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Pretreatment and Designing Energy Crops: Technological Innovations and Prospects

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ABSTRACT

The key determinants for developing commercially viable bioethanol production are pretreatment, saccharification and fermentation processes. This review discusses the outcome of current research and future prospects in pretreatment technologies and designing biofuel crops for utilization of lignocellulosic biomass. High sugar yield and integration of solutions for overcoming problems related to inhibitory compounds in the biological conversion systems can play the vital role. In the first section, current advances in pretreatment technologies, including high sugar yields, production of inhibitors has been discussed, along with the future prospects of developing energy crops with altered plant cell wall structure, which can significantly reduce the pretreatment and hydrolysis costs.

Key words: Lignocellulose, bioethanol, ionic liquid pretreatment, combinatorial pretreatment, energy crops

INTRODUCTION

Potential of renewable feedstocks like lignocellulosic biomass for bioethanol productions has recently boosted the research on pretreatment technologies, saccharifying hydrolytic enzymes and genetically engineered yeasts for conversion of biomass to ethanol. In whole lignocellulosic ethanol production process, pretreatment is an essential and cost intensive step besides saccharification and fermentation (Mussatto *et al*., 2010). For industrial lignocellulosic bioethanol production, a detailed understanding of pretreatment technologies is essential to achieve high production efficiency. Till date, a number of review articles have been published on current pretreatment technologies (Sindhu *et al*., 2015; Sun and Cheng, 2002; Eggeman and Elander, 2005; Wyman *et al*., 2005; Alvira *et al*., 2010; Parisutham *et al*., 2014), however, there is a paucity of information on interaction of inhibitors with saccharifying enzymes and yeast. To facilitate commercial bioethanol production, study must focus on low cost pretreatment technologies that can integrate with biological conversion systems, without affecting fermentation efficiency. The first section of the study overviews the current developments in pretreatment technologies.

Current emerging genetic engineering strategies in planta, including altering lignin structure, increasing polysaccharide content, expressing cell wall degrading or modifying enzymes in plants can aid in the development of bioenergy crops (Sticklen *et al*., 2014; Guerriero *et al*., 2015; Turumtay, 2015; Welker *et al*., 2015). Thus, the second section addresses the directions of research in plant biology with regards to developing future energy crops to substantially decrease the production cost.

PRETREATMENT CHEMISTRY AND TECHNOLOGIES

Biomass pretreatment is not only the important step to overcome the recalcitrance of lignocellulosic biomass but a critical cost limiting step in bioethanol production; considered as the second most expensive unit cost, only preceded by enzyme cost. Hence, any improvements in the research and development can have potential impact on the improvement of efficiency and lowering the process costs (Mosier *et al*., 2005). Different pretreatment technologies have varied modes of interacting with and breaking down the cell wall components. Feedstocks such as grasses, softwoods and hardwoods vary in their physical and chemical properties; hence, the effectiveness of pretreatments also differs. A large number of pretreatment strategies are currently being employed with different biomass feedstocks and pretreatment conditions. Depending on their effect on cell wall structure most pretreatments are aimed at lignin removal, minimal glucan loss and less inhibitor generation (Ong *et al*., 2014).

Pretreatment processes can widely be grouped as physical, physico-chemical, chemical and biological treatment. The major requirements for efficient and economical pretreatment are: (a) Low recalcitrance of cellulose for efficient enzymatic saccharification, (b) No significant degradation of cellulose and hemicelluloses, (c) Minimum amount of inhibitory compounds generation, (d) Minimum energy demand and instrumentation, (e) Low residue production after process and (f) Effective for a wide range of substrates etc. Moreover, the choice of pretreatment also has a large impact on downstream processing such as saccharification, fermentation, by product utilization and ethanol recovery etc. In this context, the current research scenarios, based on selected pretreatment technologies and their combinations are discussed below. The combination of different pretreatments has been proposed by many research workers to obtain optimal fractionation of different plant components and achieve high yields from biomass.

