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SYSTEMATIC STUDENT REVIEW

Methods for Improving Anaerobic Lignocellulosic Substrates Degradation for Enhanced Biogas Production

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Abstract A successful biogas production process depends upon adequate hydrolysis of macromolecules in the substrate and stable further conversion. The complex and rigid structure of cellulosic, hemicellulosic and lignin chain is preventing lignocellulosic biomass to reach efficient hydrolysis, therefore pretreatment of a substrate is needed for higher biogas and methane yields. There are several different physical and chemical methods of pretreatments available which include the usage of acids, alkalis, organic solvents, ionic detergents, steam, high pressure, grinding, ultrasound, and microwave irradiation. Physico-chemical pretreatments act rapidly on lignocellulose but their upscaling is very expensive in industry. Many studies have been made in finding the best combination of different pretreatment methods and also new biological techniques which could make lignocellulose pretreatment cheaper and environmentally more friendly. Using natural abilities of different fungi, bacteria or yeast to degrade lignocellulose simplifies the whole process. Also cocktails of biotechnologically produced enzymes are effective in degrading lignocellulose.

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Introduction

Energy crops and harvesting residues, manure, household waste, wood industry waste and brewery waste represent a huge source of lignocellulosic material useful for energy production. Biomass is therefore one of the most important energy sources in the world, apart from coal, oil and natural gas, because it is renewable and always available. It is ecologically friendly as its usage helps in decreasing greenhouse gasses emissions. Much less noxious emissions are produced from biomethane than from gasoline or diesel fuel [1]. There is approximately three times more energy stored in methane compared to hydrogen fuel and also transportation and storage of methane are more appropriate [2]. The prices of fossil fuels are increasing constantly so using biomass offers a great economical potential. Energy produced from biogas can be used for electricity generation, heat production or as direct fuel. This article is reviewing the obstacles in lignocellulosic substrates degradation and pointing out chemical, physical, and biological techniques of substrate pretreatments to increase the level of substrate degradation during anaerobic digestion for maximizing the yield of produced biogas.

Biogas Production Process

Anaerobic digestion of lignocellulosic materials is too slow in nature to be applicable in biogas production at industry level. Poor lignin degradation, production of toxic digested

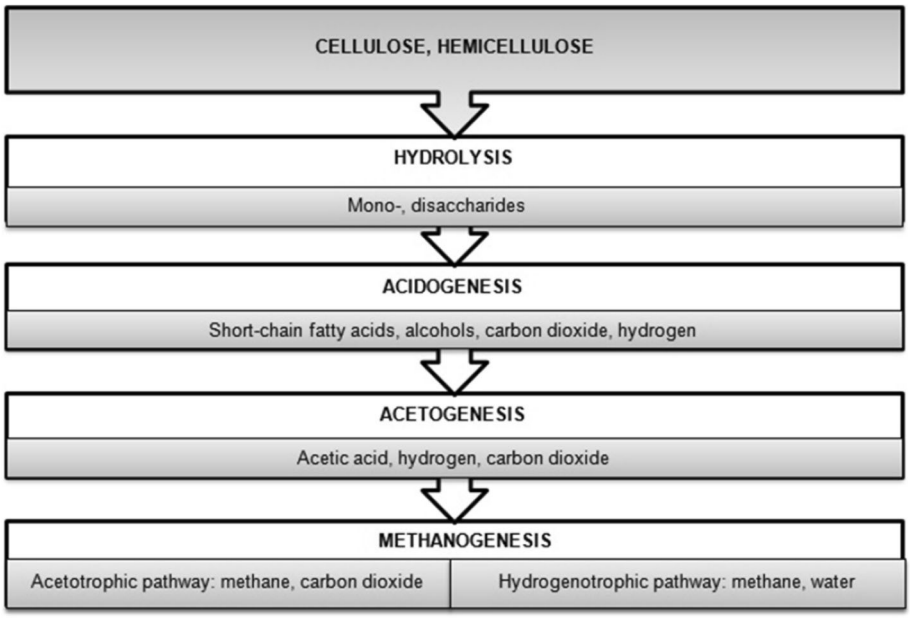

Fig. 1 Schematic overview of biogas production process from cellulose and hemicelluloses describing four main steps: hydrolysis, acidogenesis, acetogenesis and methanogenesis (summarized from [10–12])

intermediates and crystalline cellulose are the reasons for poor hydrolysis and unsuccessful biogas production which is an anaerobic multi-step bioprocess where bacterial and archaeal microbial communities interact [3]. Biogas is the main product of this process and it consists of methane (55–65 %), carbon dioxide (35–45 %), and trace gasses such as hydrogen (1 %), hydrogen sulfide, nitrogen, and carbon monoxide [4]. The content of methane correlates to the usage of biogas. Biogas containing at least 60 % of methane is usually burned for heating. When biogas is purified, it can gain the same composition as natural gas and can be used as a pure fuel. Methane concentrations up to 95 % can be achieved by the separation of gas mixture with special-purpose gas-treating systems and membrane modules, and this biogas can be used in the electricity generation and in combustion engines [1, 5–7].

