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Current perspectives in enzymatic saccharification of lignocellulosic biomass

Sunil K Khare[a,*], Ashok Pandey[b], Christian Larroche[c]

[a] Department of Chemistry, Indian Institute of Technology Delhi, New Delhi 110016, India
[b] Biotechnology Division, CSIR-National Institute for Interdisciplinary Science and Technology, Trivandrum 695019, Kerala, India
[c] Polytech Clermont-Ferrand, Université Blaise Pascal, Campus des Cézeaux 4, Avenue des Landais, BP 20206 63174 Aubière Cedex, France

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ABSTRACT

With the depletion of fossil fuel reserves, there is an urgent need to search for renewable and cost effective strategies for biofuel production. Lignocellulosic biomass has been perceived as a potential feedstock, wherein effective pretreatment and saccharification is necessary prerequisite for developing viable biofuel processes. Recent approaches in this context are, (i) studying enzymes from extremophilic organisms, particularly thermophiles which are gaining importance in this aspect as they are found to be stable and catalytically more effective under harsh conditions; (ii) usage of ionic liquids for pretreatment is emerging as a greener technology due to their non toxic nature. Developing/screening for ionic liquid tolerant lignocellulosic enzymes in order to attain simultaneous pretreatment and saccharification, offer an interesting option; and (iii) engineering/manipulating the existing lignocellulosic enzymes for desirable traits and viable saccharification and biofuel generation processes. The review encompasses these approaches and the focus on the recent development in the area.

1. Introduction

Considering the fast depleting fossil sources, there has been a major impetus worldwide to develop alternate source of fuels and bio-based chemicals. In this context, lignocellulosic feedstocks have been perceived as most potential and sustainable source for producing alternate fuels and platform chemicals [1,2]. These include agricultural by-products such as wheat and rice straw, husks, corn stover, corn cobs, bagasse, oilseed cakes, wood, grasses and dedicated energycrops such as miscanthus and switchgrass [3,4]. Extensive researches are underway for developing efficient processes to utilize them for viable production for fuels and chemicals.

The basic lignocellulosic utilization strategy involve three essential and inter-dependent steps namely (i) pretreatment of the biomass, necessary for disrupting lignocellulosic interactions to make cellulose and hemicellulose, and other carbohydrate polymers better accessible for enzymatic hydrolysis in the next step commonly called as saccharification; (ii) saccharification of pretreated material by hydrolases such as cellulases, xylanases, and other carbohydrases; and (iii) appropriate fermentation of monosaccharides, generated out of the saccharification, for production of desirable product viz., biofuels or other platform chemicals. All three processes have been extensively reviewed in the recent years [5–7]. It has been generally agreed that effectiveness of pretreatment and saccharification determines the viability and yield of the fuel or other products in the fermentation step. Number of excellent treatment methods such as acid or alkali treatment, hot water washing, steam explosion and ammonia fibre expansion have been developed in the past [8]. Since composition and structure of biomass varies from source to source, the efficacy of the pretreatment method also varies for each biomass and no single method can be said to be suitable in generic manner. Nonetheless, optimum pretreatment have been standardized for most of the commonly available biomass, which can be recommended and used with required variation.

The sacchrification step still remains as one of the critical bottlenecks. An ideal process should generate stoichiometric amount of fermentable monomeric sugars out of the lignocellulosic complex. Yield attainable so far has been less than satisfactory and rather poor. Lower amount of the fermentable sugar makes product yield unviable in next fermentation step [9,10]. The major issue which needs to be resolved are (i) better access and cellulolytic hydrol-

* Corresponding author at: Enzyme and Microbial Biochemistry Lab, Department of Chemistry, Indian Institute of Technology, Hauz-Khas, New Delhi 110016, India. Tel.: +91 11 26596533x26596597.
E-mail addresses: skhare@rocketmail.com, skkhare@chemistry.iitd.ac.in (S.K. Khare).

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ysis, which means search for kinetically more efficient cellulases and carbohydrases; (ii) whether cellulases can sustain highest catalytic activity under the pre-treatment acidic, alkaline or steam heat conditions. Since other aspects of lignocellulosic utilization such as pretreatment and fermentation have been well discussed in the past [11–13]. This paper specifically focuses on the status of available lignocellulosic enzymes, their hydrolytic efficiency and conversion yield with respect to common lignocellulosic biomass. It further encompasses the recent strategies employed to search for new carbohydrases from (i) extremophilic sources; (ii) enzyme engineering efforts; and (iii) medium engineering such as ionic liquid toward achieving simultaneous saccharification.