Physical pretreatment: Ball milling, two roll milling, colloid milling, hammer milling and vibro energy milling are the commonly used physical pretreatment methods. Physical pretreatment is based on the principle of particle size reduction by mechanical stress and thus increase in surface area. Moreover, it leads to decreased degree of polymerization and decrystallization of feedstock. However, this process is not economically feasible due to high energy requirement. Combination of physical pretreatments and other pretreatment is usually used to overcome this.

Physio-chemical treatment: Microwave assisted pretreatment, Ammonia Fiber Explosion (AFEX), steam explosion, liquid hot water and wet oxidation pretreatment comes under the physiochemical pretreatment methods. The major drawbacks of these physiochemical technologies are high operational cost, high energy requirements and inhibitors generation.

Microwave assisted pretreatment: Microwave pretreatment employs microwaves to cause localized heating of biomass leading to destruction of cell wall components thus improving cellulose and hemicellulose accessibility to enzymatic hydrolysis.

Zhu *et al.* (2005) compared the combination pretreatment of rice straw using microwave and alkali and its comparison with the alkali-alone pretreated process. They found that rice straw pretreated by microwave/alkali had a higher hydrolysis rate and glucose content in the hydrolysate in comparison with the one by alkali alone. Hu and Wen (2008) also demonstrated that microwave assisted alkali treatment is an efficient way to improve the enzymatic digestibility of switchgrass with 58.5% sugar yield.

Peng *et al.* (2014) studied the efficiency of particle size, treatment condition and reaction time for bioconversion of microcrystalline cellulose using alkali pretreatment with microwave irradiation. They found that the high concentration of alkali or temperature was necessary in cellulose degradation. However, the viability of using microwaves for lignocellulosic pretreatment requires the critical assessment for economic estimations of the operation costs and benefits.

Ammonia fiber explosion: In AFEX, biomass is treated with liquid anhydrous ammonia at temperature (60-100°C) and high pressure (250-300 psi) for several minutes (<30 min) followed by rapid pressure release. Ammonia fiber explosion process is considered a potential technique for agricultural residue and herbaceous crops. In the AFEX pretreatment, rapid expansion of ammonia causes disruption of lignin-carbohydrate linkage and decrease the cellulose crystallinity. Recently, Cha *et al.* (2014) found 93.6% glucose recovery from rice straw after combined treatment with CO_2 and ammonia. High cost, safety issues and environmental issues are the major drawback of AFEX pretreatment. Suitable condition has to be maintained for an efficient ammonia recovery and recycling to prevent its leakage to the environment.

Steam explosion, liquid hot water and wet oxidation: Steam explosion, liquid hot water and wet oxidation are water based physiochemical pretreatment methods for destruction of cell wall components. In steam explosion method biomass is subjected to pressurised steam for a period of time ranging from seconds to several minutes and then suddenly depressurised. Liquid hot water uses water at high temperature (160-230°C) and pressure (>5 MPa) in order to maintain water in the liquid state and in contact with biomass. While, in wet oxidation air or oxygen is employed in combination with water at elevated temperature and pressure.

A 74% of glucose yield was obtained from hemp (*Cannabis sativa*) after combined pretreatment of steam explosion and dilute acid (Kuglarz *et al*., 2014). Combination of wet oxidation and dilute acid was found effective for the pretreatment of *Miscanthus* with 82.4 and 63.7%, xylose and glucose yields respectively by Sorensen *et al.* (2008). Biswas *et al.* (2014) studied the optimal conditions of wet oxidation pretreatment for sugarcane bagasse and found wet oxidation pretreatment at 185°C with oxygen (0.6 MPa) yielded 87.4% glucose from sugarcane bagasse.

Chemical pretreatment: In this pretreatment most of the pretreatment technologies are represented which differ in their chemistries and their mode of cell wall destruction and modification. Many reports are available on chemical pretreatments with wide range of feedstocks variability. Leading chemical pretreatment technologies such as acidic, alkaline and ionic liquid pretreatments are included in this section.