Biogas production process consists of four steps: hydrolysis, acidogenesis, acetogenesis, and methanogenesis (Fig. 1). Hydrolysis represents degradation of complex organic substances to smaller units which is driven by the excreted enzymes of hydrolytic and fermentative bacteria. Acidogenic bacteria digest the hydrolyzed substrates and produce short chain fatty acids (SCFA) and hydrogen. Acetogenic bacteria oxidize alcohols and SCFA with more than two carbons and produce acetate, carbon dioxide and hydrogen. The biogas process is terminated by methanogenic archaea which convert acetate to methane and carbon dioxide or reduce carbon dioxide with hydrogen to produce methane [8]. In anaerobic digesters, about 70 % of methane is produced via acetotrophic methanogenesis pathway, while the rest is generated via hydrogenotrophic methanogenesis pathway [9].

Structure of Lignocellulosic Substrates and Their Degradation

Plant biomass consists of cellulose, hemicelluloses, lignin, organic soluble extracts (terpenes, fatty acids, esters, triglycerides, waxes, alcohols, tannins, flavonoids), lipids, proteins, sugars, starch, water, carbohydrates, pectins and inorganic compounds. Hard wood contains more cellulose and hemicelluloses, whereas soft wood contains more lignin. Cellulose and hemicellulose are large carbohydrates that form a lignocellulosic fraction together with lignin [13]. The ratio of their content varies and is specific for each lignocellulosic feedstock but in general, there is about 45 % of cellulose, 30 % of hemicelluloses and up to 25 % of lignin [14–16]. Methane potential of lignocellulosic substrates is correlated to the sum of their cellulose and lignin content. The higher the sum of cellulose and lignin, the lower is the methane potential [17].

Cellulosic microfibrils are attached to each other by hemicelluloses and amorphous polymers (sugars, pectin), stabilized by hydrogen bonds and covered by lignin. The structure of these microfibrils is so tightly packed that enzymes or even water molecules cannot enter the lignocellulosic net. Complex structure of 4-*O*-β-D-glucopyranosyl-D-glucose bonded with β-(1,4)-glycoside bond makes cellulose generally insoluble and impedes the access of acids and enzymes. The amorphous parts of cellulose, which have a less-ordered structure, and parts with low degree of polymerization are more vulnerable to biodegradation than crystalline regions with high molecular weight and highly ordered tertiary structure [18]. While cellulose is assembled almost exclusively of glucose monomers, hemicellulose consists of

different monosaccharide units (arabinose, xylose, mannose, galactose and rhamnose). Hemicelluloses are soluble in alkali and acid and are the most thermal-chemically sensitive part of lignocellulosic material; therefore they are easily hydrolyzed [15, 19, 20].

Many bacteria and fungi produce cellulolytic enzymes like endoglucanases, exoglucanases and β-glucosidases, which represent the degradation system of enzymes that act synergistically and catalyze cellulose hydrolysis. Endoglucanases cleave inner bonds of cellulose chain, while exoglucanases cleave the ends of the chain and as a result cellobiose is released. β-glucosidases act on oligosaccharides and cellobiose and release monomers of glucose. Endo-1,4-β-xylanases, β-xylosidases, α-glucuronidases, α-L-arabinofuranosidases and acetilxylan esterases are among enzymes which degrade xylan present in hard wood, while glucomannan from soft wood is degraded by β-mananases and β-mannosidases [21].

Lignin, the third most abundant polymer in nature right after cellulose and hemicelluloses [15], is not composed from sugars, therefore it stays intact and presents a big challenge to discover the degradation process for the energy-producing industry. Lignin is a complex hydrophobic polymer of aromatic compounds where phenylpropane units (syringyl, guaiacyl and *p*-hydroxyphenyl) are bond together into a three-dimensional structure. These units are three monolignol monomers *p*-coumaryl alcohol, coniferyl alcohol and sinapyl alcohol [22] which are methoxylated to various degrees and their ratio varies among different plants, wood tissues and cell wall layers [14]. In hard wood, lignin is covalently bonded to xylan and to galactomannan in soft woods [19]. Recalcitrance of lignocellulosic biomass depends on lignin content, amount of ferulate cross-linking in lignin, subunit composition of lignin and the degree of ester linkages between lignin and carbohydrates [23, 24]. Low-molecular alcohols, dioxanes, acetone, pyridine and dimethyl sulfoxides are few solvents that partly solubilize lignin. Rigidity of lignin structure is also disrupted at higher temperatures which is often applied in depolymerization reactions with acids or alkalis [25].

