production in detail, very few have systematically compared combined pretreatment processes. To the best of our knowledge, biological–chemical combined pretreatment, especially for biogas production, has not been discussed, as research is not widely conducted. Only three research studies to date (as shown in Table 1 have utilized a biological–alkaline combined pretreatment strategy to enhance AD of lignocellulosic biomass, both methods support the breakdown of lignin.

Li et al. (2018) [30] studied the impact of individual components of lignocellulosic biomass on cumulative methane production. Apart from providing a physical barrier to hydrolytic enzymes, lignin predominantly degrades only in an aerobic environment, whereas it can persist in an anaerobic environment. Consequently, lignin offers the maximum resistance in the biomass and is negatively correlated with biomethane potential (BMP) [18,30,31]. Generally, holocellulose is the portion converted to methane by the mixed anaerobic culture; therefore, its increased degradation results in increased carbon dioxide content. Therefore, pretreatment of recalcitrant lignocellulosic biomass without losing a major portion of holocellulose is essential to achieve a high biogas yield [32]. Without prior treatment, hydrolysis slows down, requiring a longer retention time to produce a sufficient amount of biogas [33]. The hydrolysis process is interdependent on pH. Acidogenesis and methanogenesis are efficient only in the optimal pH range of 5.5–6.5 and 6.5–8.2, respectively [34]. Alkaline pretreatment helps to solubilize lignin and neutralize the acidic products released from lignocellulosic biomass. Additionally, because the residual bases remaining in the pretreated biomass may help to prevent a reduction in pH during the acidogenesis phase and help to increase the efficiency of methanogenesis, it is considered to be more compatible with the anaerobic digestion process [35]. The pH value of the AD substrates influences the growth of methanogenic microorganisms and the dissociation of some compounds relevant to the AD process, i.e., ammonia, hydrogen sulfide, and organic acids [36]. According to the results of previous studies, NaOH is the most widely used alkali pretreatment for lignocellulosic biomass to improve biogas yield [37–39]. Nevertheless, the disadvantage of this method is the possibility of the production of Na^+ ions, which can result in the inhibition of methanogenesis and lead to negative environmental impacts with respect to the disposal of the effluent [19]. Fungal pretreatment has been known as a more environmentally friendly method to improve the methane yield of lignocellulosic biomass [40–42]. Research on the effect of fungal pretreatment on methane yield has indicated that the results strongly depend on the feedstock (its lignocellulosic content) and the fungi used, as well as on some key operational parameters, such as the incubation time, the moisture content, etc. [32]. Three groups of researchers (as shown in Table 1) have studied the combination of fungal and alkali pretreatment to improve the methane yield of lignocellulosic biomass.

Table 1. Comparison of combined fungal/enzyme—alkaline pretreatment based on biomethane yields.

Reference	Substrate	Step 1	Step 2	% Increase Compared to Sole Biological Pretreatment	Methane Yield
Ali and Sun (2015) [43]	Park waste (dry and fresh leaves) + cattle manure	2.5 % NaOH and 2.5% NH_4OH (15 d)	*Aspergillus terreus* and *Trichoderma viride* (25 °C, 7 d)	30 (compared to untreated biomass)	79.8 L/kgVS (125.9 L/kgVS biogas production)
Alexandropoulou et al. (2017) [32]	Willow sawdust	*Leiotrametes menziesii* (27 °C, 30 d)	1% (*w*/*v*) NaOH (80 °C, 24 h)	48.9	142.2 ± 0.3 L/kg TS (L. menziesii)
		Abortiporus biennis (27 °C, 30 d)		50.1	205.3 ± 0.3 L /kg TS (*A. biennis*)
Zhao et al. (2018) [44]	Maize straw	1% (*w*/*v*) NaOH (room temperature, 48 h)	*Aspergillus* sp. (30 °C, 10 d)	13.34 *	276.29 L/kg TS
			T. harzianum (30 °C, 10 d)	22.88 *	261.63 L/kg TS
			T. harzianum & *Aspergillus* sp. (30 °C, 10 d)	31.77 *	277.99 L/kg TS
			Enzyme from *Aspergillus* sp. (KY644131) (50 °C, 10 d)	25.02 *	300.85 L/kg TS
			Enzyme from *T. harzianum* (KY644130) (50 °C, 10 d)	4.14 *	285.09 L/kg TS
			Enzyme from *T. harzianum* & *Aspergillus* sp. (50 °C, 10 d)	−6.71 * (decrease)	258.45 L/kg TS

* Calculated by the authors according to Equation (1).

The results presented in Table 1 are represented as [L CH_4/kg TS] or [L CH_4/kg VS], where TS and VS are total solids and volatile solids content in kilograms (kg), and L CH_4 represents a liter of methane. The percentage increase in methane yield in combined pretreatment as compared to the single pretreatment step is calculated according to Equation (1).

$$\% \text{ increase} = \frac{\text{BMP of combined pretreatment} - \text{BMP of sole pretreatment}}{\text{BMP of sole pretreatment}} \times 100 \quad (1)$$

Ali and Sun (2015) [43] observed a 30% increase in methane yield and an 11.9% reduction in CO_2 after combining pretreatment and co-digestion of park waste and cattle manure. By using the fungal pretreatment (*L. Menziesii* and *A. biennis*) as the first step in the combined pretreatment, Alexandropoulou et al. (2017) [32] were able to use a lower concentration of NaOH for the treatment in the subsequent step compared to that used by Ali and Sun (2015) [43]. Although *L. menziesii* exhibited higher lignin degradation than *A. biennis*, it produced less BMP than *A. biennis* as the portion of the holocellulose (higher observed cellulose degradation), which could be converted to methane when the mixed anaerobic culture was degraded, resulting in increased CO_2 content. This indicates that low cellulose uptake efficiency is equally as important as lignin degradation [32]. Zhao et al. (2018) [44] compared fungi (*T. harzianum* and *Aspergillus* sp.) and their secreted enzymes as a biological pretreatment step after NaOH treatment of maize straw. In this combined pretreatment study, the enzymes achieved better performance than their fungi counterparts in producing methane yield. Zhao et al. (2018) [44] remarked that in general, when fungi are used, nutrients from the lignocellulosic substrate are consumed for their growth, thus reducing the methane yield, whereas when enzymes derived from the fungi are used,