Acidic pretreatment: In acidic pretreatment, at high temperature hemicellulose gets hydrolyzed which increases the porosity and improves hydrolysis of cellulose. Several acidic pretreatment methodologies have been studied. Sulphuric acid is the most commonly used acid while other acids such as hydrochloric and nitric acids were also tested for pretreatment of biomass.

Industrially, dilute acid are favoured over concentrated acid pretreatment as it generates lower amounts of inhibitors and reduces operational cost. Dilute acid pretreatment typically using sulphuric acid is done at high temperature (e.g., 180°C) for a shorter retention time or at lower temperature (120°C) for a longer period of time. Using the same acid, Hsu *et al.* (2010) achieved 83% of sugar yield and 70% hydrolysis efficiency from rice straw. They also correlated their findings with structural properties of biomass using FTIR analysis. Chiesa and Gnansounou (2014) compared the dilute acid and dilute alkali pretreatment on Empty Fruit Bunches (EFBs) from oil palm tree and found higher sugar yield of 85.5% from dilute acid compared to 42.6% from alkali pretreatment. This result proved that favourable pretreatment conditions reduced the inhibitors formation.

Optimization of pretreatment conditions with an aim to lower inhibitors production was conducted for rapeseed straw using dilute sulphuric acid at high solid content with lower inhibitors generation (Lu *et al*., 2009). Similar to this, recently Rajan and Carrier (2014) have also reported higher sugar yield with 37% reduction of inhibitors production from wheat straw using dilute sulphuric acid. At the high temperature and pressures often used in the industrial process, generation of furfural, HMF, formic acid and levulinic acid compounds can have negative effects on the downstream processes. Moreover, acid pretreatment requires washing of the cellulose rich slurry fraction after pretreatment and or detoxification of the hydrolyzate before fermentation.

Alkaline pretreatment: In alkaline pretreatment biomass is treated with alkaline catalyst such as calcium hydrooxide (lime), ammonia, potassium or sodium hydroxide at normal temperature and pressure. In this process lignin-carbohydrate ester linkage and hemicelluloses acetyl groups are removed and thus the accessibility of hydrolysis gets enhanced. Combination of 2% NaOH/121°C (15 psi)/60 min achieved the highest delignification (39.34%) from Sawtooth Oak (*Quercus acutissima*) shell as well as the highest sugar release of 426.36 mg g^{-1} pretreated material (Yang *et al*., 2015). Combination of NaOH (0.1 g g^{-1} of raw biomass) and lime (0.2 g g^{-1} of raw substrate) with a residence time of 6 h were used to treat switchgrass and a 59.4% glucose and 57.3% xylose yield were achieved (Xu and Cheng, 2011). Low cost of lime and reducing NaOH loading reduces the chemical cost.

Combination of dilute hydrochloric acid and lime pretreatment of corn stover using two stage pretreatment process was studied and 78.0% glucose and 97% xylose yield were reported (Zu *et al*., 2014). In one of our study while comparing the pretreatment methods of acid, alkali and biological pretreatment for weedy biomass *Parthenium*, alkali pretreatment (1% NaOH) was found more effective with releasing 513.1 mg gds^{-1} of reducing sugar (Pandiyan *et al*., 2014).

Alkaline pretreatment are operated at lower temperature and does not require complex reactors. But for industrial scale development possible loss of fermentation sugars and production of inhibitory compounds must be taken in consideration to optimize the pretreatment conditions.

Ozonolysis and organosol: Other widely used chemical pretreatment methods are ozonolysis and organosol. Organosol process usually involves extraction of lignin from plant biomass using organic solvents and with or without the presence of an acidic catalyst. Ethanol, methanol, acetone, ethylene glycol and tetrahydofurfuryl alcohol, are the commonly used organic solvents. This pretreatment causes breaking of internal lignin and hemicelluloses bonds thus making cellulose more accessible to hydrolysis. The main drawbacks of this process are high operational and dowstream processing cost and strict safety measures as organic solvents are inflammable. In ozonolysis, ozone is used to treat biomass which causes degradation of lignin by attacking aromatic ring structures. This pretreatment usually does not lead to formation of inhibitory compounds as it is performed at room temperature and normal pressure.