Lignin degradation can occur either in aerobic or anaerobic conditions. Aerobic degradation of lignin is an oxidative process, which is a part of secondary metabolism of white and brown rot fungi where extracellular phenol oxidases (lignin peroxidases, manganese peroxidases, hybrid peroxidases, laccases, alcohol oxidases, glyoxal oxidases, aryl-alcohol oxidases, versatile peroxidases and quinone reductases) [26] produce hydrogen peroxide as a byproduct, which also acts on cellulose [27]. Other degradation byproducts are phenoxic radicals of phenols which are problematic for the stability of microbial community in a bioreactor since phenols inhibit certain methanogenic microorganisms [28].

Depolymerization of lignin by extracellular enzymes demands oxygen, therefore this type of lignin degradation under anaerobic conditions is impossible. It has long been assumed that lignin appears inert and recalcitrant to microbial decomposition in anoxic environments [29]. Tarvin and Buswell (1934, cited by [30]) were the first who indicated that aromatic structures can be degraded to methane by mixed sewage sludge digester cultures within anaerobic metabolism. The anaerobic pathway for breakdown of the aromatic ring is quite distinct from the aerobic pathway [30]. Pareek et al. [31] and Ko et al. [32] reported a successful degradation of β-O-4 lignin bond under sulfate-reducing conditions. Kim et al. [33] discovered that lignocellulose degrades under anaerobic conditions by sulfate-reducing bacteria. Methanogenesis is determined by the stability of anaerobic conditions, redox potential, and by the concentration of terminal electron acceptors like sulfate. Sulfate-reducing bacteria have a stronger electron affinity for competitive substrates like hydrogen and acetate than methanogenic archaea, therefore methanogens compete with sulfate-reducing bacteria for the products of fermentation [34]. Due to the complex structure of lignin, microorganisms are incapable to use it directly as a substrate. They degrade it during co-metabolism, while using easily degradable substrates like small sugars [35]. Anaerobic lignin degradation is a process termed oxidation with radicals that are produced during cellulose degradation [27]; therefore degradation of cellulose is needed for further lignin degradation processes. Ko et al. [32] described anaerobic degradation of lignin in three steps: depolymerization, oxidation of side chains, and demethylation.

Improving Degradation of Lignocellulose by Substrate Pretreatment

Direct microbial enzymatic hydrolysis of lignocellulose produces less than 20 % of glucose from cellulosic fraction [36] and methane yields usually don't exceed 60 % of the theoretical value (475 L CH_4/kg_{VS}), therefore pretreatment of the substrate is needed to make the holocelluloses more accessible to microbial attack and to enhance hydrolysis. Cleaving of lignin–carbohydrate complexes (LCC) bonds and removing of lignin still remain the major obstacle to the biodegradability of lignocellulosic substrate. When LCC bonds are cleaved, cellulose and hemicelluloses are much more accessible for enzymatic hydrolysis and fermentation [15]. There have been plenty of reports about different substrate pretreatments made over the past 30 years [37, 38]. Improved anaerobic degradation of lignocellulose would result in increased methane yield, the main product of a biogas producing system. The limiting

factor in lignocellulose anaerobic degradation is the initial step of hydrolysis, which is influenced by pH and temperature, substrate chemical composition and the actual substrate surface available for enzymatic attack. Therefore, substrates composed of large particles are slowly degraded due to the reduced surface area [39]. Pretreatment effects on lignocellulose depend on the substrate composition and lignin content [40], which depends on plant species and time of harvest [41].

There are five types of action that pretreatment can offer: particle size reduction, solubilisation, biodegradability enhancement, formation of toxic compounds and loss of organic material [42]. On the other hand, pretreatment can also alter substrate characteristics and, therefore, decrease biodegradability. Namely, thermal method with temperatures above 160 °C can induce the formation of toxic compounds like furfural, hydrolymethylfurfural and soluble phenolic compounds [43] and resulting reduction in organic material content leads to poor methane yield [37]. To make substrate more available to degrading enzymes, structure of lignocellulosic biomass needs to be changed. Pretreating lignocellulosic substrates offers enlargement of the substrate surface and improved porosity, changes the structure of lignin and enables its removal. Pretreatments also facilitate depolymerization of hemicellulose or remove it from the core of the substrate and reduce cellulose crystallinity. The level of degradation is highly associated to the level of crystallinity, which represents one of the major obstacles in lignocellulosic biomass degradation in energy industry. Many of pretreatment techniques have been tested already but only a few have been further developed into applicable technologies. Pretreatment applicability and efficiency are evaluated by the cellulose degradation level and adequacy of the pretreatment process using different substrates [20].