they help to convert cellulose and hemicelluloses into reducing sugars and other small molecules that are readily used by microorganisms in the AD process, thereby increasing the methane yield. An exception was noted when a combination of *T. harzianum* and *Aspergillus* sp. and a combination of their secreted enzymes were used in combination with NaOH pretreatment. The two fungi inhibited each other and slowed the decomposition of the substrate, whereas the combination of their enzymes showed a contrasting effect, resulting in increased hemicellulose removal. This resulted in an irregularity, whereby the combination of two fungi with NaOH produced improved methane yield relative to their secreted enzymes [44]. However, it has been observed that even if there is a possibility of some drawbacks associated with the combined pretreatment, it is an efficient process to improve biogas and biomethane content.

The hydrolysis of lignocellulosic biomass is dependent on the lignin-to-cellulose ratio. Low lignin to cellulose (L/C) ratio implies a high degree of digestibility [45]. An improvement was noted in the L/C ratio from 0.13 for untreated maize straw to 0.10 for 1% NaOH-treated maize straw [44]. According to the values reported in a study by Alexandropoulou et al. (2017) [32], the L/C ratio of willow sawdust was calculated to be 0.81. When treated with *Leiotrametes menziesii* and NaOH, the ratio was reduced to 0.67, whereas treatment with *Abortiporus biennis* and NaOH further reduced the ratio to 0.52. The methane yield values obtained for the same combination (as seen in Table 1) confirm that the lower the L/C value, the higher the degree of digestibility and therefore the higher the obtained methane yield. Accordingly, such synergetic pretreatment methods should be further formulated and developed to enhance biogas yields in a more cost-effective and sustainable manner compared to conventional methods.

3. Comparison of Combined Pretreatment Based on Bioethanol Yields

Saccharomyces cerevisiae is the most employed strain for fermentation of enzymatic hydrolysate to ethanol because it can tolerate high temperatures and a wide range of pH values (with the acidic pH being the optimum), which makes its fermentation less susceptible to contamination than bacteria. It is also known to tolerate ethanol better than other ethanol-producing microorganisms. *S. cerevisiae* shows a broad substrate utilization, which is important for commercially viable ethanol production. In general, the media used for fermentation consist of yeast extract, peptone, NH_4Cl, KH_2PO_4, and $MgSO_4$, as supplementation of exogenous nitrogen sources enhances sugar utilization and ethanol production. *S. cerevisiae* is also GRAS (generally regarded as safe) for human consumption and therefore easier to handle [27].

Separate hydrolysis and fermentation (SHF) is conventionally used, as the optimal temperatures for enzymatic hydrolysis and fermentation differ. While efficient hydrolysis occurs in the temperature range of 45–50 °C, the optimal temperature range for fermentation is 28–35 °C. However, simultaneous saccharification and fermentation (SSF) has taken precedence in recent research due to its advantages. SSF is considered a less capital-intensive process due to its lower overall processing times, eliminating the need for separate reactors and providing higher ethanol yields, which correspond to increased conversion of xylose to xylitol under SSF conditions [46].

Table 2 shows that ethanol yields are either detected using HPLC or GC-FID techniques. The percentage of the theoretical yield is calculated by dividing the amount of ethanol obtained (g) by the amount of hexoses in the pulp (g), assuming that all the hexoses were available for fermentation, with a fermentation stoichiometric yield of 0.51 g (ethanol)/g (hexose), and multiplying by 100% [47]. The ethanol yield increase is calculated as shown in Equation (2).

$$\text{Ethanol yield increase} = \frac{\text{Ethanol conc. after combined pretreatment} - \text{Ethanol conc. after sole pretreatment}}{\text{Ethanol conc. after sole pretreatment}} \times 100. \quad (2)$$

Table 2. Ethanol yields of various biological–chemical/physicochemical pretreatment strategies.