Perez-Cantu *et al*. (2013) studied the comparison of three water based pretreatments (Organosol, steam explosion and liquid hot water pretreatment) for bioethanol production from rye straw. They compared the mass balances for cellulose, hemicelluloses and lignin. Organosol showed the best performance followed by steam explosion and liquid hot water with similar yields. The main drawback of this technology is high cost of solvent and catalysis and inhibitors generation. Safety measures are also required for organic solvents as they are inflammable. Combined pretreatment of Japanese cedar using ozonolysis and wet disk milling resulted in higher sugar yield of 68.8% glucose and 43.2% xylose (Miura *et al*., 2012). Disadvantage of this process is the high cost which makes this process economically unviable.

IONIC LIQUID (IL) AND BIOLOGICAL PRETREATMENT AS NON-CONVENTIONAL PRETREATMENT METHODS

Recently, the use of ionic liquids for pretreatment has gained much attention. The ILs are organic salts typically composed of large organic cations and small inorganic anions. At high temperatures (100-150°C) ILs forms hydrogen bonds with cellulose, as a result of this, the intrinsic network of non covalent bonds between cellulose, hemicelluloses and lignin gets effectively disrupted. Many ILs have been reported for their cellulose dissolution capabilities such as 1-allyl-3-methylimidazolium-chloride ([AMIM]Cl), 1-ethyl-3-methylimidazolium-acetate ([EMIM]Ac), 1-butyl-3-methyl imidazo lium-chloride ([BMIM]Cl) and 1-ethyl-3-methylimidazolium di ethyl phosphate ([EMIM] DEP). The ILs are considered as "Green" solvents since no toxic or explosive gases are formed (Zhu *et al.,* 2006). The advantages of ILs pretreatments such as mild operational conditions, high thermal and chemical stability and easily recycle of solvent, etc., offer great potential for future industrial scale applications. Despite its versatility and efficiency, the usage of IL pretreatment is limited because of its high cost involved. For the large scale application of ILs pretreatments, development of energy efficient recycling methods and toxicity to enzymes and fermentative process needs to be investigated.

Similarly, biological pretreatment is attracting a lot of attention as a safe and environmental friendly method for lignin removal from lignocelluloses (Sindhu *et al*., 2015). Unlike other conventional pretreatment methods, biological pretreatment employs microorganisms (mainly white and soft rot fungi) to treat biomass. These organisms degrade lignin through the action of lignin degrading enzymes such as peroxidase and laccases and expose the holocellulose. White Rot Fungi (WRF) have shown varying saccharifying enzymes and rates at which they degrade lignin and carbohydrates in different biomass. Besides WRF, some actinomycetes such as *Streptomyces* and bacteria such as *Azospirullum lipoferrum* and *Bacillus subtilis* also posses lignolytic enzymes (Saritha *et al*., 2012). For biological pretreatment, selective lignin degrading microorganisms with lower cellulase activity are considered as good candidates. Biological pretreatment is considered as environment friendly and have various advantages like low capital cost, lesser energy and chemical intensive. Most lignin degrading microbes typically grow slowly and also consume some part of carbohydrates, so the efficiency and long residence time are the main disadvantages of this pretreatment. In our study pretreatment of weedy biomass *Parthenium* by fungus *Trametes hirsuta,* higher reducing sugar yield of 485.64 mg gds^{-1} (than controls) in 24 h of saccharification with enzyme Accellerase® 1500 was recorded (Rana *et al*., 2013). A new lignolytic micromycete fungus *Myrothecium roridum* LG7 was identified as a potential microbe for biological delignification, which generated reducing sugar yield of 455.81-509.65 mg gds^{-1} from pretreated biomass of *Parthemium* and paddy straw after enzymatic hydrolysis (Tiwari *et al*., 2013a). In our exploration for new sources of hydrolytic enzymes, the sectretome of phytopathogenic fungus *Phoma exigua* was found to possess a cocktail of enzymes. The supplementation of the secretome with commercial β-glucosidase resulted in the significantly higher reducing sugar yield of 651.04 and 698.11 mg gds^{-1} from biopretreated *Parthenium* and paddy straw, respectively (Tiwari *et al*., 2013b). Combinatorial pretreatment of biological pretreatment with other mild pretreatments need to be used to overcome the challenges of biological pretreatment (Yu *et al*., 2009; Ma *et al*., 2010; Zhong *et al*., 2011; Wang *et al*., 2012).