2. Recalcitrance in lignocellulosic structure and need for pretreatment

Since each biomass has variable composition and structure, the cellulolytic enzymes cannot act uniformly in all the cases. Nigam et al. [14] have excellently compiled the composition of common lignocellulosic feedstocks. It is quite evident that biomass in general consists of 40–50% cellulose, 25–30% hemicellulose and 15–20% lignin and small amount of other extractives [15].

However, the structural alignment of these component make the lignocellulosic biomass quite recalcitrant against hydrolases, and cellulases in particular, affecting enzyme accessibility, activity and resulting into slow kinetics. It is therefore relevant to briefly consider the structural aspect herein.

The main component of lignocellulose is cellulose, with glucose as monomeric units linked to each other by β (1–4) glycosidic bonds. Each of this polysaccharide chain is hydrogen bonded with the other and remain arranged as different layers. This arrangement contributes to the crystallinity in the cellulose, which impedes the hydrolysis. The hemicelluloses, some amorphous cellulose and other polysaccharide especially pectin form cementing layers with core crystalline cellulose. This structure is finally enveloped by lignin. Each unit is referred as microfibril and many such microfibril together constitute the lignocellulose. The hemicelluloses in the biomass are generally heteropolymer of xylose, arabinose, mannose, glucose, and galactose whereas lignin is composed of three major phenolic components, namely p-coumarylalcohol, coniferyl alcohol and sinapyl alcohol [10,16]. The strong covalent bonds between lignin and cellulose and poor access to cellulose core and its crystalline nature together make the enzymatic action kinetically slow with poor yield of fermentable monomeric sugars [17].

This necessitates effective pretreatment to loosen the lignin-cellulose association and making the cellulose accessible during enzymatic hydrolysis. Thus the pretreatment becomes very crucial step in the lignocellulosic biomass utilization. During the past few decades, several approaches have been used for developing low cost pretreatments for generating sugar syrups from cellulose and hemicellulose [18,19]. Pretreatments for lignocellulosic biomass include biological, mechanical, chemical methods and various combinations thereof [20]. The choice of the optimum pretreatment process depends on the feedstocks. There are a number of reports on pretreatment options for various biomass types available with each having its own merit and demerits [21,22].

Menon and Rao [20] provided an excellent overview of different pretreatments. If assessed in terms of sugar yield, minimum inhibitor formation and by- product formation, scalability and generic application to large number of biomass, acid, alkali, liquid hot water, steam explosion, and Ammonia fiber expansion (AFEX) have been considered quite useful to meet most of these criteria. However, acid and liquid hot water pretreatments have individual drawbacks, steam explosion suffer from high inhibitor formation whereas alkali generates less inhibitors but needs longer treatment time with high salt formation. In this context AFEX seems better but success at pilot scale need to be established in more cases.

Nonetheless, methods are available for effective pretreatment to facilitate next step of enzymatic hydrolysis.

3. Enzymatic saccharification of lignocellulosic biomass

Cellulases are primary enzymes for cellulose hydrolysis. These comprises of three predominant activities viz., *exo*-1,4- β-glucanase, *endo*-1, 4-β- glucanase and cellobiase. The balanced and appropriate combination of these activities is what determines the

Table 1
Key cellulases and hemicellulases and saccharification efficiency.

S.No	Enzyme	Biomass treated	Saccharification %	References
1	*Trichoderma reesei* Cellulase	Sugarcane tops	90%	[27]
2.	*T. reesei* cellulases	Rice straw, eucalyptus treated with NaOH and H_2SO_4 and hydrothermal treatment	80%, 100, 100 and 90%, 100, 100 respectively	[28]
3.	*Chrysoporthe cubensis:* *Penicillium pinophilum* 50:50 (v/v)	Alkali treated sugarcane bagasse	Glucan hydrolysis efficiency reached an excess of 60% and xylan conversion exceeded 90%	[29]
4.	Cellulase, hemicellulase and xylanase produced by *Aspergillus* sp. and *A. niger* and *Thermomyces lanuginosus* respectively	Alkali pretreatment of *Miscanthus sacchariflorus* *var*. No. 1	220 mg glucose/g	[30]
5.	*Chrysoporthe cubensis*	Alkaline pretreated sugarcane bagasse	56% and 90% of glucose and xylose, respectively	[31]
6.	White-rot fungi *Pycnoporus sanguineus*	Alkali-treated sugarcane bagasse	60% reducing sugars	[32]
7.	*Aspergillus oryzae* ITCC 4857.01	Alkali treated sugarcane bagasse	9%	[33]
8.	Commercial cellulase	Sugarcane tops treated with dilute acid	0.685 g/g of reducing sugar was produced per gram of pretreated biomass	[34]
9.	Commercial cellulase from Fluka, Onozuka and cellulase from *T. viride* CMIT3.5	Pretreated corn stover, *Miscanthus*, and wheat straw	52%, 59%, and 61% in wheat straw, corn stover, and *Miscanthus* respectively	[35]
10.	Commercial Xylanase XL and Novozyme 188	Steam pretreated wheat straw	Increase from 40 to 50%	[36]