Substrate	1st Step	2nd Step	Hydrolysis and Fermentation (Ethanol Concentration Detection Technique)	Ethanol Yield Increase (%)	Ethanol Concentration (% Theoretical Ethanol Yield)	Reference
Biological—Alkaline Pretreatment						
Pinus Radiata	*Gloeophyllum trabeum* (27 °C, 28 d)	25% *w*/*w* NaOH (180 °C, 5 h)	SHF: 5% substrate consistency. Celluclast (20 FPU/g) and β-glucosidase (40 UI/g) (50 °C, 24 h, 150 rpm). 3 g/L *Saccharomyces cerevisiae* IR2-9a (40 °C, 96 h, 150 rpm). (GC-FID)	−37.5% * compared to alkaline alone	99.55 mL/kg wood (33.98%)	Fissore et al. (2010) [48]
Wheat straw (*Triticum aestivum*)	*P. subvermispora* (28 °C, 21 d)	0.1% NaOH (5% *w*/*v*) (50 °C, 1 h, 165 rpm)	SHF: Cellulase (15 FPU/g) and xylanase (30 U/g) (50 °C, 60 h, 165 rpm). 0.5 g/L *Saccharomyces cerevisiae* (Fermentis LPA 3035) (32 °C, 24 h, 200 rpm). (GC-FID)	79.41% * compared to alkaline alone	122 ± 8 mg/g substrate (62%)	Salvachúa et al. (2011) [49]
	I. lacteus (28 °C, 21 d)			80.88% * compared to alkaline alone	123 ± 5 mg/g substrate (62%)	
Biological—Acid Pretreatment						
Water hyacinth (*Eichhornia crassipes*)	*Echinodontium Taxodii* (28 °C, 10 d)	0.25% H_2SO_4 (100 °C, 1 h)	SHF: 2% substrate consistency; Cellulase (30 FPU/g) (50 °C, 48 h). 0.3% *v*/*v* activated *Saccharomyces cerevisiae* (40 °C, 72 h, 100 rpm for first 8 h). (HPLC)	31.51% * (1.34-fold increase) compared to acid alone	0.192 g/g of dry material (sole acid = 0.146 g/g of dry material)	Ma et al. (2010) [50]
Glycyrrhiza uralensis Fisch. Ex DC (GUR)	*Phanerochaete chrysosporium* (28 °C, 21 d)	2.5% H_2SO_4 (100 °C, 2.5 h)	Cellulase (30 FPU/g) (50 °C, 48 h); heterotrophic cultivation of *C. prototheocoides* for microalgal oil production. (28 °C, 7 d, 200 rpm) (GC-MS)	1.34-fold increase relative to acid treatment alone	1.66 g/L (oil content)	Gui et al. (2013) [51]
Glycyrrhiza uralensis Fisch. Ex DC (GUR)	*Phanerochaete chrysosporium* (28 °C, 21 d)	2M acetic acid (100 °C, 3 h)	Cellulase (40 FPU/g) (50 °C, 48 h); heterotrophic cultivation of *C. prototheocoides* (28 °C, 120 h, 200 rpm) (GC-MS)	1.54 *-fold increase relative to acid treatment alone	1.91 g/L (oil content)	Gui et al. (2014) [52]
Oil palm empty fruit bunches (OPEFB)	*Pleurotus floridanus* LIPIMC996 (31 °C, 28 d)	Ball-milled at 29.6/s for 4 min; phosphoric acid treatment (50 °C, 5 h)	SSF: Enzymatic hydrolysis (60 FPU/g cellulose). *Saccharomyces cerevisiae* CBS 8066 (35 °C, 48 h, 130 rpm) (HPLC)	7.39% * increase relative to acid treatment alone	21.8 g/L (62.8%)	Ishola et al. (2014) [53]
Water hyacinth	*Phanerochaete chrysosporium* (30 °C, 60 h, 150 rpm)	1% H_2SO_4 (100 °C, 1 h)	6 g/L *Saccharomyces cerevisiae* (30 °C, 24 h, 120 rpm) (GC)	8.61% increase relative to acid treatment alone	1.40 g/L	Zhang et al. (2018) [54]
Biological—Organosolv Pretreatment						
Sapwood of beech (*Fagus crenata*)	*C. subvermispora* FP90031 (28 °C, 56 d)	60% (*v*/*v*) ethanol solution (180 °C, 2 h)	SSF: Cellulase (10 FPU/0.25 g). 10% *v*/*v* *S. cerevisiae* AM12 (40 °C, 96 h, 100 rpm) (GC-FID)	1.6-fold increase relative to ethanolysis treatment alone	0.176 g/g of wood	Itoh et al. (2003) [55]

Table 2. *Cont.*

Substrate	1st Step	2nd Step	Hydrolysis and Fermentation (Ethanol Concentration Detection Technique)	Ethanol Yield Increase (%)	Ethanol Concentration (% Theoretical Ethanol Yield)	Reference
Pinus radiata wood 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 min; hot alkaline wash: 1% NaOH (75 °C, 1 h)	SHF: Cellulase (20 FPU/g glucan) and β-glucosidase (40 CBU/g glucan) (50 °C, 72 h, 150 rpm); *Saccharomyces cerevisiae* Y-1528 (30 °C, 48 h, 150 rpm) (GC-FID); SSF: 2% substrate consistency; Cellulase (20 FPU/g glucan) and β-glucosidase (40 CBU/g glucan) (50 °C, 72 h, 150 rpm). *Saccharomyces cerevisiae* Y-1528 (37 °C, 48 h, 150 rpm) (GC-FID)	SHF: 61.90% *; SSF: 458% * compared to control	SHF: 136 g/kg wood (37%) SSF: 162 g/kg wood (44%)	Muñoz et al. (2007) [47]
Acacia dealbata wood 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 min; hot alkaline wash: 1% NaOH (75 °C, 1 h)	SHF: Cellulase (20 FPU/g glucan) and β-glucosidase (40 CBU/g glucan) (50 °C, 72 h, 150 rpm); *Saccharomyces cerevisiae* Y-1528 (30 °C, 48 h, 150 rpm) (GC-FID); SSF: 2% substrate consistency; Cellulase (20 FPU/g glucan) and β-glucosidase (40 CBU/g glucan). *Saccharomyces cerevisiae* Y-1528 (37 °C, 48 h, 150 rpm) (GC-FID)	SHF: −7.14% *; SSF: 4.28% * compared to control	SHF: 143 g/kg wood (48%) SSF: 195 g/kg wood (65%)	Muñoz et al. (2007) [47]
Pinus radiata	*Gloeophyllum trabeum* (27 °C, 28 d)	60% ethanol in water solvent (200 °C, 1 h)	SSF: Celluclast (20 FPU/g) and β-glucosidase (40 UI/g) (50 °C, 24 h, 150 rpm); 3 g/L *Saccharomyces cerevisiae* IR2-9a (40 °C, 96 h, 150 rpm) (GC-FID)	44.6%	210 mL/kg wood (72%)	Fissore et al. (2010) [48]
Pinus radiata wood chips	*Gloephyllum trabeum* ATCC 11539 (25 °C, 21 d)	Biopulp: 95% ethanol in water solvent (60:40 *v*/*v* ratio) with 0.13% H_2SO_4 (*w*/*v*) (185 °C, 18 min) Control pulp: 95% ethanol in water solvent (60:40 *v*/*v* ratio) with 0.13% H_2SO_4 (*w*/*v*) (200 °C, 32 min)	SSF: 10% substrate consistency; Celluclast (20 FPU/g), β-glucosidase Novozymes 188 (40 IU/g); 6.0 g/L *Saccharomyces cerevisiae* IR2T9 (40 °C, 96 h, 150 rpm) (GC-FID)	Similar yield in both control pulp and biopulp	161 g/kg wood (63.8%)	Monrroy et al. (2010) [46]
Japanese cedar *(Cryptomeria japonica)*	*Phellinus* sp. SKM2102 (28 °C, 56 d)	Ethanol/lactic acid/water (40:10:50, *w*/*w*) (190 °C, 30 min)	SSF: Meicelase (10 FPU/0.25 g); 10% *v*/*v* *S. cerevisiae* AM12 (35 °C, 72 h, 100 rpm) (GC-FID)	NA	8.94 g/L (28.3%)	Baba et al. (2011) [56]
	C. subvermispora FP-90031-sp (28 °C, 56 d)	Ethanol/lactic acid/water (40:10:50, *w*/*w*) (200 °C, 1 h)			9.82 g/L (31.1%)	