A comparison of leading pretreatment technologies and their sugar yield is presented in Table 1. Inhibitor generation is important factor in evaluating the pretreatment efficiency. Du *et al.* (2010) studied effect of inhibitors formation on corn stover, poplar and pine under eight different chemical conditions. They analyzed forty different inhibitory compounds which

Table 1: Current leading pretreatment technologies

Process	Substrate	Pretreatment conditions and remark	Sugar yield	References
Microwave pretreatment	Switchgrass	Microwave assisted alkali pretreatment (0.1 g/g of alkali loading)	58.5 %	Hu and Wen (2008)
	Rice straw	Optimization of microwave pretreatment conditions. Microwave Intensity (MI) 680 W, Irradiation Time (IT) 24 min and substrate concentration (SC) 75 g L^{-1}, were found to be optimal conditions	37.8, 20.2 and 31.8%cellulose, hemicellulose and total maximal saccharification efficiencies, respectively	Ma *et al.* (2009)
	Water hyacinth	Microwave assisted dilute acid pretreatment (1% v/v H_2SO_4 at 140°C for 15 min)	94.6% of total reducing sugar	Xia *et al.* (2013)
AFEX	Empty palm fruit bunch fiber	AFEX pretreatment at 135°C for 45 min	90% total reducing sugar	Petri and Schmidt-Dannert (2004)
	Rice straw	CO_2 added Ammonia explosion as an effective pretreatment method	93.6 % glucose	Cha *et al.* (2014)
Steam explosion	Industrial hemp (*Cannabis sativa)*	Combined steam explosion and dilute acid (1% w/v sulfuric acid at 180°C)	74 % glucose	Kuglarz *et al.* (2014)
Liquid hot water (LHW) pretreatment	Soyabean straw	Pretreated at 210°C for 10 min	64.55% glucose	Wan *et al.* (2011)
Wet oxidation	*Miscanthus*	Combination of dilute acid soaking (0.75% sulphuric acid at 100°C for 14 h) followed by wet oxidation using both atmospheric air and hydrogen peroxide as the oxidizing agent for 5 min at 170°C	82.4% xylose, 63.7% glucose	Sorensen *et al.* (2008)
	Rice husk	Combination of alkaline (1% w/v H_2O_2 solution) at room temperature followed by wet oxidation pretreatment	21% total glucose	Banerjee *et al.* (2011)
	Sugarcane bagasse	Wet oxidation pretreatment at 185°C with oxygen (0.6 MPa) was found optimal method in pilot scale pretreatment	87.4 % glucose	Biswas *et al.* (2014)
Acid pretreatment	Wheat straw	Dilute acid pretreatment (0.75% v/v H_2SO_4 pretreatment at 121°C for 1 h)	75% of total sugar yield	Saha *et al.* (2005)
	Rice straw	Dilute acid pretreatment using H_2SO_4 (1% w/v at 160°C or 180°C for 5 min)	83% of total sugar yield	Hsu *et al.* (2010)
	Oil palm	Dilute acid pretreatment with 1.5 % v/v H_2SO_4 at 161°C		
		Comparison of dilute acid and dilute alkali pretreatment methods	85.5% glucose	Chiesa and Gnansounou (2014)
	Rapeseed straw	Optimization of H_2SO_4 catalyzed hydrothermal pretreatment conditions at high solid content of 20% 1% (w/w) H_2SO_4 treatment at 180°C for 10 min was found more effective pretreatment with lower inhibitors generation	63.17% glucan,75.12% xylan	Lu *et al.* (2009)
	Wheat straw	Dilute acid pretreatment using 10 dm^3 m^{-3} H_2SO_4 at 140°C for 30 min Optimizing pretreatment conditions reduced inhibitors by 37%	89% of total sugar yield	Rajan and Carrier (2014)
Alkali	Bermuda grass	0.75% w/v NaOH pretreatment at 121°C for 15 min	90.43% glucan, 65.11% xylan	Wang *et al.* (2010)
	Switchgrass	Combination of NaOH (0.10 g /g raw biomass) and Ca $(OH)_2$ (0.02 g/g raw biomass) with a residence time of 6 h	59.4% glucose, 57.3% xylose	Xu and Cheng (2011)
	Sugarcane bagasse	Dilute ammonia (15% w/v) pretreatment at 170°C for 60 min	82.7 % glucose, 47.3 % xylose	Zhang and Wu (2014)
	Wheat straw	Combining alkaline and oxidizing reagents (alkaline peroxide) as an effective pretreatment method (5% w/w H_2O_2, in a solid:liquid ratio of 1:20, pH adjusted to 11.5 with 2 M NaOH)	63.7% glucose, 55.2 xylose	Toquero and Bolado (2014)
	Corn stover	Two stage pretreatment process using dilute HCl at 60°C for 12 h followed by Ca $(OH)_2$ loading of 0.1 g/g of substrate	78.0% glucose, 97.0% xylose	Zu *et al.* (2014)
	Parthenium sp.	Comparison of acid (10% w/v H2SO4 solution for 20 min at 121°C), alkali (1% sodium hydroxide for 1 h) and biological (using *Marasmiellus palmivorus*) pretreatment	61.2, 60.2 and 47.6% total sugar from acid, alkali and biological pretreatment, respectively	Pandiyan *et al.* (2014)