Chemical and Physical Pretreatments

Chemical Pretreatments

Chemical approach is one among the most studied pretreatment possibilities and originally it was developed for delignification of cellulosic materials in paper industry. They usually include sodium hydroxide, perchloric or peracetic acid, organic solvent, acid hydrolysis with sulfur or formic acid and ammonium fiber explosion (AFEX). In combination with enzyme hydrolysis, efficient lignin removal strategy is prior processing by organic solvents like ethanol, methanol or acetone at high temperatures. Pretreatments with alkali hydrolysis are preferred for lignin removal ([20, 44]), though steam and combinations of steam with acids or alkalis are also useful techniques [21], but chemicals make the whole process more expensive and environmentally less friendly. Mok and Antal [45] showed that hot water and high pressure can unchain up to 22 % of cellulose, 60 % of lignin and all of hemicelluloses. Pretreating the substrate with ionic solvents is a novel method with great perspective [46, 47], though it has not been evaluated for its effect on upscalling yet. Almost 100 % hemicelluloses removal can be achieved by dilute-acid pretreatment [44].

Physical Pretreatments

The physical pretreatment methods comprise ball milling, compression milling, cryo-milling, ultrasound and steam treatments, to name just a few. The common feature is reducing the substrate particles size and therefore enlarging the substrate surface available for enzymatic degradation. The reduced size after chipping is usually 10–30 and 0.2–2 mm after milling or grinding [15]. Grinding lignocellulosic substrate promotes the rate and extent of enzymatic hydrolysis by reducing the size of substrate and degree of crystallinity of lignocelluloses [44]. Ball milling improves hydrolysis to similar yields as steam explosion [48], where substrate is subjected to high temperatures by steam injection, while pressure is quickly released into the tank. Untreated wheat straw cellulose solubilisation resulted in less than 4 % glucose hydrolysis yield, while ball milling and also steam explosion resulted in enhanced cellulose solubilisation and greater hydrolysis yields (approximately 40 %). Thermal pretreatment with high pressure and the addition of oxygen, termed wet oxidation, is most commonly used for pretreating lignocellulosic substrates preceding bioethanol fermentation [49], although it is known to cause the loss of organic material [37]. Improved anaerobic digestibility of lignocellulose by microwave irradiation has also been proved to be successful [50].

The formation of microbial cellulase inhibitors is among the reported negative effects of physical pretreatments [51]. The energy input in the physical pretreatments is higher than the theoretical energy content available in the biomass, therefore physical pretreatments are expensive and difficult to scale-up [14, 20].

Effectiveness Comparison

Bruni et al. [52] compared the effectiveness of different pretreatment procedures on the enhancement of biogas production from biofibers separated from digested manure. The highest methane yield (66 % higher compared to untreated biofibers) was obtained by chemical pretreatment (addition of CaO at 15 °C for 25 days). Tedesco et al. [53] examined the effectiveness of a Hollander beater mechanical pretreatment of macroalgae *Laminaria* spp.

biomass on biogas and methane yield. 10 min of beating and incubation at 50 °C resulted in 52 % higher biogas yield and 53 % methane yield improvement compared to untreated substrate.

Sežun et al. [54] tested the influence of mechanical and chemical pretreatments on anaerobic digestion of brewery spent grain. Mechanical pretreatment included homogenization of brewery spent grain with Ultraturrax (10 min at 7,200 rpm), which fragmented substrate particles to the mean particle size below 0.5 mm. Chemical pretreatment included maceration of alkalized (addition of 20 % NaOH, pH 10.0) or acidified (addition of 37 % HCl, pH 2.0) substrate for 5 days at room temperature and combination of chemical processing with thermal pretreatment, where acidified substrate was heated to 140 °C for 2 h in pressurized thermo-chemical stirred vessel. They concluded that different pretreatment procedures did not prevent the inhibition of intermediate lignocellulosic biodegradation products (*p*-cresol) on methanogenic process in biogas production. Mechanical and thermo-chemical pretreatment only delayed the inhibition for about 60 days in comparison with chemically pretreated substrate. Over 90 % of lignin degradation was achieved with chemical pretreatment, while there was very little effect at mechanical and thermo-chemical pretreatments [55]. They agreed that the degree of lignin degradation adversely affects the digestion efficiency as lignin degradation products, released during thermo-chemical pretreatment, are stronger inhibitors to anaerobic degradation than lignin itself.