Table 2. *Cont.*

Substrate	1st Step	2nd Step	Hydrolysis and Fermentation (Ethanol Concentration Detection Technique)	Ethanol Yield Increase (%)	Ethanol Concentration (% Theoretical Ethanol Yield)	Reference
Biological—Steam Explosion Pretreatment						
Sawtooth oak, corn, and bran	*Lentinula edodes* (120 d)	Steam explosion (214 °C, 5 min, 20 atm)	SSF: 0.1 g enzyme/g substrate Meicelase (45 °C, 48 h, 140 strokes/min); *Saccharomyces cerevisiae* AM 12 (40 °C, 24 h, 100 rpm) (HPLC)	49.68% * increase compared to spent shitake mushroom medium	23.8 g/L (87.6%)	Asada et al. (2011) [57]

* As calculated by the authors using the data provided in the research article. GC-FID: gas chromatography detector; HPLC: high-performance liquid chromatography.

3.1. Combined Biological–Alkaline Pretreatment

Fissore et al. (2010) and Salvachúa et al. (2011) [48,49] studied biological followed by alkaline treatment of wood and wheat straw, respectively. Brown rot pretreatment of *Pinus radiata* wood chips led to carbohydrate degradation, whereas lignin is not severely attacked. The alkaline pretreatment of bio-treated wood chips further favored the removal of short cellulose chains, leaving a high amount of residual lignin in the pulp. The low pulp yield and low carbohydrate retention led to a decrease in enzymatic hydrolysis and fermentation efficiency of the wood in biochemical treatment as compared to alkaline pretreatment alone [48]. *I. lacteus* and *P. subvermispora* pretreatment of wheat straw showed intermediate levels of glucose consumption, and no inhibitors of the fermenting yeast were generated in the process of alkaline pretreatment. Consequently, a 90% conversion of glucose to ethanol was achieved, resulting in an ethanol yield of 62% in both cases [49].

3.2. Combined Biological–Acid Pretreatment

Zhang et al. (2018) [54] compared the combination of biological and mild acid pretreatment with the combination of biological and dilute alkaline pretreatment for water hyacinth biomass. They reported that although alkaline treatment showed the most effective lignin removal ability, combined biological—acid treatment resulted in increased reducing sugar content, as more cellulose was preserved [54]. Ma et al. (2010) [50] also confirmed that combined biological–acid pretreatment stimulated improved ethanol fermentation by increasing glucose concentration, decreasing the fermentation inhibitors, or producing fermentation accelerants. Therefore, microbial–acid pretreatment is considered to be superior for ethanol production from water hyacinth. Ma et al. (2010) [50] studied SHF, whereas Zhang et al. (2018) [54] studied SSF with *Saccharomyces cerevisiae* on water hyacinth pretreated with combined biological–acid treatment. By adding cellulase enzyme and conducting hydrolysis and fermentation subsequently (SHF) under optimized conditions, Ma et al. (2010) [50] obtained a greater increase in ethanol yield as compared to Zhang et al. (2018) [54]. Pretreatment of oil palm empty fruit bunches (OPEFB) using fungal pretreatment, phosphoric acid pretreatment, and the combination of both methods produced maximum ethanol concentrations of 6.8 g/L, 20.3 g/L, and 21.8 g/L, respectively. However, the percentage of the theoretical yield of ethanol was 27.9% in 72 h in the case of fungal pretreatment and 89.4% and 62.8% in 48 h in the case of phosphoric acid and combined pretreatment, respectively. In the case of combined pretreatment, a material loss of 63.6% occurred, resulting in a decreased ethanol yield. However, superior theoretical yield can be obtained by phosphoric acid alone or in combined pretreatment than by fungal pretreatment alone [53]. Gui et al. (2013 and 2014) [51,52] investigated combined *Phanerochaete chrysosporium*—acid pretreatment (2.5% H_2SO_4 and 2M acetic acid, respectively) on *Glycyrrhiza uralensis Fisch. Ex DC* (Chinese licorice). Biomass growth and oil production by *C. protothecoides* were higher in the cotreated samples than in samples acid-pretreated samples alone. Combined biological and acetic acid achieved slightly better results than

combined biological and sulfuric acid pretreatment, possibly due to either a reduction in the number of inhibitors generated or due to an increase in the production of certain growth accelerants (such as proteins, amino acids, or other components) during combined pretreatment [51,52].

3.3. Combined Biological—Organosolv Pretreatment

Fungal–organosolv pretreatment is the most studied biological–chemical pretreatment for ethanol production. Itoh et al. (2003) [55] compared four strains for fungal pretreatment of sapwood of beech and their ethanol yield. They reported that fungal treatments with *D. squalens* and *C. subvermispora* improved the ethanol yield, whereas *P. ostreatus* and *C. versicolor* did not result in a significant increase. The yield of ethanol increased with an increase in ethanolysis temperature, but the combination with fungal treatment helped to decrease the ethanolysis temperature to below 200 °C, thereby saving 15% of the electricity needed for ethanolysis. Fungal pretreatment with *C. subvermispora* improved the separation of cellulose and hemicellulose components in ethanolysis at 180 °C, leading to 82% recovery of carbohydrates and therefore a significant increase in ethanol yield [55].

