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Review

Advances and Challenges in Biocatalysts Application for High Solid-Loading of Biomass for 2nd Generation Bio-Ethanol Production

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Abstract: Growth in population and thereby increased industrialization to meet its requirement, has elevated significantly the demand for energy resources. Depletion of fossil fuel and environmental sustainability issues encouraged the exploration of alternative renewable eco-friendly fuel resources. Among major alternative fuels, bio-ethanol produced from lignocellulosic biomass is the most popular one. Lignocellulosic biomass is the most abundant renewable resource which is ubiquitous on our planet. All the plant biomass is lignocellulosic which is composed of cellulose, hemicellulose and lignin, intricately linked to each other. Filamentous fungi are known to secrete a plethora of biomass hydrolyzing enzymes. Mostly these enzymes are inducible, hence the fungi secrete them economically which causes challenges in their hyperproduction. Biomass's complicated structure also throws challenges for which pre-treatments of biomass are necessary to make the biomass amorphous to be accessible for the enzymes to act on it. The enzymatic hydrolysis of biomass is the most sustainable way for fermentable sugar generation to convert into ethanol. To have sufficient ethanol concentration in the broth for efficient distillation, high solid loading ~<20% of biomass is desirable and is the crux of the whole technology. High solid loading offers several benefits including a high concentration of sugars in broth, low equipment sizing, saving cost on infrastructure, etc. Along with the benefits, several challenges also emerged simultaneously, like issues of mass transfer, low reaction rate due to water constrains in, high inhibitor concentration, non-productive binding of enzyme lignin, etc. This article will give an insight into the challenges for cellulase action on cellulosic biomass at a high solid loading of biomass and its probable solutions.

Keywords: bioenergy; biofuel; cellulase; biomass; lignin; high solid loading; hydrolysis

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1. Introduction

The increasing demand for fossil fuels has threatened the environment in terms of greenhouse gas (GHG) emissions [1]. About 80% of the world's energy requirement is being fulfilled by fossil fuels even today [2]. Depletion of fossil fuel resources, rapid industrialization, increasing energy demand, and environmental concerns such as increasing GHG emissions are among the major driving forces which have enabled society to

look for alternatives to fossil fuels; especially renewable and sustainable solutions for fulfilling the needs of energy [3,4]. Among renewable energy sources; bioethanol has attained a significant level of popularity. In various parts of the World its being produced by different feedstocks. It includes starchy crops such as corn and sugarcane; cellulosic biomass such as agricultural residues, forest wood residues, and microalgal as well as macroalgal (aquatic seaweed) biomass [5,6]. Hence, any biomass converted to glucose can be employed as feedstock for conversion to ethanol. Though starchy biomass can be easily converted into its monomers, glucose; it creates competition between food/feed and energy [7]. In the US, corn crops are employed for ethanol production, which may not be feasible in developing countries.

Lignocellulosic (LC) biomass is the most abundant and ubiquitous renewable resource on Erath [8]. LC biomass does not find applications for food and feed, can be employed as renewable raw material for biofuel production. Chemically it consists of majorly three components namely; cellulose, hemicellulose and lignin. Holocellulose (cellulose + hemicellulose) part can be hydrolyzed by acids or by enzymes into fermentable sugars which can be further converted into ethanol via yeasts such as *Saccharomyces cerevisiae* or *Kluveromyces* [9]. To deal with biomass containing lignin is challenging especially when our target is the holocellulosic fraction. Nature has designed plant biomass in such a way that it can withstand microbial attack and stand erect on soil which is a home for millions and billions of microorganisms [10]. Thus, the boon for them becomes the major challenge in its microbial degradation due to its recalcitrant nature [11].

As the technology for 2nd generation bio-ethanol has reached a so-called matured stage; still it requires researchers to revisit the need for further research. The shutdown of several commercial plants globally reiterated the need to understand the reasons for failure, whether technical solutions are needed or it's just political or policy-related issues [3,4]. The logistic of biomass from fields to the site is also a challenge which adds cost to the technology as well as the availability of biomass in bulk, throughout the season is one of the major limitations. To solve these issues to an extent, an integrated configuration of bioethanol production has been adopted in most instances where the industry is set at a place having easy availability of biomass and at the same site, cellulase is being produced employing pretreated biomass as a carbon source. The same pretreated biomass is converted into ethanol by employing produced enzymes [12]. Consolidated bioprocessing appears quite promising for the future looking at the advancements reported recently, wherein a single pot the whole steps are performed simultaneously from cellulase production to ethanol conversion [13]. High solid loading of biomass is the crux of the overall technology to have sufficient sugars in the medium for effective conversion to ethanol to have economically feasible ethanol distillation.

This article focus on the challenges that arise for the application of biomass-degrading enzymes to hydrolyze biomass such as the selection of efficient pretreatment method of biomass making it more amorphous by removing lignin preferably, to ensure the best hydrolytic efficiency of enzymes, generation of inhibitors during pretreatment causing hindrance to enzymatic hydrolysis or fermentation for ethanol production, high-solid loading of biomass to obtain higher sugar concentration to ensure sufficient ethanol concentration for effective distillation, however; causing less space or limited medium required for substrate enzyme reaction, non-productive enzyme-lignin binding and feedback inhibition of cellulases. The solutions or advances to tackle a few of these challenges such as a sequential biomass loading, adding additives, simultaneous saccharification and fermentation to overcome feedback inhibition of cellulase, achieving higher ethanol conversion efficiency, etc. have also been discussed.