Table 1: Countinue

Process	Substrate	Pretreatment conditions and remark	Sugar yield	References
Ionic Liquid (IL) pretreatment	Switchgrass	Comparison of dilute acid (1.2% w/w sulfuric acid) and IL pretreatment (1-ethyl-3-methylimidazolium acetate ([C2 mim][OAc])	96.0% glucan	Li *et al.* (2010)
	Pine Eucalyptus	Comparison of dilute acid (1.2% w/w sulphuric acid) pre-soaking and IL pretreatment (using ([C2 mim][OAc])	96 % total sugar yields from switchgrass	Li *et al.* (2013)
	Switchgrass Rice straw	Combined method using 10% ammonia and IL (1-ethyl-3-methy limidazolium acetate ([Emim]Ac)	82% cellulose recovery	Nguyen *et al.* (2010)
	Switchgrass	Use of biphasic system using aqueous ammonia to facile recycling of IL and rapid recovery of the sugars	53% glucose, 88% xylose yield	Sun *et al.* (2013)
	Switchgrass	1-ethyl-3-methylimidazolium acetate ([C2mim][OAc]) pretreatment and saccharification in a single vessel	81.2% glucose, 87.4% xylose	Shi *et al.* (2013)
Organosolv pretreatment	Rye straw	Organosolv (using ethanol as organic solvent) pretreatment was found more effective methods amongst steam explosion and liquid hot water pretreatments	89.7% sugar recovery	Perez-Cantu *et al.* (2013)
Ozonolysis	Japanese cedar	Combined ozonolysis and subsequent wet disk milling	68.8 % glucose, 43.2 % xylose	Miura *et al.* (2012)
Biological pretreatment	*Parthenium*	Biological pretreatment using *Trametes hirsuta* for 7 d followed by saccharification using commercial enzyme Accellerase® 1500	52.65 % total sugar yield	Rana *et al.* (2013)
	Parthenium Paddy straw	Biological pretreatment using lignolytic fungus *Myrothecium roridum* LG7 and hydrolysis using commercial enzyme Accellerase® 1500	84.9, 59.21% sugar yield from paddy straw and *Parthenium*, respectively	Tiwari *et al.* (2013a)
	Paddy straw	Biological pretreatment using lignolytic fungus *M. roridum* LG7 and hydrolysis using cocktail of fungus *Phoma* secretome supplemented with commercial commercial β-glucosidase	85.86, 60.18% sugar yield from paddy straw and *Parthenium*, respectively	Tiwari *et al.* (2013b)
	Rice hull	Combined pretreatment with H_2O_2 (2%, 48 h) and fungus *Pleurotus ostreatus* (18 d)	49.6% glucose	Yu *et al.* (2009)
	Water hyacinth	Combination of mild acidic (0.25% H_2SO_4) and biological pretreatment (*Echinodontium taxodii*)	36.6 % total reducing sugar	Ma *et al.* (2010)
	Populus tomentosa	Combination of biological pretreatment using *Lenzites betulina* C5617 with liquid hot water pretreatment	60.26% glucose	Wang *et al.* (2012)
	Corn stover	Microbial pretreatment with *Ceriporiopsis subvermispora* with effects of particle size and moisture content	66.61% glucose, 38.30% xylose	Wan and Li (2010)
	Paddy straw	Biological pretreatment using actinomycete isolate *Streptomyces griseorubens* ssr38 and hydrolysis using commercial enzyme Accellerase® 1500	60.43% total sugar yield	Saritha *et al.* (2013)