Muñoz et al. (2007) [47] studied bio-organosolv pretreatment on both *P. radiata* and *A. dealbata* wood chips. Due to the low lignin content in *A. dealbata*, the glucan-to-glucose conversion was rapid, and therefore, fermentation led to a higher ethanol yield relative to that of *P. radiata.* Overall, there was still a low wood-to-ethanol conversion rate due to low pulp yield in the case of *A. dealbata* and due to the high residual lignin content in *P. radiata* pulp. The low ethanol yield was not associated with the action of inhibitors but with low pulp consistency during enzymatic hydrolysis. This could be concluded because most of the yeast inhibitors generated from the pretreatment step are removed during the washing step. During SHF and SSF of both *P. radiata* and *A. dealbata,* the effect of ethanol catabolism was observed, which was attributed to low pulp consistency (2%), leading to low concentrations of fermentable carbohydrates in the medium to be used by the yeast. The low sugar concentration causes substantial catabolic ethanol oxidation by the yeast and therefore low ethanol yields due to 'diauxic shift'. The diauxic shift occurs due to a change in the metabolism of *S. cerevisiae* from fermentation to respiration when glucose is exhausted. Comparatively, SSF produced higher ethanol yields of bio-organosolv-pretreated *P. radiata* and *A. dealbata* wood chips after a longer processing time as compared with SHF. This could be a consequence of a lower enzymatic hydrolytic rate under SSF conditions. However, untreated *P. radiata* wood chips produced higher ethanol yields under SHF conditions than under SSF conditions, which was attributed to high lignin content in *P. radiata*, which deterred the enzyme action at the low temperature (37 °C) used in SSF as compared to the optimized SHF conditions in (hydrolysis at 50 °C and fermentation at 30 °C) [47]. Similarly, biotreatment was found to improve the hydrolysis rate and result in a higher glucan-to-glucose conversion rate in the case of a study conducted by Fissore et al. (2010) [48]. Moreover, organosolv pulping was superior to alkaline pulping of biotreated samples, resulting in 72% of the maximum possible ethanol yield due to the selective delignification action by organosolv, which helped to retain more carbohydrates in the pulp, whereas alkaline delignification favored the removal of short cellulose chains [48]. Monrroy et al. (2010) [46] conducted a similar study to that of Fissore et al. (2010) [48] and found that when subjected to an organosolv process, biotreated and control pulps produced similar ethanol yields. However, the organosolv process conditions required for biotreated pulp were much less severe due to the improvement in solvent accessibility. As stated in many previous reviews, physical structural features that are relevant for conversion of LCBs to liquid fuel, such as reduction in lignin content, decrease in cellulose crystallinity, increase in pore volume, and decrease in particle size, are better in biotreated biomass compared to controls, which improves solvent accessibility [46]. Similarly, when white-rot fungi were studied in combination with mild ethanolysis without acid, a significant improvement was observed in the ethanol yield of softwood [56].

3.4. Combined Biological–Steam Explosion Pretreatment

Fungi–steam explosion-pretreated biomass produced a water-extractive fraction that contained 5-hydroxymethylfurfural (HMF) and furfurals, as well as sugars and organic acids. Therefore, to achieve efficient ethanol production, water extraction was necessary to remove the HMF and furfurals, which are inhibitors of alcohol fermentation. In a study by Asada et al. (2011) [57], without water extraction, glucose accumulation was observed during SSF, although no ethanol was produced. A probable explanation is that *S. cerevisiae* was inhibited by water-soluble lignin, furfural, HMF, and organic acids. Consequently, a water extraction step was included, and to reduce the cost of ethanol separation from the fermentation broth, SSF was conducted with a high substrate concentration. Furthermore, an ethanol conversion rate of 87.6% was achieved at 100 g/L of substrate concentration, whereas a further increase in substrate concentration to 300 g/L produced the highest ethanol yield of 38.8 g/L after 60 h of incubation. On the other hand, *S. cerevisiae* stopped the uptake of glucose after 35 h of incubation, after which, with an increase in incubation time, only glucose was accumulated in the fermentation broth. Additionally, beyond a substrate concentration of 100 g/L, the ethanol conversion rate declined as lignin separated at a high concentration inhibited the growth of S. cerevisiae. Therefore, a substrate concentration of 200 g/L was estimated to be optimal to produce the maximum ethanol concentration.

According to the various articles analyzed in this section, fermentation efficiency and production costs depends on various factors, such as substrate consistency, total enzyme load-to-substrate ratio, and glucose loss due to fungal decay. A high substrate concentration inhibits enzyme action due to high residual lignin content and increased inhibitory substance concentration and faces problems such as mixing and mass transfer in the fermentation process. The hydrolysis rate depends on the total enzyme-to-substrate ratio, so lowering the substrate consistency while increasing the enzyme load increases the yield, as well as the cost of the process. Therefore, fermentation strategies need to be improved to achieve high substrate concentration to produce increased ethanol yields per batch while reducing the cost. Considering the cost of ethanol separation from the fermentation broth, strategies to continuously remove inhibitory materials in the fermentation broth need to be developed [48,57].

4. Properties of the Employed Microorganisms in the Pretreatment Step

According to the articles reviewed by Meenakshisundaram et al. (2021) [8], 40 microbial strains have been used for combined biological–chemical/physicochemical pretreatment studies, of which 33 are fungi and 7 are bacteria (represented in green in the phylogenetic tree in Figure 1. The use of bacteria for the degradation of lignin has not been extensively studied compared to fungal degradation. Bacteria produce secondary metabolites and use extracellular enzymes for the breakdown of lignocelluloses, although delignification has been classically reported as slower and more limited [58]. Bacteria from phyla Proteobacteria, Actinobacteria, and Firmicutes are known to produce ligninolytic enzymes and are major decomposers of lignocelluloses in soils. Actinobacteria and Proteobacteria use lignin depolymerization, aromatic compound catabolism, and specific product biosynthesis for lignin breakdown [59]. Actinobacteria are not represented in Figure 1 Because they are mostly studied for enzyme production by growing on pretreated biomass rather than being used in the pretreatment step for LCB degradation. This is because they are mostly cellulolytic bacteria. Similarly, *Bacillus* sp., which belongs to Firmicutes, although known for its lignin-degrading abilities, is increasingly exploited for cellulolytic enzymes because its mechanism is still unknown and its lignin-degrading rate is generally lower than that of fungi [60,61]. *Pseudomonas putida* of the phyla beta-Proteobacteria and *Cupriavidus basilensis* B-8 of the gamma-Proteobacteria phyla are well known for their degrading abilities by producing manganese peroxidase. Bacteroides, such as *Sphingobacterium*, produce manganese superoxide dismutase, which uses a hydroxyl radical mechanism to oxidize lignin. These findings with respect to the mechanism of LCB degradation indicate that the use of bacteria for lignocellulosic biomass pretreatment

could prove to be more important than previously thought, especially because bacterial growth time is lower than that of fungi. It is interesting to study more bacteria and their enzymes for biofuel production, as they can be engineered and can exist under a range of environmental conditions [58,59,62].