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Table 2
Lignocellulosic enzymes from extremophiles.

Organism	Classification	Enzyme	Component of biomass acted upon	Enzyme characteristics	References
Thermotoga strain FjSS3-B.1	Thermophile	Cellobio-hydrolase	Carboxymethylcellulose; amorphous cellulose	Highly thermostable with pH_{opt} 7, T_{opt} 105 °C and $t_{1/2}$ of 70 min at 108 °C	[44]
Thermotoga maritime	Hyperthermophile	Cellulase II	Crystalline cellulose; carboxymethylcellulose; ß-glucan; PNP-cellobioside	pH_{opt} 6–7.5, T_{opt} 95 °C with $t_{1/2}$ of 30 min at 95 °C	[45]
Alicyclobacillus acidocaldarius	Thermophile and acidophile	Endo-glucanase (*celA*)	Carboxymethylcellulose; cellooligosaccharides; lichenan; xylan	Thermostable endoglucanase with T_{opt} 70 °C, pH_{opt} 5.5 and $t_{1/2}$ of 30 min at 75 °C	[46]
A. acidocaldarius	Thermophile and acidophile	Endo-glucanase (*celB*)	Carboxymethylcellulose; swollen cellulose; xylan	Highly thermostable (T_{opt} 80 °C) with stability maintained over a wide range of pH values from pH 1.0 to 7.0.	[47]
Sulfolobus solfataricus	Extremely thermophilic and acidophilic crenarchaeon	Endo-glucanase (*SSO1949*)	Carboxymethyl cellulose; cellooligosaccharides	Extremely acidophilic and thermophilic enzyme with pH_{opt} 1.8, T_{opt} 80 °C and $t_{1/2}$ of 8 h at 80 °C	[48]
S. solfataricus	Extremely thermophilic and acidophilic crenarchaeon	ß-D xylosidase/ α−L arabinosidase (*xarS*)	Arabinofuranoside; xylopyranoside	Thermostable enzyme, pH_{opt} 6.5.T_{opt} 80 °C with $t_{1/2}$ of 10.5 hr at 90 °C for xylosidase activity	[49,50]
S. solfataricus	Extremely thermophilic and acidophilic crenarchaeon	Endoglucanase/ xylanase (*SSO1354*)	Xylan, arabinan, carboxymethyl cellulose	Extreme thermostability T_{opt} 95 °C, pH_{opt} 3.5-4, $t_{1/2}$ of 53 min at 95 °C with activity on broad range of substrates	[51,52]

efficiency of saccharification though it varies with the types of biomass to be pretreated.

Xylanases are next important enzymes which act/hydrolyze hemicelluloses components of biomass. Cellulases and hemicellulases and their applications in biomass hydrolysis have been extensively reviewed by others [23–26].

Some of the key cellulases and hemicellulases are summarized in Table 1.

Van Dyk and Pletschke [37] summarized various commercial cellulose preparations and their constituent enzymes. These are mainly supplied by Novozyme (Celluclast, Novozyme 188 etc.) and Genencor (Spezyme and Accelarase 1500 etc.). Former have been very effective for switch grass, whereas later have been most efficient for douglas fir and spruce reaching almost 100% saccharification. As of now the novozyme has been most suitable for saccharification.

Lignins complexed cellulose becomes quite recalcitrant and less amenable to degradation. unless provided with suitable pretreatment [38]. As for as biological treatment are concerned, white rot fungi especially *Phenerochaete chrysosporium* is most effective in degrading the lignin part of lignocellulosic complex [39]. Lignin degradation is assisted by lignin peroxidase, manganese - dependent peroxidase and laccase enzymes by attacking phenolic part of lignin [40].