highlighted the need for careful selection of pretreatment technologies. Uppugundla *et al.* (2014) published interesting data while comparing three pretreatment technologies, AFEX, dilute acid and IL pretreatments on corn stover. The AFEX treatment showed no significant change, while 85% hemicellulose solubilisation and 90% lignin removal were observed in dilute acid and IL pretreatment, respectively. Fermentation efficiency showed that dilute acid and IL pretreated hydrolysates required exogenous nutrient supplementation while AFEX pretreated hydrolysate did not require nutrient supplementation.

Further insight into biodegradability, hemicellulose accessibility and hydrolysis enzyme adsorption was studied by Kumar and Wyman (2009). Pure avicel glucan and poplar solids were pretreated by AFEX, Ammonia Recycled Percolation (ARP), dilute acid and lime digested to various degrees by cellulase together with β-glucosidase enzymes. Glucan and xylan accessibility were found to be dependent on the pretreatment system applied to poplar solids; however, the specific activity did not decline as drastically as reported in the literature, suggesting that enzyme features and the chemical/physical environment are mainly responsible for the reduced rates in the conversion process. The reduced degree of polymerization on poplar solids from different pretreatment methods suggested that xylan removal had a more severe impact on cellulose chain length than lignin removal.

Despite advances in different pretreatment methodologies in recent years, combination of different pretreatment methods seems to hold promise for the future, as a result of economic viability and higher ethanol yield. Moreover, basic research to understand the plant cell wall structure and pretreatment chemistry are important for developing effective pretreatment technologies. Interestingly, genetic engineering approaches to alter plant cell wall structure can play a crucial role in reducing the pretreatment and hydrolysis costs of biomass. A review on engineering future energy crops to deconstruction of plant cell wall has been discussed further in the second section of this review.

Engineering energy crops: Other than purchasing cost, pretreatment economics is strongly affected by total sugar yields and inhibition of downstream processes caused by sugar degradation products. Modification in plant cell wall either by reduction in recalcitrance, increase in carbohydrate content or expression of hydrolases can greatly influence pretreatment and hydrolysis cost. Recently, several researchers including Sticklen (2008) and Mood *et al.* (2013) have suggested genetic manipulation of energy crops as a promising future prospect of pretreatment. A number of researchers have reported “Engineering future energy crops” for deconstruction of plant cell wall in recent years (Li *et al*., 2008; Taylor *et al*., 2008; Yuan *et al*., 2008; Abramson *et al*., 2013). The strategies to deconstruct plant cell wall can be grouped broadly as lignin modification (content, monolignol composition and degree of polymerization), increasing and altering polysaccharides (content, composition and degree of polymerization) and expressing cell wall-degrading or modifying enzymes in planta.

Modification of lignin: Chen and Dixon (2007) analyzed the relationships between lignin content/composition and chemical/enzymatic saccharification. Weng *et al.* (2008) concluded that the emerging genetic engineering strategies in planta including manipulation of lignin biosynthesis at the regulatory level, controlling monolignol polymerization enzymes and modification of lignin polymer structure, together with the exploration of lignin degradation enzymes from other organisms should aid in the optimization of biofuel production and the development of bioenergy crops.