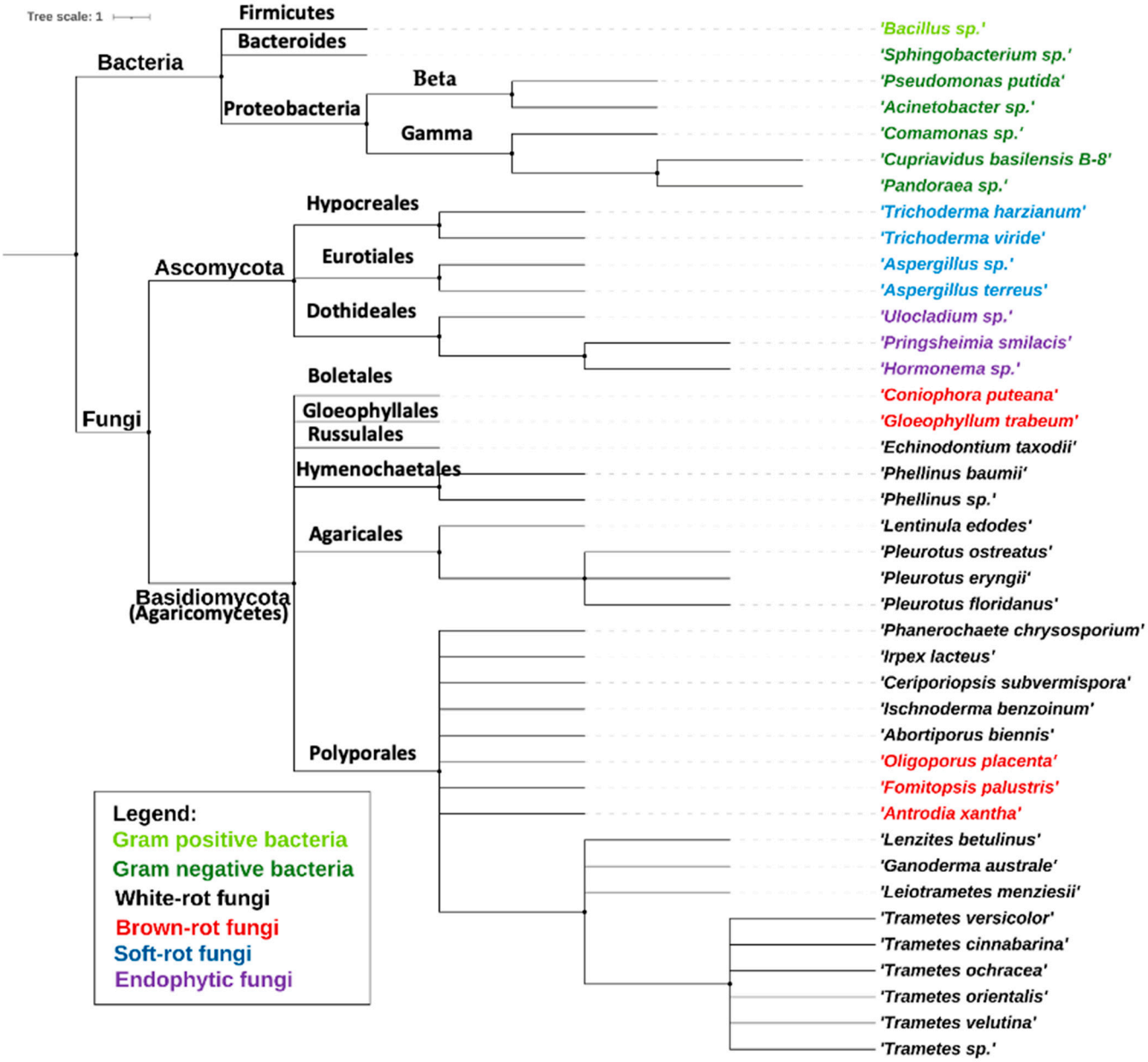

Figure 1. Phylogenetic tree of the microbes used in combined pretreatment of lignocellulosic biomass (only the microbes used in the studies compared in the review of Meenakshisundaram et al. (2021) [8] are represented here). The data were input in the NCBI taxonomy browser, a PHYLIP tree file was generated, which uploaded on the iTOL website, and the phylogenetic tree image was generated.