4. Use of extremophilic enzymes

In recent years, there has been an emerging trend to use extremophilic enzymes for biomass pretreatment and saccharification. Extremophiles are the class of microorganisms which inhabit under harsh environmental conditions for example hyperthermophiles/thermophiles thrives at high temperatures, psychrophiles at low temperature, halophiles at high salt, acidophiles and alkalipailes at low and high pH; respectively. Its logical that their physiological and metabolic activities take place under respective extremes. This necessitates that their enzymes must be functional and catalytically active under these extreme conditions and perform the reactions accordingly. Their enzymes dubbed as extremozymes, are uniquely adapted to tolerate extremes in pH, temperature and environmental change better than mesophilic microbes and their enzymes [41]. Thus it needs to be explored whether extremophiles may possess robust and highly thermostable lignocellulose degrading enzymes viz., cellulases, xylanases, ligninases which can be more effective in saccharifications. Concomitant addition of tolerant cellulases and fermentative microorganisms may also be a possibility for simultaneous saccharification and fermentation (SSF) [42]. Thus use of extremophiles and their enzymes as potential biocatalyst seems quite promising but remains to be investigated at a viable scale. The class thermophiles and acidophiles are considered to be best suited for such applications due to the fact that the preceding biomass pretreatment is often performed under these conditions. Thermophilic and hyperthermophilic enzymes and microorganisms have been extensively reviewed in the past by Kelly and colleagues [43]. Table 2 summarizes various extremophiles which have been investigated for potentially useful enzymes.

Blumer-Schutte et al. [53] have in fact categorized lignocellulosic hydrolases into two broad classes namely “Primary” and “Secondary” cellulolytic enzymes. Primary cellulolytic enzymes are ‘free-acting’ cellulases from thermophiles, e.g., that from *Caldicellulosiruptor saccharolyticus* which are functional at about 70 °C [54], and enzymes from *Anaerocellum thermophilum* which work even at higher temperature of 75 °C [55]. The cellulases of these two microbes have distinct cellulose binding domain and efficiently act on crystalline cellulose. Secondary cellulases or other glycosyl hydrolases are those which do not directly act on crystalline cellulose but exhibit various glucosyl hydrolases activities viz., glucanses for cellulose hydrolysis. They lack a Cellulose Binding Domain (CBD). As a representative example, *T. maritime* is cited which is not able to utilize cellulose even though it expresses several β-1, 4-glucanases but is devoid of CBDs. So far, only those microorganisms possessing primary enzymes have been observed to utilize

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crystalline cellulose as substrate. These are mostly thermophilic anaerobes viz. *Anaerocellum*, *Thermoanaerobacter* and *Caldicellulosiruptor* sp.

Among the aerobic thermophiles which have been well characterized for their cellulosomes, *Thermotoga* genus is most represented in these cases. They exhibit α-glucanase, β-glucanase, cellobiase and hemicellulase activities but do not utilize crystalline cellulose. They also lack CBD and operate at 60–65 °C [45].

Among hyperthermophiles, archaea have been investigated quite in depth. Main genera among them are *Pyrococcus* and *Sulphobolous* sp. They grow in the temperature range of 85 °C and above as high as 100 °C in case of *P. furriosus*. They possess α-glucanase and β-glucanase activity but are not as effective in cellulosic saccharification [56].

Prospects of extremophiles in effective biomass saccharification have been reviewed recently by [57]. They have described the detail characteristics of three *endoglucanases* from *Sulfolobus solfataricus* namely SSO1354, SSO1949 and SSO2534. Their enzymes are both acidic and thermophilic. The SSO1949 enzyme is both extremely acidophilic and thermophilic, with optimum activity at pH 1.8, 80 °C. The SSO1949 gene product does not possess cellobiohydrolase activity and is inactive on crystalline cellulose [48]. The SSO1354 has better xylosidic activity. Optimal pH values for cellulose and xylanase activities are reported to be pH 3.5 and 4.0, respectively. These are most active at 95 °C with reasonably good half-life [52]. The extreme thermostability and pH for activity on a broad range of substrates are quite suggestive of its usefulness because lignocellulosic biomass contains a significant fraction of hemicelluloses in addition to the cellulose.

Alicyclobacillus acidocaldarius also produces acid and heat stable endoglucanase designated as CelA, CelB and CelG, however in this also crystalline cellulose is also not acted upon [47].

