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Emerging trends in high-solids enzymatic saccharification of lignocellulosic feedstocks for developing an efficient and industrially deployable sugar platform

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Abstract

For the techno-commercial success of any lignocellulosic biorefinery, the cost-effective production of fermentable sugars for the manufacturing of bio-based products is indispensable. High-solids enzymatic saccharification (HSES) is a straightforward approach to developing an industrially deployable sugar platform. Economic incentives such as reduced capital and operational expenditure along with environmental benefits in the form of reduced effluent discharge make this strategy more lucrative for exploitation. However, HSES suffers from the drawback of non-linear and disproportionate sugar yields with increased substrate loadings. To overcome this bottleneck, researchers tend to perform HSES at high enzyme loadings. Nonetheless, the production cost of cellulases is one of the key contributors that impair the entire process economics. This review highlights the relentless efforts made globally to attain a high-titre of sugars and their fermentation products by performing efficient HSES at low cellulase loadings. In this context, technical innovations such as advancements in new pretreatment strategies, next-generation cellulase cocktails, additives, accessory enzymes, novel reactor concepts and enzyme recycling studies are especially showcased. The review further covers new insights, learnings and prospects in the area of lignocellulosic bioprocessing.

Keywords: Lignocellulosic biomass; Cellulases; High-solids enzymatic saccharification; Fermentable sugars; Bio-based products

Abbreviations

2G- Second-generation	IU- International Units
AE-Accessoryenzymes	LCA- Life-cycle assessment
AFEX- Ammonia fiber expansion	LCB- Lignocellulosic biomass
BALI-Borregaard Advanced Lignin	LCC- Lignin-carbohydrate complex
BR- Biomass recalcitrance	LMW- Low molecular weight
BSP- Bagasse sulphite pulp	LPMO- Lytic polysaccharide monooxygenase
CAPEX- Capital expenditure	LWH- Liquid Hot water
CBU- Cellobiase units	MSSP- Minimum sugar selling price
CELF- Co-solvent enhanced lignocellulosic fractionation	OPEX- Operational expenditure
CS- Corn Stover	PEG- Polyethylene glycol
CrI- Crystallinity Index	PHP- Phosphoric acid plus hydrogen peroxide
DA- Dilute Acid	PPEH- Periodic peristalsis enzymatic hydrolysis
DDR- Deacetylation and disc refining	RaBIT - Rapid Bioconversion with Integrated recycle Technology
DM- Dry matter	RS- Rice straw
DMR- Deacetylation and mechanical refining	RSM- Response Surface Methodology
DP- Degree of polymerisation	SCB- Sugarcane bagasse
DryPB- Dry acid pretreatment and bio-detoxification	SE- Steam Explosion
EA- Extractive Ammonia	SHF- Separate hydrolysis and fermentation
ED- Ethylenediamine	SPORL- Sulphite pretreatment to overcome recalcitrance of lignocellulosics
EtOH- Ethanol	SSF – Simultaneous saccharification and fermentation
FPU- Filter paper units	SScF- Simultaneous saccharification and co-fermentation
GHG- Green house gas	TEA- Techno-economic analysis
HRR- Horizontal Rotating reactor	THF- Tetrahydrofuran
HSES - High-solids enzymatic saccharification	VSTR- Vertical stirred tank reactor
HT- Hydrothermally	WS- Wheat Straw
	USD- US dollars

Introduction

The world is witnessing a gradual transition in energy to overcome the shortfalls of fossil fuels such as their non-renewable nature, import dependence, and environmental pollution, including global warming due to greenhouse gas (GHG) emissions [1]. Sustainability and energy-secured future is envisaged from renewable energy sources as such solar, water, wind, geothermal and biomass. In particular, lignocellulosic biomass (LCB) is an inexpensive, biodegradable, abundant and non-edible energy-dense material popularly termed as second-generation (2G) feedstock. It is a repository of fermentable sugars preserved as structural carbohydrates in the form of cellulose and hemicellulose that are intertwined and sealed by a complex and heterogeneous aromatic polymer known as lignin by covalent interactions forming Lignin-Carbohydrate Complex (LCC) [2-4]. LCB stands uniquely distinct as it can be transformed into diversified products ranging from energy, power, fuels, chemicals, polymers, carbon fibres to enzymes, composites, adsorbents, functional pharmaceutical ingredients depending on technology platforms used [3,5,6].