The fungi that include species from Ascomycota and Basidiomycota phyla are well-known for their decaying capacities using ligninolytic enzymes. Most soft rot fungi (shown in Figure 1 in blue and purple) belong to Ascomycota, and they typically attack the outer surface of wood in relatively wet environments, leading to softening of the wood. The enzymes laccases and peroxidases, which are produced by soft rot fungi, are unspecified and more limited in function, and very little is known about their degradation mechanism [63]. Endophytic fungi, on the other hand, do not cause any harm to the host plant

and require healthy plants to sustain their life while helping the host to resist diseases and droughts. Endophytes are an emerging group of fungi used for biofuel production, as they produce hydrocarbons and use plant components. Their contribution to the formation of crude oil has been proven. Therefore, it is interesting to study the pathways through which endophytes aid in biofuel production and to utilize the secondary products for industrial applications [64]. The Basidiomycota phylum is predominant, particularly the class Agaricomycetes (as shown in Figure 1, which are well known as wood-decomposing fungi. Agaricomycetes are mushroom-forming fungi that display two main modes of deadwood decay, i.e., white rot and brown rot. White-rot fungi degrade lignin, as well as parts of hemicelluloses and cellulose, leaving a bleached residue. Brown rot, on the other hand, attacks the hemicellulose and cellulose, with only minor modifications to lignin, leaving a brownish residue [65]. Brown rot fungi are the major component of forest soils and litter, and to metabolize the holocellulose component of wood, they secrete both enzymatic and non-enzymatic degradative metabolites. The molecular size of secreted enzymes is too large to penetrate the pore structure of wood. Therefore, it is hypothesized that low-molecular-weight compounds diffuse into the cell wall and are used to catalyze the production of hydroxyl radicals in a Fenton-like mediated reaction. Most brown-rot fungi produce endoglucanases to cleave the β-1,4 glucosidic linkages and β-glucosidases to hydrolyze cellobiose or other short oligosaccharides. They also produce several endo-xylanases and β-xylosidases, which are required for the breakdown of hemicelluloses. Brown-rot fungi, such as *Coniophora puteana*, can produce cellobiohydrolase, an exo-cleaving enzyme that acts on cellulose. Owing to their selective holocellulose-degrading ability, only five fungi used in the studies reviewed here are brown rot (shown in red in Figure 1. White-rot fungi also use both enzymatic and non-enzymatic systems to preferentially attack the hemicellulose and lignin in wood. White-rot fungi use enzymes such as lignin peroxidase, manganese peroxidase, and laccase for the ligninolytic activity, whereas endo-glucanases and exo-glucanases are both produced to synergistically act on crystalline cellulose. The metals and radical ions generated through enzymatic action aid in the non-enzymatic penetration mechanism into the wood [58,65–68]. The majority of strains studied for lignocellulose degradation belong to the order of Polyporales within the class Agaricomycetes. Six strains of Polyporales belong to the *Trametes* genus, which belongs to white-rot fungi. The abundance of studies using white-rot fungi is due to the preference for highly selective lignin biodegradation to ensure a cellulose-rich substrate for biofuel production. Considering that the type of lignocellulolytic enzymes produced is limited to the type of strain, one of the best approaches to improve biofuel production of pure culture pretreatment is to combine it with other pretreatment methods [68].

5. New Trends in Microorganisms Used in Downstream Process

Existing commercial fungal pretreatment technology for the AD process only uses aerobic fungi, which could impose facility-related costs, mainly for sugar production due to low sugar efficiency and high retention time. On the other hand, using anaerobic fungi could reduce the long retention time, owing to their capacity for simultaneous biological pretreatment and AD processing. This would also help to reduced capital investments required for a separate aerobic pretreatment reactor. Potential anaerobic fungi for lignocellulosic biomass degradation can be selected among those found in digestive tracts and feces of ruminant and non-ruminant herbivores [14]. For example, Dollhofer et al. (2015) [69] studied anaerobic fungi isolated from rumen fluid of a cow and of a chamois, such as *Neocallimastigomycota*, which aid in in the decay of the major portion of consumed fodder. The lignin is mechanically disintegrated by the growth and expansion of the rhizoids of Neocallimastigales, making cellulose and hemicellulose accessible for further attacks. Furthermore, they possess highly efficient cellulases and several enzymes needed to catabolize hemicelluloses. The carbohydrates are further metabolized to produce compounds that provide energy in the form of ATP for fungal growth and that are also possible substrates for methanogens. This syntrophic interaction is advantageous because

the energy in biomass is captured and converted mostly to methane without loss by respiration as occurs during an aerobic pretreatment process. Although these anaerobic fungi have been observed to accelerate the degradation of dry matter and produce an initial increase in biogas production, it is followed by an increase in the concentration of volatile fatty acids (VFAs), leading to a requirement for separate hydrolytic and fermentation phases in an AD process. Therefore, anaerobic lignocellulolytic fungi could be used as a cost-efficient method to circumnavigate the bottlenecks associated with hydrolysis [69]. Cheng et al. (2009) [70], Jin et al. (2011) [71], and Procházka et al. (2012) [72] all reported an increase in methane production within 3–7 days when a simple coculture of anaerobic fungus and methanogens was used to treat LCBs. Another promising strategy that has demonstrated improved methane yield from LCBs is the addition of rumen fluids, which contain several microbial communities that are able to simultaneously secrete multiple digestive enzymes, such as cellulase, hemicellulose, and β-glycosidase. These enzymes help to gradually degrade the LCB components to VFAs [73]. These projects have not been translated to large-scale applications due to the difficulty of using strictly anaerobic microbes and keeping the digesters strictly anaerobic throughout the fermentation. Further research is required to determine the optimal conditions for such cocultures [70]. Conventionally, oxygen is known to inhibit the AD process; however, recently, microaeration, which introduces a very limited supply of oxygen, has been proposed as an alternative technique to improve AD efficiency. Microaeration enhances the abundance of facultative bacteria, such as phylum Firmicutes, during the hydrolysis phase, and this increased growth rate results in increased content of hydrolytic enzymes. Consequently, a shorter lag phase and improved hydrolysis rate are achieved. The introduction of limited amounts of oxygen was not lethal to methanogens but led to an increase in oxytolerant genera due to acclimatization. These shifts in the microbial community structure are responsible for the improvement of the anaerobic digestion efficiency of cellulosic substrates [74].

The direct addition of selected strains or mixed cultures to anaerobic digesters is called bioaugmentation, which helps to improve the catabolism of resistant material, such as lignocellulosic biomass. It is also an environment-friendly and cost-effective form of biological pretreatment, although some view it as an improved inoculation method to increase methane yield [68]. Considering bioaugmentation as a pretreatment step, Hu et al. (2016), Mulat et al. (2018), and Sträuber et al. (2015) [75–77] combined bioaugmentation and chemical pretreatment to enhance biomethane yields. Although the maximum methane yield was only slightly improved in these studies, the lag phases were slightly shorter than their non-bioaugmented counterparts [76].