2. Key Biocatalysts for Biomass Hydrolysis for 2nd Generation Bio-Ethanol Production

Enzymes are the biocatalysts that can accelerate biological reactions which are highly specific in nature. Interest in biocatalysts is increasing with time as they are the most

sustainable catalysts. Especially, for bioconversions of biomass into biochemicals biocatalysts in comparison to solid catalysts or chemocatalysts have the excellent capability of catalyzing the reactions under mild conditions due to which not much energy is consumed. It also leads to minimizing greenhouse gas emissions thus leading to environmental sustainability. Hydrolysis of microcrystalline cellulose with solid catalysts such as Al_2O_3-B_2O_3 solid catalysts took place at 180 °C [14] which is energy consuming whereas in presence of cellulase the hydrolysis occurs at 50 °C [15] which is far milder. In the pharmaceutical industry, chemocatalyst may not be replaced completely with biocatalysts as it is difficult sometimes for multistep complex reactions [16]. Biocatalysts do not possess any threat to the environment when disposed of, unlike solid catalysts.

As enzymes are environmentally benevolent and sustainable; are preferred for hydrolyzing biomass into fermentable sugars [17]. Here cellulases are the major biomass hydrolyzing enzymes/biocatalysts that are mostly produced by microorganisms like bacteria and fungi. Filamentous fungi like *Trichoderma*, *Penicillium*, *Humicola* and *Aspergillus* are among the most studied and exploited ones for cellulase production [18–20]. *Trichoderma reesei* is the model microorganism for cellulase production and most of the commercial enzymes available are produced by these fungi either as a whole or as a component of cellulase [21]. Cellulase is a complex enzyme having multiple components like endoglucanase, exoglucanase/cellobiohydrolase, beta-glucosidase, and auxiliary enzymes like LPMOs as well [17]. Cellulose fibers are attacked by endoglucanase initially to produce a nick in between and then exoglucanase or cellobiohydrolase attacks the reducing and non-reducing ends to produce smaller oligosaccharides mainly cellobiose which are hydrolyzed by β-glucosidase (BGL) [22]. Thus β-glucosidase is the show stopper as finishes off the reaction by giving the end product as glucose monomers [23,24]. Thus, each component of cellulase acts synergistically to hydrolyze cellulose completely which has been depicted in Figure 1. Beta-glucosidase is the rate-limiting enzyme as the end product glucose itself can inhibit it by feedback inhibition. *Trichoderma reesei* is regarded as the hyper producer of cellulases which can secrete extracellularly all the other components of cellulases; however, it is deficient in beta-glucosidase. Several efforts have been taken to either supplement beta-glucosidase to make a cocktail of enzymes or heterologously express *bgl* gene in *T. reesei* itself so as to have the optimum amount of all the components in secreted enzymes [25–27]. *Penicillium* and *Aspergillus* are known to produce comparatively higher BGL and are now giving tough competition to all-time winners, '*Trichoderma*' for cellulase production [28]. Though the enzyme cost has been reduced drastically in the last few decades, still there is space for further reduction as it may increase the economic feasibility of 2nd generation bio-ethanol technology. Bioprocess advancements for cellulase production have been drastically attended since last decade and several research articles are being published regularly on the application of new strategies for bioprocess improvement such as employing micro-nano bubble technology, resolving the issue of high DO requirement [29], however; most of them are based on lab-scale studies, necessitating the data of pilot-scale studies for scale-up [12].

Biomass hydrolysis efficiency increases in the presence of accessory enzymes like xylanase. Along with cellulase, xylanase also plays an extremely important role as the biomass contains hemicellulose along with cellulose. Xylanase is also a complex enzyme similar to cellulase and acts on xylan fractions to hydrolyze it. As the xylanase acts on xylan/hemicellulose part, the cellulose becomes more amorphous and accessible to cellulase which is the reason for the enhanced efficiency of biomass hydrolysis in presence of xylanase. Most of the fungi like *Trichoderma* and *Penicillium* employed for cellulase production produce xylanase also along, making these filamentous fungi an excellent source of biomass hydrolyzing biocatalysts [30]. Even though if the enzyme is good enough there are many other challenges needs to be tackled during hydrolysis at high solid loading of biomass which is depicted in Figure 2.

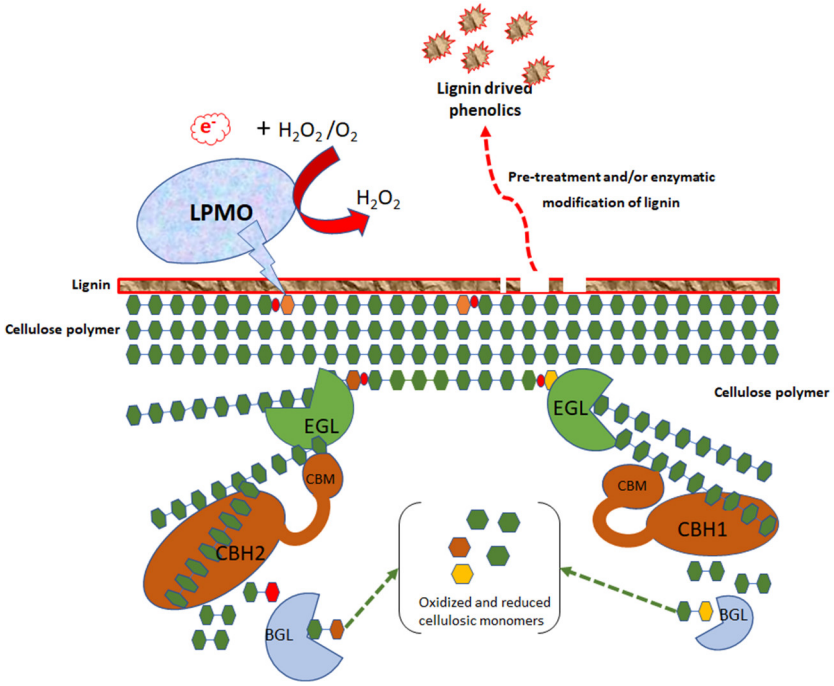

Figure 1. Action of biocatalysts on cellulose polymer to hydrolyse into its monomers.