Biological Pretreatments

Biological pretreatments involving wood degrading white-, brown-, soft-rot fungi, bacteria or enzymes that selectively degrade lignin, cellulose and hemicelluloses are more favorable than chemical or physical pretreatments on account of lower process expenses, less energy needs, big product yields, reduction in quantity of digestate [56–58], no chemical requirement and no byproducts are generated which would represent a potential threat to the environment. Time of biological pretreatment is usually longer and requires strict control of growth conditions and that is a major drawback in applying fungi and microorganisms or their enzymes in enhancing lignocellulose substrate degradation. Many microorganisms are capable of degrading and using cellulose and hemicellulose as a source of carbon for energy, and are useful for substrate pretreatments for their ability to improve enzymatic hydrolysis. Lignolytic enzymes are able to increase the substrate surface area, alternate lignin structure, and solubilize lignin, while xylanases can solubilize hemicelluloses [15].

There are different process configurations of pretreatment possible. Hydrolysis and fermentation can be separated where each step can be carried out at its optimum conditions. Saccharification and fermentation can also be simultaneous where integration of process steps results in lower process cost and efficient process design.

Essential Hydrolytic Enzymes

Only microorganisms with sufficient levels of extracellular cellulolytic system, like endoglucanases (1,4-β-D-glucan-4-glucanohydrolases, exoglucanases (1,4-β-D-glucan glucanohydrolases, also known as cellodextrinases), cellobiohydrolases (1,4-β-D-glucan cellobiohydrolases) and β-glucosidases (β-glucoside glucohydrolases), are efficient cellulose degraders [59–61]. Usually the levels of β-glucosidases are lower compared to other degrading enzymes, so they are often added to process sludge. Endoxylanases and β-xylosidases degrade xylan into xylose, endomannanases and β-mannosidases degrade mannan and pectinases degrade pectin (Fig. 2) [62]. Cellobiose, a hydrolyzed product of cellulose, has been found to inhibit the synthesis of endo- and exoglucanases via feedback loop [63], therefore it is best to construct a special bioreactor where cellobiose would be continuously removed from hydrolysis sludge and degraded to glucose separately [21]. Several fungi and bacteria can be used for lignin removal from lignocellulosic substrates and also for the removal of specific components such as antimicrobial substances [64].

Cellulose degradation is affected by different physical and chemical factors as cellulases are inducible enzymes. Optimal pH is unique for each microorganism producing cellulases, but in general it is best between pH values 4.6 and 7. Generally 50–65 °C is the optimal temperature for degrading cellulose, while 25–50 °C is best for the growth of microorganisms. It has been reported that temperatures above 65 °C cause reduction of cellulase activity due to the enzyme denaturation [21]. Some thermophilic fungi grow best at 45–50 °C, and their cellulases are most active at 50–78 °C [66]. Steiner et al. [67] report that the addition of nitrogen into the process not only increases the amount of cellulases produced but also increases the price of the whole process. Kumar et al. [21] showed that also phosphorus and carbon are important in the process as potassium dihydrogen phosphate and increasing the concentration of substrate (source of carbon) accelerated cellulase production. Among phenolic compounds, there are certain phenols which induce laccase production (like gallic, tannin, maleic and salicylic acid), but others have an inhibitory effect [68]. Sheweita et al. [69] discovered that sugars like cellobiose, glucose, xylose, saccharose, sorbose, methylglucosides and sugar alcohols increase synthesis of cellulases. To certain concentrations, these sugars