All above studies indicate many potential thermostable *endo*-ß-glucanases and ß-glucosidases, from thermophiles and hyperthermophiles and that their 'secondary' cellulolytic enzymes are capable of hydrolysing amorphous substrates but not the crystalline cellulose. Potential sources of cellobiohydrolases or other exocellulases from hyperthermophilic have not been reported so far. An extensive screening from this view point would be useful.

5. Recombinant cellulases and hemicellulases

Use of microbial cellulases and hemicellulases for biomass saccharification suffer from the drawback owing to the high cost of enzymes due to their low productivity. Overexpression of these enzymes has been attempted in quite a many instances. Some of the interesting recombinant cellulases and xylanases with heterologous expression are summarized in Table 3.

Escherichia coli has been the most widely accepted systems for recombinant expression of the endoglucanase or xylanases. The overexpressed enzyme in most cases acquired enhanced thermal stability reaching up to 80–90 °C. In some cases, purification and further characterization has not been reported. The recombinant *C. thermocellum* GH5 cellulase and GH43 hemicellulase genes expressed in *E. coli* cells were simply grown in repetitive batch mode, with the aim of enhancing the enzyme production [64]. Hetzler et al. [65] expressed six different cellulase genes from *Cellulomonas fimi* ATCC 484 and *Thermobifida fusca* DSM43792, thereby enabling *Rhodococcus opacus* PD630 to degrade cellulosic substrates to cellobiose. These recombinant strains also hydrolyzed cotton, birch cellulose, copy paper, and wheat straw. The status of recombinant cellulases though remains to be promising but yet to be perfected.

Table 3
Representative examples of some recombinant cellulases and xylanases.

Source	Host organism	Enzyme	Purification	Mass (kDa)	pH opt.	pH stability	Temperature opt. (°C)	Thermal stability	References
Geobacillus sp. 70PC53	*E. coli*	Endo-glucanase	His-Trap affinity chromatography	43	5.0	4.0-9.0	65	Thermostable; 90% activity retained after heating at 65 °C for 6 h	[58]
Geobacillus sp. MT-1	*E. coli*	Xylanase	Ammonium sulphate precipitation	36	7.0	5.5-10.0	70	Stable at 55 and 60 °C; $t_{1/2}$ 50 min at 65 °C	[59]
Bacillus subtilis strain I15	*E. coli*	Cellulase	Ammonium sulphate precipitation and Sephadex G-100 gel filtration chromatography	52	6.0	-	60	Retained > 90% of the activity at 65 °C after 2 h	[25]
Bacillus subtilis	*E. coli*	Endo-glucanase	–	55	6.5	-	50	Retained 70% activity at 75 °C after incubation for 30 min	[60]
Bacillus sp.	*E. coli*	Endo-glucanase	Gel filtration and ion-exchange chromatography	65	7.0	6.0–8.0	60	Stability up to 70 °C	[61]
Thermoanaerobacter tengcongensis MB4	*E. coli*	Endo-glucanase	Superdex 75 gel filtration	-	between pH 6.0–6.5	5.5-11.0	Between75 and 80 °C	$t_{1/2}$ 30 min at 82 °C	[62]
Actinomadura sp. S14	*E. coli*	β-1,4-xylanase	IMAC Sepharose 6 fast flow column followed by Gel filtration on a Superdex 200 1	21	6.0	5.0-11.0	80	Stable at 60 °C and 70 °C but activity loss beyond 80 °C after 2 h	[63]

Table 4
Studies using ionic liquids and sccharification efficiency.

Ionic liquid used	Biomass treated	% Saccharification	References
1,3-dimethylimidazolium dimethylphosphate	Corn stover	Sugar content raised to 92.7% after ionic liquid mediated pretreatment	[74]
1-ethyl-3-methylimidazolium-acetate	Sugarcane bagasse	Increased glucose content from 80.0–83.3 to 91.6–92.8% of sugarcane cellulose	[75]
1-ethyl-3-methylimidazolium acetate	Rice straw	75% glucose yield	[81]
1-ethyl-3-methylimidazolium acetate	Switch grass	80% saccharification efficiencyiy	[82]
1-Ethyl-3-methylimidazolium acetate	Wood	Saccharification of 65 wt%	[83]
1-H-3-methylimidazolium chloride	Soybean straw and corn straw	Yield of reducing sugar was up to 53.27 mg from 0.2 g of soybean straw and 50.03 mg from 0.2 g of corn straw.	[84]
1-ethyl-3-methylimidazolium acetate	Switch grass	81.2% glucose and 87.4% xylose	[85]
1-ethyl-3-methylimidazolium acetate + peracetic acid	Pine biomass	250 fold higher glucose formation	[86]
1-ethyl-3-methylimidizolium acetate	Corn stover	80% glucose and 50% xylose	[87]
1-ethyl-3-methylimidazolium acetate	*Miscanthus giganteus*	Saccharification efficiency of 90%	[88]
Ionic liquid-water mixtures of 1-butyl-3-methylimidazolium methylsulfateand1-butyl-3-methylimidazolium hydrogensulfate	*Miscanthus giganteus*, (*Pinus sylvestris*)	Up to 90% of the glucose and 25% of the hemicellulose	[89]