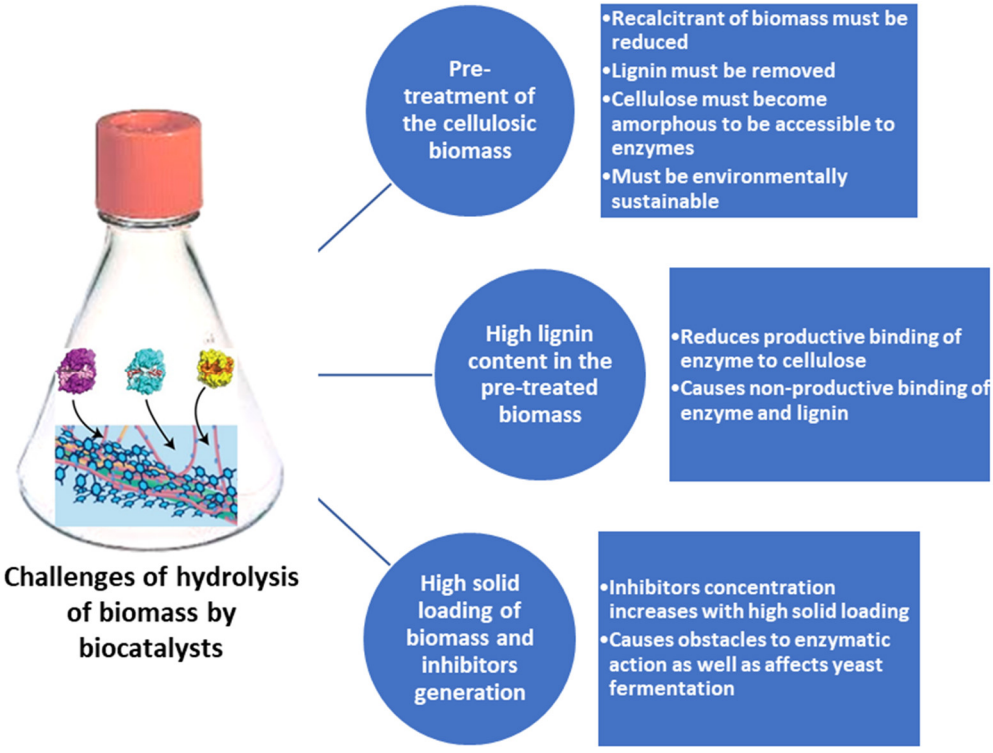

Figure 2. Challenges during biomass hydrolysis via application of biocatalysts.

3. Challenges in the Application of Biocatalysts for Hydrolysis of LC Biomass

3.1. Pretreatment of the Cellulosic Biomass

Lignocellulosic biomass is mainly comprised of polysaccharides (cellulose, hemicellulose), lignin, ash, protein, and extractives. Polysaccharides in lignocellulosic biomasses are glued with lignin and other cell wall components, making a strong recalcitrant matrix [31,32]. The extraction of polymeric cellulose and hemicellulose is highly desired for their transformation into renewable fuels, chemicals, and materials in biotechnological

conversion processes. Pretreatment is thus a necessary step to overcome this natural recalcitrant and make them accessible for enzymatic attack for downstream transformation into high value-added chemicals [33]. The selection of pre-treatment method has the utmost significance in biomass hydrolysis which majorly depends on the type of biomass, its composition as well as the intended end product which is cellulose or holocellulose in case of bioethanol production [11]. The lignin content and the type of lignin, its degree of acetylation and the presence of sugar-derived inhibitory compounds generated during pretreatment as well as oligomers also impacts enzymatic hydrolysis of biomass by affecting enzymatic activity [12].

Many pretreatment methods such as chemicals, green solvents, and mechanical or biological have been used and developed todate to break the inter-and intra-molecular linkages between carbohydrate sugars and lignin to hydrolyze, depolymerize and open up the complex structure of biomass. Different pretreatment methods perform differently by targeting the particular component of the biomass. Table 1 shows the significance of various pretreatment methods and its action on biomass and its components. For instance, dilute acid pretreatment hydrolyzes the hemicellulose fraction, while the other reduces the crystallinity and degree of polymerization. Similarly, hydrothermal pretreatment involves the treatment of biomasses at elevated temperature (100–250 °C) in presence of liquid water, steam, and heat for a short treatment time and is considered eco-friendly, low cost and economically viable process. It is known that at high temperatures, water acts as catalytic media and liberates hydronium ions leading to polymerization of polysaccharides through selective hydrolysis of ether bonds and cleavage of acetyl groups [34,35].

Table 1. Pretreatment type, mode of action on specific cell wall components and their benefits.

Pretreatment	Mode of Action	Benefits	Limitations	References
Liquid hot water	-Remove extractives -Solubilise acetal groups from hemicellulose	-Increase surface area and porosity -No chemical intake thus free from the neutralization step	-Low cellulose digestibility and lignin solubilization High water and energy demand	[36]
Dilute acid	-Catalyze hemicellulose acetal linkage and retain cellulose structure	-High xylose release increases surface area and increases cellulose digestibility -Commercially viable	-Generate fermentative toxins and requires high metallurgy and neutralization step -Sugar losses in terms of toxins and pseudo lignin formation	[37]
Ammonia Fiber explosion	-Catalase lignin breaking of ß-ß and ß-O-4 cleave in lignin -Solubilize hemicellulose	-Efficient in lignin removal -Increase surface area, and porosity and reduces overall crystallinity -Less formation of inhibitors -Low energy demand	-High ammonia cost -Recycling issues -Less efficient for softwood biomasses -Oligomers formations	[38]
Alkali treatment	-Attribute lignin solubilization, lignin structure change and improves hemicellulose solubilization	-Improves biomass swelling, suitable for all types of biomasses, and reduces overall biomass crystallinity -Low sugar loss, high lignin removal with amorphous cellulose production	-High dose input, harsh condition requirement, -generate oligomers, washing and neutralization step requirement, wastewater generation	[39]