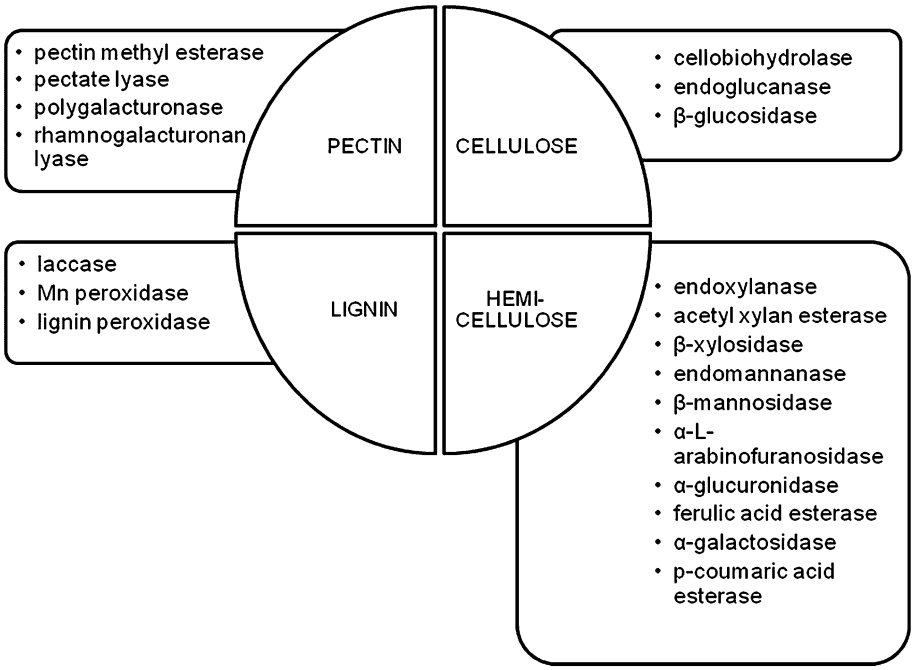

Fig. 2 Principal enzymes degrading lignocellulosic substrates containing cellulose, hemicelluloses, lignin and pectin (summarized from [65])

induce the cellulose regulatory protein, termed cellulose activator molecule (CAM), but higher concentrations are inhibitory.

Lignocellulolytic Fungi

It has been proved that brown- and soft-rot fungi attack cellulose and induce only minor alterations in the lignin structure, while white-rot fungi are more efficient in degrading lignin and are, therefore, the most studied biomass-degrading microorganism [70, 71]. Soft-rot fungus *Trichoderma reesei* is most frequently reported as a suitable source of cellulases because of its efficient extracellular cellulase enzyme system [72], but it also has low glucosidase activity which results in incomplete cellobiose hydrolysis and inhibition of the enzymes [73]. Filamentous fungi (*Aspergillus niger*, *Humicola insolens*, *Thermomonospora fusca*, *Trichoderma reesei*, *Trichoderma longibrachiatum*, *Trichoderma koningii*) are known as efficient producers of xylanases, which are excreted into the medium, and their enzyme levels are higher than those of yeast and bacteria (*Bacillus* sp.) [74]. Anaerobic fungi are capable of degrading cellulosic materials by cellulolytic enzymes from cellulosomes. Also *Chytridiomycetes* like *Anaeromyces*, *Caecomyces*, *Cyllamyces*, *Neocallimastix*, *Orpinomyces*, and *Piromyces* produce lignocellulytic enzymes [75] like lignin peroxidase, manganese peroxidase and laccase which delignify the substrate [76–78]. Experimental results of Akin and Borneman [79] showed that anaerobic fungi from rumen are superior hemicellulose degraders to bacteria and that co-cultivation of anaerobic fungi and methanogens enhances cellulose degradation. Co-cultivation results in a higher yield of digestive enzymes produced and is therefore more efficient for improved lignocelluloses degradation.

Biological pretreatments involving white-rot fungi are usually carried out by solid-state fermentation, where the production of ligninolytic enzymes is higher than in submerged fermentation [80]. Factors like fungal strain, nutrient composition, moisture, aeration, pH and temperature affect the enzyme activity and lignin degradation, therefore monitoring those is needed for an optimal pretreatment and successful performance of white-rot fungi [76].

Lignocellulolytic Bacteria

Also bacteria, which are used in lignocellulosic substrate pretreatment, produce cellulases and ligninases. Anaerobic bacteria like *Clostridium stercorarium*, *Butyrivibrio fibrisolvens*, *Acetivibrio cellulolyticus*, *Bacteroides cellulosolvens*, *Ruminococcus albus*, *Ruminococcus flavefaciens*, *Cellulomonas fimi*, and *Pyrococcus furiosus* produce cellulolytic and hemicellulolytic enzymes. In some anaerobes, like cellulolytic ruminal bacteria, highly active cellulolytic and hemicellulolytic enzymes are combined in extracellular multienzyme complexes, cellulosome [81]. The substrate breakdown is optimized by synergistic action of different catalytic activities and potent binding domains [82–84]. Fungal cellulosomes are less studied than bacterial [75, 85, 86]. It has been proved that degrading potential of cellulosomes is greater than that of free enzymes [65].

Hetero-fermentative bacteria are a better choice as an inoculum for a bacterial pretreatment of lignocellulosic substrate [57]. They facilitate the production of intermediates for methanogens and are therefore more beneficial for successful anaerobic digestion compared to homo-fermentative bacteria [87].