The biochemical approach for LCB valorisation invariably generates a sugar platform comprising principally glucose and xylose. It involves multistage process modules, namely pretreatment, hydrolysis, fermentation and downstream processing [2,7,8]. Commercial exploitation of this route is highly dependent on the cost-effective production of clean fermentable sugars with no or minimal amount of inhibitors and their efficient transformation to bio-based fuels and chemicals. High-solids enzymatic saccharification (HSES) is one of the readily deployable strategies to attain industrially relevant titres of fermentable sugars [9,10]. The substrate loading in HSES is generally >15%, where practically "no free water" is available at the start of the reaction [10]. However, it has been observed that the sugar release is often disproportionate to increased solid loadings. Therefore, to expedite hydrolysis and attain improved biomass conversion rates, researchers tend to perform HSES at high enzyme loadings ($\geq$ 25 FPU or 25 mg protein g^{-1} glucan). Yet, the significance of enzyme and operating cost during a longer duration of hydrolysis cannot be undermined while achieving higher sugar yields [11]. Moreover, earlier studies confirm that cost of enzymes is one of the bottlenecks in industrialisation LCB-based biorefineries, e.g. cellulase alone accounts for 25-30% of the operational cost in a 2G biorefinery [12]. Therefore, in the last decade, global efforts have been made to simultaneously reduce the enzyme loadings during HSES and achieve high product titres. However, before addressing the issue of "enzyme cost," it is equally essential to understand the role of competing factors that play a vital role during HSES. Figure 1 illustrates the technical hurdles associated with HSES and depicts the broad areas of improvement in processes and enzyme functioning targeting these barriers.

An in-depth analysis on the exogenous and endogenous constraints of HSES has already been done by da Silva et al. [10]. The present review stands remarkably distinct by discussing emerging trends in various process modules that not only circumvented the bottlenecks of HSES but successfully demonstrated the development of an industrially deployable sugar platform (($\geq$ 85 g L^{-1} fermentable sugars or $\geq$ 40 g L^{-1} bio-based products) at enzyme loadings < 17.5 FPU or 17.5 mg protein g^{-1} glucan. This enzyme loading was chosen based on the fact that a good pretreatment step invariably increases the glucan content up to ≥55% and the cellulase doping restricted below 10 FPU or 10 mg protein g^{-1} dry matter (DM) is considered as low enzyme loadings. Some notable works where no chemical pretreatment was done are discussed with exceptions. The last part of the review highlights the new insights gained from existing literature and prospects in this area.

General approaches promoting HSES

This section discusses general approaches that promote HSES. For instance, the water constraint at high-solids ($\geq$ 15%) makes it inevitable for the researchers to perform saccharification in a fed-batch mode due to the mass and heat transfer problems in such a viscous broth with limited water activity. Multi-step substrate feeding overcomes the mass and heat transfer limitation. It enhances the diffusivity of the cellulases in the reaction slurry leading to an early onset of biomass liquefaction, a significant drawback with a single substrate feeding regime [13]. However, an efficient fed-batch strategy largely relies on a number of factors including type of lignocellulosic feedstock, choice of pretreatment, solid loading targeted, composition of cellulase cocktail, dosage selection, enzyme kinetics, fortification with accessory enzymes/additives and duration of hydrolysis. Similarly, after attaining concentrated sugar solutions, there are three routes to ferment sugars to desired products. These routes are known as separate hydrolysis and fermentation (SHF), simultaneous saccharification and fermentation (SSF) or simultaneous saccharification and co-fermentation (SScF). In the first process, after hydrolysis, the sugar-rich liquid stream is separated from the residual biomass and is then fermented to various products. In the latter approaches, microbial fermentation is commenced after brief pre-hydrolysis, where microbes consume sugars in the heterogeneous slurry amidst the presence of inhibitors. In SSF, the microbe can assimilate only one carbon source, whereas, in SScF, the strain can consume one or more carbon sources sequentially or simultaneously. The last two processes have gained importance in the recent past owing to several advantages. SSF and SScF can successfully eliminate the problem of "product inhibition" exhibited by cellulases, as the sugar produced during hydrolysis is continuously diverted for fermentation. Reduced capital expenditure (CAPEX), operating expenditure (OPEX) and low effluent discharge give an extra edge to these

processes [14]. These are some typical process variables to negate the pitfalls accompanying HSES and are an integral part of LCB bioprocessing while exploiting the biochemical platform.