Steam explosion	-Break hemicellulose-cellulose-lignin linkages -Reduces cellulose crystallinity	-Remove hemicellulose, and extractives and reorganize the lignin structure -Increase biomass surface area -Enhance cellulose digestibility by rupturing cell wall recalcitrant matrix	-High pressurized equipment requirement -Generate toxins compounds -Results in pseudo lignin formation -Partial cellulose and hemicellulose recovery	[40]
Hydrothermal	-Catalyze the breaking of hemicellulose and lignin -Deconstruct cellulose chemical structure	-Dissolve hemicellulose and part of lignin in the aqueous phase -Increases enzymatic digestibility of cellulose	-High water and energy consumption -Low lignin removal	[41]
Ionic liquid	-Selectively solubilize lignin, hemicellulose and cellulose through cleavage of ether bonds and H-bonds in lignin–carbohydrate complexes -Alter cellulose crystalline structure to amorphous phase -Decompose β-O-4, β-β, β-5 bonds	-Mild reaction conditions and can deal with a variety of biomasses, Benefited from the no-toxins formation -High cellulose digestibility -Biodegradable and biocompatible	-High production cost and challenging recycling, -High ionic liquid toxicity towards enzyme -High viscosity and lack of separation technology	[42,43]
Deep eutectic solvents	-Efficiently solubilize lignin and hemicellulose -Selectively change the crystalline phase of cellulose,	-Increases pore size and pore volume, High digestibility of pretreated biomass -Benefited from high biomass loading -Reduced inhibitor formation	-High production cost -Cellulose degradation	[44]
Biological (Fungi/bacteria)	-Biologically catalyze the depolymerization of lignin linkages through laccase, peroxidase enzymes secreted from fungi and bacteria) -Alter cellulose structure	-Degrades lignin and hemicellulose, benefited with reduced energy consumption with no inhibitor formation	-Slow reaction rate -High enzyme inputs -Requires high surface area -Requires strict culture and is not suitable for industrial scale	[10,45]
Organosolv	-Selective solubilization of	-Recover pure lignin and cellulose	-Use of costly solvents and faces recovery issue	[46]

	lignin and hemicellulose -Depolymerize lignin structure -Break β-O-4, β-β, β-5 linkage	-Suitable for hardwood and softwoods -Reduces crystallinity and enhances biomass surface areas, high digestibility	-Enzymatic deactivation due to organic solvent -Requires a high-pressure reactor	
CO_2 explosion	-Solubilize hemicellulose -Increases surface area and reduces crystallinity	-Less toxic and non-flammable gas -Economically and environmentally favourable	-High pressure and temperature requirement -Not suitable for industrial scale -Low lignin solubilization	[47]
Irradiation (Microwave, ultrasound, plasma, hydrodynamic cavitation, electric field)	-Loosing of biomass components through ultrasonic wave -Free radicals induce oxidation -Disrupts hydrogen and ether linkage	-Improves lignin removal hemicellulose solubilization -Promotes higher surface area, and porosity and loosens biomass component for higher enzymatic attack	-Energy-intensive process, economically not viable for large scale -Low sugar recovery and assisted with high OPEX and CAPEX	[48]

Alternatively, alkali pretreatment involves the treatment of biomass with sodium, potassium, calcium, and ammonium hydroxide in varying concentrations leading to the breaking of ester linkages with lignin cellulose causing cellulose swelling, and partially recrystallize cellulose and partially solubilizing hemicellulose [49]. Pulping industries use acid or alkali to catalyze the digestion of biomass at 130–160 °C for the desired temperature for lignin solubilization. Sodium sulphite treatment with or without alkali leads to the attachment of sulfonate moiety in lignin solubilization [50]. Another pretreatment method known as the Kraft process, employs sodium sulfide and sodium hydroxide at temperatures up to 170 °C, leading to the efficient separation of lignin from biomass, and enhancing the enzymatic yield [46]. Besides, high-pressure treatment during the retreatment process results in the breaking of inter-linkage of hemicellulose bonds, thus leading to solubilization of xylose. The steam explosion is generally employed either with chemicals or without chemicals and under a high-pressure system, which is then blasted to break the structure of biomass order and effectively remove hemicelluloses. Furthermore, dilute acid-catalyzed steam explosion can further enhance the degradation of hemicellulose structure and partially dissociate lignin while increasing overall enzymatic yield [51].

Dilute acid pretreatment is a well-established method for pretreatment of lignocellulosic biomasses, which employs organic and/or inorganic acids for the deconstruction of lignocellulosic biomasses. Dilute acid pretreatment leads to the breaking of acetal linkage in hemicellulose, partially solubilizing lignin fragments, increasing the surface area and porosity of pretreated biomass, thus increasing the cellulase accessibility for enzymatic hydrolysis. Moreover, strong mineral acids, such as H_2SO_4, lead to the generation of HMF, acetic acid, furfural, and formic acid, which are inhibitory to downstream enzymatic hydrolysis and fermentation steps. Therefore, acid pretreatment requires an additional step for detoxification. Moreover, in comparison, organic acid, acetic acid, and formic acid demonstrated higher pretreatment efficiency, as they have specific presentations of acid groups that mimic hydrolytic enzymes and can promote cellulose degradation [52]. Hot water pretreatment at (110–320°) promotes hemiacetal linkages in lignocellulosic biomass, releasing acids during the hydrolysis step. The acids produced during pretreatment further facilitated the breakdown of the ether linkages of lignin.