Fu *et al.* (2011) observed encouraging results on the lignin deconstruction and down-regulation of the switchgrass caffeic acid O-methyl transferase gene which decreases lignin content modestly, reduces the syringyl: guaiacyl lignin monomer ratio and resulted in improved forage quality and most importantly, increased ethanol yield by up to 38% using conventional biomass fermentation processes. The down-regulated lines required less severe pretreatment and lower cellulase dosages for equivalent product yields, using simultaneous saccharification and fermentation with yeast. Furthermore, fermentation of dilute acid-pretreated transgenic switchgrass using *Clostridium thermocellum* with no added enzymes showed better product yields than obtained with unmodified switchgrass. Therefore, this apparent reduction in the recalcitrance of transgenic switchgrass has the potential to lower processing costs for biomass fermentation derived fuels and chemicals significantly. Alternatively, such modified transgenic switchgrass lines should yield significantly more fermentation chemicals per hectare under identical process conditions.

Reduction of lignin in the biofuel crops by genetic methods is considered to be among the most economic means of reducing costs associated with pretreatment and hydrolysis steps. However, potential negative issues such as reduced biomass productivity also need to be considered in including them as long term goals (Hisano *et al*., 2009).

Modification of carbohydrates: Increase in cellulose content and reduction in cellulose crystallinity are potential strategies for altering the carbohydrate content of plant cell wall. The *Cel*A and *csl* have been identified as major gene super families for cellulose biosynthesis. Other genes such as *Korrigan, Cobra* and *Kobito* are also found to be involved in cellulose and cell wall synthesis (Torney *et al*., 2007; Harris *et al*., 2009; Maloney and Mansfield, 2010). Hemicellulose matrix is also found to create cell wall recalcitrants. Alteration of O-acetylation of hemicellulose may also lead to a decrease in acetate content for reduced inhibition of fermentation or altered capacity of hemicelluloses to hydrogen bond with other cell wall polymers (Gille and Pauly, 2012; Xiong *et al*., 2013).

Expression of hydrolases in planta: In planta expression of heterologous cellulases can help in the reduction of cell wall recalcitrance during pretreatment and hydrolysis. Cellobiohydrolase CBH1/CBH2, endoglucanase, exoglucanase, β-glucosidase and xylanase have been expressed in monocot plants, besides dicot plants such as tobacco, *Arabidopsis,* sugarcane, maize and rice (Lopez-Casado *et al*., 2008; Jung *et al*., 2012). Various heterologous hydrolases are expressed in plants, such as thermostable xylanase (XynB) into maize (Shen *et al*., 2012), glycoside hydrolase from *Acidothermus cellulolyticus* endoglucanase I (EI) in tobacco and maize (Brunecky *et al*., 2011), cellobiohydrolase II (*Cel*6A) in maize endosperm (Devaiah *et al.,* 2013), *Acidothermus cellulolyticus* (E1) endo-cellulase in corn plants (Park *et al*., 2011), thermostable endo-1,4-β-glucanase (E1) from *Acidothermus cellulolyticus* into rice (Zhang *et al*., 2012). Sainz (2011) reviewed the expression of cellulases and hemicellulases in biofuel crops and stated that the cellulase expression system in planta will significantly improve the process economics of cellulosic ethanol production. Mood *et al.* (2013) summarised data on the current recombinant cell-wall-deconstructing enzymes in plants and suggested ‘Engineered feedstocks’ as the future bioenergy crops.

CONCLUSION

Pretreatment technologies and understanding their chemistry are important to determine the most effective method of biomass deconstruction. Designing “Energy plants” are considered as future prospects for pretreatment. Implementation of combinatorial pretreatment technologies and designing energy crops as a single step is can be an emerging technology to reduce the cost of bioethanol production. This compilation summarises the inherent advantages and probable road blocks foreseen in the path for process integration in the success of biorefineries for bioethanol production.

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