6. Use of ionic liquids

As yet another promising approach in the recent years, the use of ionic liquids in various chemical processes has increased significantly due to their nature as the "green solvents", highly advantageous properties, thermal and chemical stability, high polarity, and very low toxicity [66]. In some cases these have been proved quite efficient in dissolving cellulosic materials as substrate [67]. As for as their chemistry goes, they are composed of a bulky asymmetric cation and a weakly coordinating anion [68]. Owing to their unique set of properties, many ionic liquids mediated processes have already been adopted at industrial scale viz. process for isobutane alkylation, BASIL (Biphasic acid scavenging utilizing ionic liquids) process which led to 8×10^4 times increase in productivity as compared to use of usual solvents [69]. Ionic solvents facilitate/enhance the saccharification by dissolving cellulose and thus making lignocellulose more accessible to enzymatic action of cellulose. The cellulose dissolution power of ionic liquids is accorded to their ability to donate hydrogen i.e., their basicity [70].

Lignocellulosic biomass has garnered a lot of attention over the years due to its conversion to biofuels and other useful chemicals for the industry [71]. For the generation of biofuels a pretreatment step is usually required to primarily disrupt the biomass structure and to increase the cellulose accessibility for lignocellulosic enzymes action during saccharification. The pretreatment methods used commonly over the past years include physical methods like, mechanical grinding etc. whereas the chemical ones include acid, alkaline hydrolysis, as well as use of organic solvents like methanol, ethanol and acetone [8]. Among the lignocellulosic hydrolysis often pose resistant to degradation due to cellulose crytallinity and insolubility [72]. In this context, use of ionic liquids is fast emerging as an efficient strategy for pre-treating recalcitrant lignocellulosic biomass [73]. Some of the recently studied ionic liquids for pretreatment processes include 1,3-dimethylimidazolium dimethylphosphate, 1-ethyl-3-methylimidazolium-acetate, 1-butyl-3-methylimidazolium chloride, 1-butyl-3-methylimidazolium methanesulfonate among many others [73–76].

The advantages cited are that ionic liquids (a) do not inhibit cellulose hydrolysis enzymes, (b) accelerate enzymatic hydrolysis at low enzyme concentrations (c) can be supplemented with antisolvent such as water, ethanol or acetone for better cellulose regeneration, and (d) can be recovered and recycled [77–80].

Some of the studies wherein the ionic liquids have increased the saccharification efficiency as well as reduction in time to achieve enzymatic hydrolysis are summarized in Table 4.

Use of 1-butyl-3- methyimidazolium chloride led to the sugar conversion in less than 5 h of enzymatic hydrolysis [90], 1-ethyl-3-methylimidazolium acetate treated pine, switch grass conversion to glucan in 3 h [91]. 1-ethyl-3-methylimidazolium-acetate pretreated sugarcane bagasse generated 91–92.8% glucose content as compared to 80–83% achieved in conventional hydrolysis [75]

All these studies prove that ionic liquids are promising solvents in biomass utilization, however, more investigations are needed on various aspects such as mechanism, development of generic processes/type of ionic liquid according to the nature of biomass, and scale ups.

7. Conclusions

Development of technology for generating biofuels and platform chemicals from lignocellulosics is attracting vigorous scientific attempts. There is potential of extremophiles for enzyme production and use of ionic liquids.

In this regard 1-ethyl-3-methylimidazolium acetate EMIM [OAc] has garnered a great deal of attention for its promising results showing upto 90% increase in saccharification efficiency. An endoglucanase from the strain *Alicyclobacillus acidocaldarius* has emerged as a potential extremozyme. Further studies are essential in these areas.

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