Ionic liquids (ILs) and or deep eutectic solvents (DES) are mainly composed of salts, comprising organic cations and organic or inorganic anions. They are endowed with their high thermal and chemical stability, intrinsic solvatochromic properties, and tunable

cation and anion [53]. In recent years, ILs and DES have been considered "green solvents" for lignocellulosic dissolution for selective recovery of hemicellulose, lignin, and cellulose [54,55]. DES can be synthesized easily by reacting with suitable hydrogen-bond acceptors and hydrogen-bond donors, which have unique solvatochromic properties to interact with h-bonding of cellulose and hemicellulose and cleavage ether and H-bonds in lignin–carbohydrate complexes. ILS/DES results in alteration in crystalline cellulose I structure transformation to amorphous cellulose II, which could be easily digestible towards enzymatic hydrolysis for production of higher sugars for value-added chemical production [42,56]. Ionic liquids (ILs) and or deep eutectic solvents (DES) are mainly composed of salts, comprising organic cations and organic or inorganic anions. They are endowed with their high thermal and chemical stability, and intrinsic solvatochromic properties and are tunable varying cation and anion. In recent years ILs and DES have been considered a "green solvent" for lignocellulosic dissolvation for selective recovery of hemicellulose, lignin and cellulose. Besides, ILS/DES results in alteration in crystalline cellulose I structure transformation to amorphous cellulose II, which could be easily digestible towards enzymatic hydrolysis for production of higher sugars for value-added chemical production. We strongly believe that hydrothermal pretreatment as well eutectic solvents may serve as best methods in present scenario where sustainability is the highest priority and somehow helps either in removal or relocation of lignin. Pretreatments opted by various researchers for various biomass have been enlisted in Table 2.

Table 2. Biomass pretreatment and its significance in terms of hydrolysis efficiency.

Biomass	Pretreatment	Hydrolysis Significance	References
Mustard stalk	Dilute acid, steam explosion and alkali pretreatment	Maximum of 65.2, 66.5 and 59.5% hydrolysis yield were achieved for alkali, dilute acid and steam explosion, respectively. Overall cellulose conversion was enhanced to 80% within 72 h of hydrolysis	[39]
Mustard stalk	Ionic liquid pretreatment	Max. 97.7% glucose yield was achieved during enzymatic hydrolysis	[42]
Poplar	Synergistic hydrothermal-DES pretreatment	Integrated pretreatment resulted in effective hemicellulose and lignin solubilization. A Maximum 96.33% glucose yield was archived.	[55]
Corncob	Binary acids (H_2SO_4 + CH_3COOH)	Results in 85.6 of hemicellulose and 81.41 of lignin removal Saccharification yielded a maximum of 55.4 mg/mL of glucose while producing 24.6 mg/mL of ethanol	[57]
Pineapple waste	Cascade pretreatment (Steam heating (LPSH) and maleic acid (MA)	A maximum of 67.8% lignin reduction was achieved. Hydrolysis results in 54.79% glucose and 69.23% xylose release	[52]
Bagasse	Sulphuric acid pretreatment followed by autoclave	SSCF results in 77.51 g/L of ethanol at 30% solid loading	[58]
Hardwood and softwood	Glycerol organosolv	Selectively fractionate biomass components and enhanced enzymatic hydrolysis for high sugar.	[59]
Bagasse, rice straw	Hydrothermal and DES	Saccharification resulted in an enhancement of glucose yield by 3.1, 3.4-fold for rice straw and sugarcane bagasse	[60]
Bagasse	Gamma radiation (25, 100, 250, 400 and 1000 kGy)	Promotes delignification and results in high xylose yield 3-fold increase in total reducing sugar	[49]
Bagasse	Ultrasound (50% amplitude, 75 °C temperature for 60 min retention time	Maximum of 78.7% lignin removal and 94% xylose and 87.8% glucose recovery Downstream fermentation resulted in 0.468 g ethanol/g holocellulose	[61]

Bagasse	Non-thermal plasma (14 kV, 60 Hz, 30 mA)	Results in 58.5% lignin removal. A maximum of 51.3% glucose and 38.3% xylose yield was achieved	[62]
Mustard stalk and wheat straw	Ionic liquids	Attributes to cellulose crystalline structure transformation to amorphous phase leading to a maximum of 97.7% of glucose were achieved	[42]
Corn stover	Steam explosion pretreatment	A maximum of 79.3% glucose recovery was obtained Nearly 83% hemicellulose solubilization was achieved	[63]
Garden biomass	Alkali pretreatment	Enhanced 30% more reducing sugars with ~81% cellulose conversion, Improved lignin and hemicellulose solubilization	[64]
Oat straw	Combine alkali and hydrothermal pretreatment	A maximum of 68% of hemicellulose was solubilized with 96% of glucan yield. ~50 g/L of ethanol was achieved from the fermentation of reducing sugars	[65]
Wetland reed grass	Supercritical water (3.5 MPa, 30 min and 1:50)	Highest cellulose yield of 35.1%. This resulted in 99.5% cellulose recovery with high lignin and hemicellulose removal	[36]

3.2. High Solid Loading of Biomass and Inhibitors Generation

For economic efficient production of sugar syrup via hydrolysis of lignocellulosic biomass at least 15% *w/w* solid loading in the reaction mixture is required for enzymatic hydrolysis steps [66]. There are a lot of benefits among which the reduction in operational and capital costs are the major ones along with obtaining higher concentration of sugars. But the overall process at higher solid loading of biomass possess many technical threats and hinderances; questioning the feasibility of the process. These challenges include rheological challenges causing difficulties in mixing and handling, lack of free water for enzymatic reaction also resulting increased inhibitors concentration affecting enzymatic efficiency and finally insufficient heat and mass transfer [67]. Reduction in hydrolytic efficiency at high solid loading is mostly attributed to water constrain [68]. Water constrain also causes the sugar monomers and oligomers generated during pretreatment or hydrolysis to be in close proximity with enzymes causing feedback inhibition. Soluble and non-soluble inhibitors are generated during pretreatment along with sugars end products whose concentration is related to structural composition of biomass, its properties, type of pretreatment and its severity [69]. Besides sugar monomers and oligomers other soluble inhibitors produced during degradation of sugars and phenols such as furan derivatives hampers hydrolysis efficiency as may be present at the significant amount due to less dilution at high solid loadings [70,71].