For ethanol fermentation, direct microbial conversion (DMC), as well as simultaneous saccharification and co-fermentation (SSCF) strategies are being tested. In DMC, a mono- or coculture of microorganisms is used for cellulase production, biomass hydrolysis, and ethanol fermentation in a single reactor, reducing the capital investment required, as bacteria, such as *Clostridium thermocellum*, and some fungi, including *Neurospora crassa*, *Fusarium oxysporum*, and *Paecilomyces* sp., have been shown to produce cellulases and aid in the direct fermentation of cellulose to ethanol. However, due to the long fermentation period required (3–12 days) and low ethanol yields, it is not yet regarded as an efficient process. SSCF differs from DMC in that it uses a combination of microorganisms sequentially in different fermentation periods for improved utilization of sugars [78]. Although *S. cerevisiae* is known to be a robust organism for ethanol production, it cannot utilize the pentose sugar xylose. Other yeasts, such as species belonging to the *Pichia* and *Candida* genera, can utilize C-5 sugars, but their ethanol production rate is very low compared to that of *S. cerevisiae*. Therefore, for LCBs such as sugarcane bagasse and rice straw, which contain more than 20% xylose, *S. cerevisiae* is employed in the first phase of fermentation for utilization of hexose sugars, followed by *Candida shehatae* in the second phase for pentose utilization. Here, the microorganism is chosen based on its compatibility with pH and temperature during the operating phase [79]. Still, high ethanol yields are not achieved; therefore, genetic engineering has been applied to develop robust strains capable of fermenting pentoses

to obtain higher yields. Several genetically modified microorganisms, such as *P. stipitis* BCC15191, *P. stipitis* NRRL Y-7124 recombinant *E. coli* KO11, *C. shehatae* NCL-3501, and *S. cerevisiae* ATCC 26603, have been developed for a wide range of monomer utilization [78]. Kun et al. (2019) [80] comprehensively reviewed the progress and possibilities of strain engineering of filamentous fungi for improved enzyme production to aid in the degradation of lignocellulosic biomass. Additional research on such genetically modified fungi strains and their application in effective submerged or solid-state fermentation processes can help to establish a bio-based economy on a large scale [80].

6. Challenges and Possible Solutions

The major challenge associated with scaling up lignocellulosic biofuels is the cost factor. Pretreatment accounts for 17% of production costs. Because pretreatment steps sometimes involve acids/alkali or solvents, as well as operation at high temperature and pressure, special corrosion-resistant equipment is required, increasing capital costs. To reduce operational costs, solvent losses need to be minimized while maximizing biomass loading. Operational parameters need to be optimized to identify process parameters that have the greatest economic impact so that these issues can be addressed. Because the energy content of LCBs is not fully utilized in the production of biogas or bioethanol alone, a combination of anaerobic digestion and fermentation processes from the same pretreated biomass could help to overcome these limits [81]. Experimental results reported by Cesaro and Belgiorno (2015) [15] show that the stillage obtained as a byproduct of fermentation to produce ethanol still has the organic potential to be transformed into methane. Moreover, fermentation acts as a pretreatment step, reducing the energy requirements to convert the stillage into biogas. Research and investigation are required to produce valuable byproducts during the pretreatment process, which could also maximize the cost-effectiveness of the process [7]. Therefore, the development of mass and energy balance could help to take complete advantage of biomass energetic potential while reducing costs [15]. A vast number of variables, such as biomass type, the interaction of the biomass with the pretreatment method, energy requirements, etc., are involved in mass and energy balance. Recently, computational tools have been increasingly used to advance the understanding of experimental pretreatment results and predict the efficiency, economic viability, and sustainability of the process. These machine learning approaches require large databases to generate predictive models of biomass pretreatment efficacy and biofuel yield. State-of-the-art visualization technologies, such as Raman spectroscopy, atomic force microscopy, and fluorescent labeling, could produce more data to contribute to the understanding of the effect of pretreatment on biomass [82]. Hence, reviews such as this article can help to fill the knowledge gap to develop simulation tools and support synergy between computational and experimental studies with respect to the development of full-scale viable lignocellulosic biomass conversion processes [83].

7. Conclusions

Lignocellulosic biomass is a sustainable bioenergy source of the future. The choice of combined pretreatment strategies for LCB degradation depends on the downstream process. Combining two fungi or two enzymes does not improve the biogas yield, owing to slow degradation due to competition between the two fungi or excessive carbohydrate removal by two enzymes. Combining fungal pretreatment with alkaline pretreatment has been reported to improve the biogas yield. However, the combination of enzyme with alkaline pretreatment was reported to achieve superior results, as fungal growth uses some nutrients that are essential for microorganisms in the AD process. Only alkaline pretreatment has been studied to date for biological–chemical combined pretreatment for biogas production. Nevertheless, the combination of biological and other chemical methods seems to provide a vast scope for research and process development for biogas production. Ethanol yield varies depending on the fermentation strategy and substrate consistency when the same type of combined pretreatment is applied. Low substrate consistency leads

to a diauxic shift, whereas high substrate consistency leads to inhibition of enzyme action. When significant delignification occurs, SSF is effective, whereas, for biomass with higher lignin content, SHF is effective. For bioethanol production, the combination of fungal and organosolv pretreatment appears to be the most studied process. Currently, strategies to co-ferment *S. cerevisiae* with other strains that utilize pentose sugars are being developed to obtain higher yields of ethanol. The use of metabolic engineering to develop strains that are applicable in a wide range of environments for biofuel production is also an emerging field of study. Furthermore, the use of bacteria for LCB degradation is interesting, as they can be easily engineered and can exist under various environmental conditions. Currently, bacteria are increasingly used for ligninolytic enzyme production, as the extent of delignification using bacteria has not reached the efficiency of white-rot fungi. Bioaugmentation with anaerobic fungi for biogas production and genetic engineering of ethanol fermenting strains are emerging fields to improve biofuel yields.

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