The most important mannanase producing bacteria are *Bacillus* spp., *Streptomyces* sp., *Colibacillus cellulovorans*, *Caldicellulosiruptor* sp. Rt8B, and *Caldocellum saccharolyticum* [88–90], although mannan degraders can also be found among yeasts and fungi [91]. Microbial mannanases are usually excreted to the medium [92].

Rumen fluid microbial communities are often used as a seeding material in anaerobic digestion to increase the hydrolysis of lignocellulosic substrate and the production of fatty acids [93]. Anaerobic fermentation of corn stover by rumen microorganisms in batch reactors resulted in 70 % of high volatile solids conversion efficiency after 240 h incubation at 25–40 °C and 66 % of volatile solids conversion was achieved by incubating lignocellulosic aquatic plant cattail (*Typha latifolia linn*) with rumen microorganisms for 125 h in batch reactors [94, 95].

Zhong et al. [96] used a community of different microorganisms for the enhancement of biogas production from corn straw: yeast, cellulolytic bacteria and lactic acid bacteria. Yeast (*Saccharomyces cerevisiae*, *Coccidioides immitis* and *Hansenula anomala*) consumed sugars in the straw and broke down the crystalline areas in cellulose with cellulose fibers exposed to celluloytic bacteria (*Bacillus licheniformis*, *Pseudomonas* spp., *Bacillus subtilis* and *Pleurotus florida*) attack. Lactic acid bacteria (*Lactobacillus deiliehii*) were added for adjusting the pH value. The results showed that the best pretreatment time was 15 days at 20 °C with 0,01 % (w/w) of microbial complex dose. They achieved 33 % higher biogas yield, 75 % higher methane yield and 35 % shorter digestion time compared to the untreated corn straw.

Yan et al. [97] achieved 10–20 % enhancement of methane content in produced biogas and 9 % higher total biogas yield from rice straw, which was pretreated with BYND-5 microbial community for 7 days at 30 °C. The BYND-5 community consisted of *Firmicutes* (6 %), *Bacteroidetes* (40 %), *Deferribacteres* (9 %), *Proteobacteria* (16 %), *Lentisphaerae* (2 %), *Fibrobacteraceae* (2 %) and uncultured bacterium (25 %).

Enzymatic Lignocellulose Pretreatment

Pretreatment of lignocellulosic substrate to facilitate the accessibility of holocellulose (cellulose and hemicelluloses) is neded to increase the biogas potential of lignocellulosic material. Adding exogenous enzymes can improve the performance of anaerobic digestion systems, though they are quite sensible as their activity is affected by substrate, incubation time, system configuration, temperature and pH [38]. Enzymatic hydrolysis has several advantages over acid-based hydrolysis methods as the very mild process conditions give high yields, without any corrosion problems and the pretreatment cost is lower [14]. The biogas yield has accelerated for 19 %, when sugar beet pulp was hydrolyzed with a mix of enzymatic preparations Celustar XL and Agropect pomade with endoglucanase, xylanase and pectinase activities and for 13 % when the spent hops were pretreated with the same enzyme mix [98]. Interestingly, the level of enzymatic hydrolysis can be doubled by ultrasound pretreatment [99]. Despite of usual lignocellulose degradable enzymes, there are also some proteins like glycoside hydrolases from family 61, expansions and swollenin [100–102] that contribute to lignocellulose degradation in way that is not completely understood yet. It is also possible to pretreat lignocellulose substrate only by adding extracellular extract of fungi which contain cellulases, hemicellulases and ligninases. Enzymes used can be either natural or synthetic and they can be immobilized on inert material or float in process sludge. Synthetic enzymes are more stable and have better activity than natural ones, but are usually too expensive for industrial application. Sometimes combinations of enzymes are best for optimal substrate degradation [100, 101] either using customized cocktails of individual enzymes or commercial, crude mixtures of enzymes [65] (some of them are listed in Fig. 2). One of the drawbacks of commercial mixtures is that its content is not fully characterized [100, 101] and they may contain many non-essential enzymes [103]. When selecting individual, purified enzymes, properties like pH and temperature optimum, stability and specific activity should be considered. High specific activity results in less enzyme used and therefore reduced process cost. There are also enzymes with cross-specifity as they act on more than one substrate [104, 105]. Using a multifunctional enzyme reduces a number of enzymes needed for full hydrolysis, thus reducing the cost of the process.

Optimal efficiency and economy of the process can be achieved only by optimal enzyme and substrate loadings. Boussaid and Saddler [106] showed that lignin substrates require higher enzyme loadings as the adsorption of enzymes to lignin is more difficult. Higher enzyme loadings are also needed when using commercial enzyme mixtures because of the lower specific enzyme activity. Amount of enzymes used for bioconversion of lignocellulolytic substrate is therefore an important factor to consider because it dictates the cost of the whole process and its efficiency is measured as grams of sugars produced per mg protein [65]. Kumar and Wyman [107] reported that enzyme loading depends on the type of substrate pretreatment used before.