It has been reported that normally, the glucan conversion efficiency gets reduced at high solid loadings which is otherwise reported to be ~70% at low or moderate solid loading which definitely depends on the type of biomass and its pretreatment and biomass hydrolyzing enzyme loading as well [72]. Also, at high solid loading significant amount of oligomers are formed and unfortunately many microorganisms lack the capacity to utilize these for their growth and metabolic activities [73].

3.3. High Lignin Content and Non-Productive Binding of Enzyme to Lignin

Lignin is bonded with carbohydrates in the biomass by covalent bonding in the plant cell wall which strongly causes physical hindrances to enzyme accession to the carbohydrate [74]. Lignin in the biomass is the major reason for biomass recalcitrance, it is considered as a major obstacle for effective hydrolysis of LC biomass via enzymes [10]. For efficient biomass conversion effective biomass hydrolysis is of paramount significance. Pretreatment of biomass which facilitates lignin removal are encouraged as the physical blockage of enzyme is mainly due to insufficient removal of lignin from the biomass as well as its redeposition on the LC biomass surface or repolymerisation of solubilized lignin in the form of droplets [75]. Lignin causes hindrance to enzymatic action on a cellulosic

fraction by binding irreversibly with enzymes giving rise to non-productive enzyme binding. These non-productive bindings between enzyme and lignin are usually via hydrogen bonds, electrostatic and hydrophobic [76]. These interactions also depend on the lignin characteristics, as lignin S/G ratio affects it inversely. Higher the G unit in lignin lower the interaction with enzyme, hence lower S/G lignin causes less non-productive binding with enzyme. This way lignin affects hydrolysis of cellulose by making enzymes unavailable for effective hydrolysis reducing its overall hydrolytic efficiency.

4. Probable Solutions for Biocatalyst Applications

4.1. Additives to Prevent Non-Productive Binding of Enzymes to Lignin

During hydrolysis of biomass via enzymes, supplementation of additives may address the blocking of unproductive binding of cellulase to lignin [77]. Few studies report that surfactants may reduce the non-productive binding of enzymes with lignin and promote efficient hydrolysis as more enzymes become available for acting on cellulosic fibres [78]. Xu et al. analysed a series of non-catalytic proteins, ionic and non-ionic surfactants as well as biosurfactant as additives during enzymatic hydrolysis of biomass [79]. Employing cheaper non-catalytic proteins such as bovine serum albumin, ovalbumin, corn steep liquor or even peptone reduces the enzyme losses due to the non-productive adsorption of cellulase on lignin [80]. The cheaper non-catalytic protein binds with the lignin allowing cellulase to act on cellulose for effective hydrolysis, thus enhancing the overall hydrolytic efficiency and yield; also saving the cost of enzyme [11].

4.2. Sequential Addition of Biomass (Fed-Batch) for Increased Hydrolysis at High Solid Loading Doses

The enzymatic hydrolysis becomes dramatically frailer at high solid loading which is a major bottleneck in biomass to ethanol technology [69]. To overcome the limitations researchers have employed fed-batch process for enzymatic saccharification where pretreated solid biomass was fed in multi-steps to obtain a relatively higher concentration of sugar and thereby ethanol [81,82]. Sequential addition or fed-batch strategies have the potential to overcome the challenges of mixing to maintain low viscosity thereby resolving the issues of mass transfer enabling low power consumption [83]. Fed-batch enzymatic hydrolysis gives higher glucose concentration and higher glucan conversion efficiency at high solid loading biomass hydrolysis [84]. Even by following this strategy, it seems hard to cross 5% wt/V ethanol concentration due to having less cellulose content and high lignin (>30%) in some biomass, hence the pre-treatment of biomass which favors lignin removal can be effective [84]. By adopting this strategy, the authors reported upto 80% glucan conversion at high solid loading thereby ethanol fermentation using Saccharomyces cerevisiae with a corresponding yield of 82.7%. Studies based on fed-batch mode have evaluated various parameters like initial solid loading, one-time or fed-batch enzyme addition, the number and time of feeding of biomass/substrate. It is necessary for the fed-batch biomass addition strategy that the initial solid loading must be highest possible that the system can hold which will promote fast liquefaction of biomass by enzyme (which will be comparatively higher per gram of biomass then in case of batch hydrolysis) releasing glucose at a faster rate. Then the biomass may be added further which will increase the total solid loading at the end [85]. Still the higher efficiencies of hydrolysis may depend on other multiple factors also like type of biomass, pretreatment, enzyme (components or the cocktail) and the enzyme dosage as given in Table 3. Higher enzyme dosage may decrease the time for liquefaction however it needs to be minimized for economic reasons [85]. Periodic peristalsis has been reported to reduce the water constrain to a greater extent during biomass hydrolysis at high solid loading [85–88]. A combination of fed-batch pattern with appropriate rotational speeds in hydrolysis reactor and initial solids loading is suggested to facilitate, scalable, energy-efficient biomass hydrolysis [89]. The authors presented that even though the hydrolysis efficiency decreases with

increasing solid loading the over glucose concentration in the broth was as high as 206 g/L at 45% solid loading [89], which indicates that upto ~10% ethanol can be produced with high efficiency fermentation making the bioethanol distillation cost effective.

Along with biomass/substrate fed-batch, enzyme fed-batch was also tried for improving hydrolysis, however the batch feeding of enzyme initially was reported to be the best [53,83]. The reason could be that the enzyme per gram of biomass remains high initially, causing quick hydrolysis and liquefaction; thereby making further addition of biomass feasible, however the stability of enzyme needs to be high so as to remain active till the end of hydrolysis. Table 3 gives an account of high solid loading of biomass achieved by fed batch mode of biomass addition and the hydrolysis efficiency. Data in Table 3 enables us to understand that there are multiple factors which are responsible for higher hydrolysis efficiencies such as biomass composition, pretreatment, solid loading, strategies of loading biomass, bioreactor employed, the enzyme dosage and type of enzyme along with reaction conditions.