Table 1 Comparison of different pretreatment abilities to enhance biogas and methane yields in biogas production from lignocellulosic substrates

Pretreatment type	Lignocellulosic substrate	Pretreatment agent	Process type	Biogas production	Methane production	References
Chemical	Manure biofibers	NaOH, steam, laccases	Batch	–	+34 %	[52]
		CaO		–	+66 %	
Mechanical	Macroalgae	Beating	Batch	+52 %	+53 %	[53]
Biological	Corn straw	Yeast, cellulolytic bacteria, lactic acid bacteria	Continuous	+33 %	+75 %	[96]
	Rice straw	BYND-5 microbial consortium	Batch	+9 %	+10–20 %	[97]
	Maize	Additive (bacteria + enzymes)	Batch	+12 %	–	[109]
		Additive (bacteria + fungi)		+10 %	–	
		Additive (bacteria + yeast)		+15 %	–	
Combined	Leaves	NaOH, cellulase	Batch	–	78 %	[111]

Substrates pretreated with alkalis needed higher enzyme loadings than pretreatments with acids or hot water for the same level of hydrolysis achieved. The reason lies in the hemicellulose that remains after alkali pretreatment and additional xylanases are needed for full degradation [108].

Further research is focused on enzymes, found in extremely thermophilic microorganisms, which are insensitive to acid and heat [14] and would be useful in improving the processing of lignocellulosic biomass.

Comparison of Different Biological Pretreatments

Divergent biological ensilage additives containing yeasts or enzymes have also been proven to enhance the hydrolysis step in the anaerobic digestion process by decomposing complex carbohydrate structures of maize [109]. 7 weeks long pretreatment of maize with a complex additive containing hetero- and homo-fermentative bacteria (Silasil Energy®) as well as enzymes α-amylase, cellulase, hemicellulase and pentosanase (Sil-all 4 × 4®) or fungi and yeasts (Microferm®) increased the biogas production from pretreated maize per organic dry matter for 12, 10 and 15 %, respectively. Adding a common microbial inoculum (Bonsilage Mais®) containing only homo-fermentative and hetero-fermentative bacteria had no effect on biogas and methane production compared to the non-pretreated substrate.

Using vital microorganisms is more efficient than free enzymes as microorganisms can regenerate and produce different useful enzymes at same time depending on the given substrate [38]. The most accelerated biogas production and the highest methane yields can be achieved by combining pretreatment types and using mixed cellulolytic cultures (Table 1). A pretreatment process where lignin is separated from cellulose and hemicelluloses is better than enzymatic hydrolysis of the lignocellulosic biomass. The removal of lignin increases the rate of hydrolysis; otherwise lignin inhibits the hydrolysis because of blocking the access to the cellulose and irreversibly binding to hydrolytic enzymes [110].

Bruni et al. [52] increased the methane yield for 34 % pretreating manure fibers with steam and NaOH (160 °C for 15 min) and subsequent enzymatic treatment with laccases (37 °C for 20 h). Juntarasiri et al. [111] found the best solution for methane production from redwood (*Pterocarpus indicus* Wild) leaves in combination with industrial wastewater, which was soaking biomass in NaOH for 24 h, rinsing and then soaking in cellulases for 24 h. This two-step pretreatment resulted in biogas containing up to 78 % methane.

Conclusion

The availability of lignocellulosic biomass as a disposal of different agri-food industries catapulted it among top ecologically friendly candidate substrates for usage in biogas industry. Limited hydrolysis of lignocellulose is the reason why this substrate presents uncertainty of techno-economic feasibility, particularly at large scale production. There have been quite some pretreatment methods developed. Mainly physical and chemical methods are nowadays in use in industrial scale, as research focused on biological pretreatment methods started much later. The advantages of slow, but cheap and environmentally friendly biological pretreatment methods are slowly displacing the disadvantages of quick and powerful but expensive physico-chemical pretreatments. Enzyme hydrolysis is still a major technological and economical bottleneck in the conversion of lignocelluloses to biogas. Combinations of pretreatment types and biological pretreatments using mixed cellulolytic cultures offer the highest yields of biogas and methane in biogas production from lignocellulosic substrates. Substrate pretreatments can represent an expensive part of the

biogas production process, but untreated substrates cost even more [112], therefore they are at a crucial step in the production of biogas from lignocellulosic biomass. The type of the substrate, economic assessment and environmental impact should be considered before choosing of the optimal pretreatment.

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