Table 3. High solid loading of biomass adopting fed-batch mode for increased sugar concentration.

Biomass Type	Pretreatment	Final Solid Loading	Vessel Type	Feeding Strategy Biomass Addition (%) and the Timings (h)	Enzyme and Its Dosage	Harvest Time	Hydrolysis Efficiency on the Basis of Holocellulose Content	Reference
Sugarcane bagasse	Formalin pretreated, acetone dried	30%	Erlenmeyer flasks	10%, 10% and 10% at 0 h, 12 h and 36 h or 48 h respectively	Cellulase from Novozymes at 10 FPU/g dry biomass	144 h	86%	[53]
Sugarcane bagasse	Formalin	20%	Erlenmeyer flasks	6.6%, 6.6% and 6.6% at 0 h, 12 h and 36 h respectively	Cellulase from Novozymes at 10 FPU/g dry biomass	144 h	80%	[53]
Sorghum straw	Milled to 20–40 mess size, Alkaline oxidative pretreatment using NaOH and H_2O_2	15%	Stirred tank reactor with three marine propellers with three blades	every 10 min, details not given	Cellic Ctec2 204 FPU/mL calculated by authors, 80 FPU/g pretreated biomass	10 h	91%	[85]
Sorghum straw	Milled to 20–40 mess size, Alkaline oxidative pretreatment using NaOH and H_2O_2	20%	Stirred tank reactor with three marine propellers with three blades	every 10 min, details not given	Cellic Ctec2, 80 FPU/g pretreated biomass	10 h	75%	[85]
Sugarcane bagasse	Alkali pretreatment (0.4% NaOH/g biomass)	22%	Not mentioned	10%, 5%, 4%, 3% at 0 h, 8 h, 12 h, 16 h respectively	Cellic Ctec3, 0.55 FPU/mg protein, 4 FPU/g dry biomass	48 h	76%	[79]
Corn stower and wheat straw	Dilute acid pretreatment (0.75% H_2SO_4)	20%	3 L stainless steel vertical reactor with helical impeller	10%, 5%, 5%, at 0 h, 3 h and 6 h respectively	Enzyme dosage 20 mg/g glucan	72 h	58%	[89]

Corn stower and wheat straw	Dilute acid pretreatment (0.75% H_2SO_4)	30%	3 L stainless steel vertical reactor with helical impeller	20%, 5% and 5% at 0 h, 3 h and 6 h respectively	Enzyme dosage 20 mg/g glucan	72 h	55%	[89]
Corn stower and wheat straw	Dilute acid pretreatment (0.75% H_2SO_4)	45%	3 L stainless steel vertical reactor with helical impeller	21%, 8%, 8% and 7% at 0 h, 3 h, 6 h and 9 h respectively	Enzyme dosage 20 mg/g glucan	72 h	48%	[89]
Sugarcane bagasse	Alkali Organosolv	20%	-	8%,4%, 4%, 4% at 0 h 6 h, 12 h and 18 h respectively	3 FPU/g biomass + xylanase (2.4 mg/g biomass) + AA9 (1 mg/g biomass)	72 h	85%	[86]
Rice straw	Dilute acid	30%	Stainless steel vessel of 250 mL working volume with a double helical ribbon impeller	10%, 10% and 10% at 0 h, 12 h and 24 h respectively	Cellic Ctec2, 15 FPU/g glucan	60 h	76%	[90]
Corn stover	Organosolv pretreatment	40%	100 mL red cap Duran, Scott bottle	12%, 7%, 7%, 7% and 7% at 0 h, 2 h, 6 h, 24 h and 48 h respectively	Cellic Ctec2 247 FPU/mL, 15 FPU/g dry biomass	96 h	89% when 16.8% was oligomers, 25% total reducing sugar was obtained	[53]
Sugarcane straw	Hydrothermal	30%	Bioreactor with 50 mL working volume, having three two-flat-blade paddle impellers	5%, 5%, 5%, 5%, 5% and 5% at 0 h, 2 h, 4 h, 8 h, 12 h and 24 h respectively	Cellic Ctec2 10 FPU/gds, added in fed batch mode	72 h	~71%	[83]
Sugarcane straw	Hydrothermal	30%	Bioreactor with 3 L working volume, having two three-blade elephant ear impellers	2.5%, 2.5%, 2.5%, 2.5%, 2.5%, 2.5%, 2.5%, 2.5%, 2.5% and 2.5% at 0 h, 1 h, 2 h, 4 h, 8 h, 12 h, 18 h, 24 h, 30 h, 36 h, 42 h and 48 h respectively	Cellic Ctec2 10 FPU/gds, added in fed batch mode	144 h	~60%	[83]

4.3. Simultaneous Saccharification and Fermentation of Biomass

Simultaneous saccharification and fermentation (SSF) is crucial for high titres of ethanol production in case of feedback inhibition of β-glucosidase. Usually enzymatic hydrolysis of cellulose into glucose is done by cellulase which comprises exo-cellulase, endocellulase and β-glucosidase. Cellulose micro-fibres are attacked first by endocellulase in between the fibres to produce small fibres with reducing and nonreducing ends and then exo-cellulase attacks on these reducing and non-reducing ends to produce glucose dimers (cellobiose) which are completely hydrolysed into glucose by β-glucosidase [22]. The synergistic action of the enzyme is crucial for efficient hydrolysis of biomass. Feedback inhibition of β-glucosidase by its end product glucose is also a major challenge, as it is a rate-limiting enzyme [91]. This feedback inhibition can be tackled when glucose is utilized as soon as it is generated which is possible via simultaneous saccharification and fermentation (SSF), where yeast utilizes glucose produced during hydrolysis and convert them to ethanol simultaneously. It was demonstrated that at 23.0% solid loading, even with low enzyme loading of ~6.5 FPU/g glucan 80.8% ethanol yield can be obtained via SSF [92]. Cellulase produced from *Trichoderma reesei* is usually deficient in β-glucosidase and glucose sensitive also [21,93] and thus *Penicillium* sp came into the limelight having optimal β-glucosidase showing significant resistance to glucose [28]. Even *Aspergillus* sp. are known to produce higher and glucose resistant β-glucosidase comparative to *Trichoderma* however lacks other counterparts of cellulase [19,24]. Thus, via designing the cocktail of cellulolytic enzymes by blending various components of cellulase from different sources, especially glucose tolerant β-glucosidase; the issue of feedback inhibition may be resolved [11,26].

SSF is an acceptable strategy to overcome feedback inhibition to obtain a higher concentration of ethanol, however, the yeast employed must be resistant to the ethanol concentration targeted. Figure 3 shows the SSF strategy for ethanol production. High inhibitor concentration due to high solid loading may be tackled by using multiple inhibitor tolerant yeast which may be developed by adaptive evolution [9].

It has been observed that batch fermentation results in lower ethanol concentration in broth and usually reaches ~4% ethanol concentration in the broth which is also not feasible economically for distillation to obtain anhydrous ethanol [12,94]. Hence it is very much required to enhance the ethanol concentration in the broth for reduction in energy consumption during ethanol recovery [83]. Low ethanol concentration in broth is primarily due to low solid loading and may be pentose sugar presence which remains unfermented. Hence to obtain higher ethanol concentration, increasing solid loading is necessary but cellulosic biomass are low-density bulk volume material with high strong hygroscopicity which causes difficulty to handle slurries exceeding 15% solid concentration. Also, the broth usually contains pentoses and hexoses together and pentose fermenting yeast could significantly enhance the ethanol production efficiency. There are leads on genetically modified yeast being able to ferment hexoses and pentoses simultaneously.

SSF of solid LC biomass have the capacity to improve the economics of the 2nd generation ethanol process as well as reduce the complexity by consolidating the steps of the process and combating to an extent the end-product inhibition of enzymes which is prominent in separate hydrolysis and fermentation.

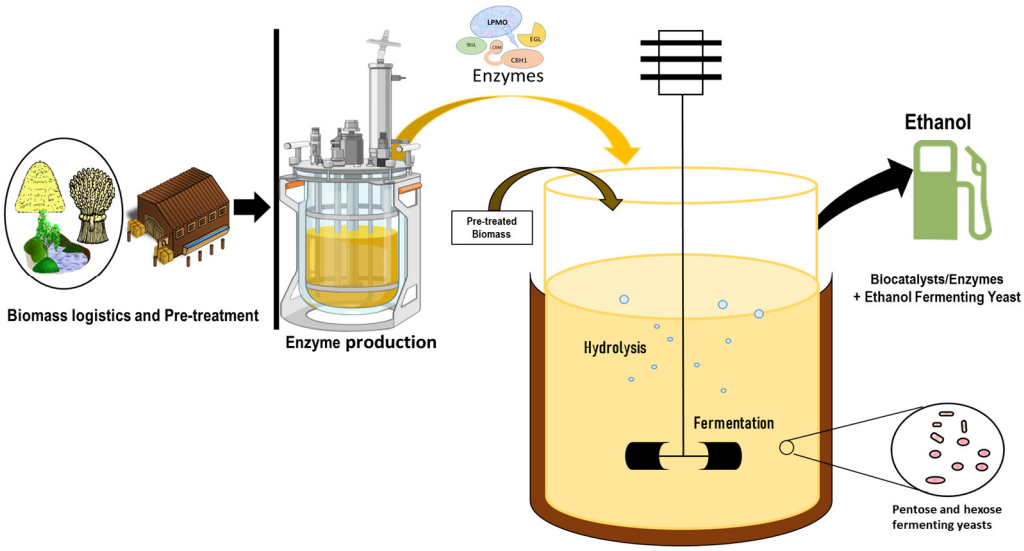

Figure 3. Simultaneous saccharification (hydrolysis) and fermentation for ethanol production.

5. Future Perspectives and Conclusions

Bioethanol from lignocellulosic biomass is an alternative to petroleum fuel which is environmentally sustainable. Along with significant benefits it offers in comparison to petroleum fuels there are challenges associated with it. Holocellulose present in biomass can be converted into fermentable sugars by employing biocalaysts which is the most acceptable route for biomass conversion. The major bottlenecks at this stage is the feasibility of hydrolysis which needs to be at high solid loading of biomass so as to have descent fermentable sugar concentration to be converted to ethanol. Solid-loading above 20% of pretreated biomass is required to have more than 4% ethanol concentration to have economically feasible distillation. Challenges are associated with high solid loading of biomass, low hydrolysis efficiency due to mass transfer issue, high inhibitors concentration, nonproductive lignin-enzyme binding, feedback inhibition of enzyme, etc. which can be resolved to an extent by adding additives as noncatalytic protein or surfactants, adding biomass in fed-batch mode in multiple splits to the bioreactor, employing simultaneous saccharification and fermentation mode, employing cocktail of enzyme with glucose resistant β-glucosidase, etc. Bioreactor design and novel bioprocess strategies have the capacity to revolutionise this step which is critical for the success of the 2nd generation bioethanol technology. Pretreatment of biomass supporting lignin removal or relocalisation like hydrothermal pretreatment and deep eutectic solvent methods must be employed which are eco-friendly also. Improved inhibitor tolerant and highly efficient enzymes, improved pretreatment strategies along with better hydrolysis bioreactor design may bring pivotal changes in the overall picture. Though bioethanol plants are coming up globally, still there are space for improvement in the technology which needs to be addressed seriously. Bioprocess for cellulase production as well as properties of produced cellulase such as temperature tolerance would be beneficial [95]. Bioprocess engineering, better enzyme cocktail with synergistic acting components, additives for lignin blocking, may change the overall efficiency of the process and paves the way for efficient bioconversion of biomass via biocalaysts for bioethanol production.

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