Effective strategies to promote HSES at low enzyme loadings

Physicochemical pretreatment

Significant reduction in biomass recalcitrance (BR) is essential to unlock the potential of LCB and extract the carbohydrate fraction successfully. This inherent feature of any biomass is the culmination of several factors associated with it. The primary determinants include the distribution of LCB's principal components, the chemical composition of lignin, types of LCC linkages and their predominance, degree of polymerisation (DP) and crystallinity of the cellulose, presence of lipids, proteins, pectin, extractives and mineral content, which in turn control the physical properties of the biomass [15]. Thus, BR acts as a barrier and shields the embedded polysaccharides of LCB from enzyme attack. Hence, a rational design of an efficient and low-cost physicochemical pretreatment is highly desirable for HSES [16]. Pretreatment should ideally disrupt the LCC linkages leading to effective removal of ash and lignin, fractionation of disaggregated biopolymers, reduced crystallinity index (CrI) and improved surface characteristics of biomass, making it accessible for enzymatic hydrolysis. The forthcoming sections discuss successfully employing various conventional and emerging pretreatment methods for producing industrially relevant titres of fermentable sugars or their fermented products at cellulase loadings ≤17.5 mg protein or FPU g^{-1} glucan.

Conventional pretreatment strategies

Generally, the conventional strategies involve single-stage use alkali (NaOH, KOH, NH_3, Na_2CO_3), acids (mainly inorganic acids such as H_2SO_4, H_3PO_4, HCl, HNO_3) or water (hot water, hydrothermal, sub-critical water, steam explosion) for biomass pretreatment [17]. NaOH usage is the most economical, preferred, popular and industrially deployable method that facilitates lignin solubilisation, mercerisation of cellulosic fibres, increases biomass porosity, swelling characteristics, and water holding capacity [17, 18]. However, the major disadvantage of these high digestible pretreated carbohydrate-rich biomasses is the inefficient utilisation of xylose fraction. Interestingly, many industrial microbes such as *S. cerevisiae* lack the genes for the xylose metabolism or even if genes are present, owing to carbon catabolite repression, glucose is the preferred substrate and catabolism of xylose is repressed [19,20].

On the contrary, water, steam and typically dilute acids (DA) cleaves the thermolabile acetyl groups attached to the hemicellulose backbone releasing acetic acid. This weak acid, in turn, facilitates selective hydrolysis of amorphous hemicellulose and the release of pentose-rich sugars in the aqueous fraction [17, 21]. But the

drawbacks associated with these processes include partial melting of lignin and its re-condensation as pseudo-lignin onto pretreated biomass which blocks the pores, restricting the accessibility of cellulases and increasedbiomass crystallinity [22, 23]. Many investigators prefer to adopt a minimum of two-step chemical pretreatment to circumvent the flaws accompanying each of these traditional approaches. Generally, such processes involve the xylan removal by its hydrolysis in the first step and partial or complete delignification in the next. Table 1 gives a summarised account of various single or multistage traditional pretreatment methods that led to polysaccharide enrichment, biomass delignification, and the subsequent impact on product titres as well as conversion yields.

As evident from Table 1, most of the NaOH pretreatment studies led to ≥65% biomass delignification and displayed product bioconversion yields not exceeding 66%. Only one study is an exception, where 84.5% glucan conversion was achieved, with sugar productivity being 3.47 g L^{-1} h^{-1} [29]. By performing pretreatment and fed-batch hydrolysis at high solids, 530g of fermentable sugars were extracted from one kg sugarcane bagasse (SCB) at the expense of 1.32 USD/kg sugar and contributed 1.57 CO_2 equiv. in terms of climate change. Thus, the group could decipher the cost of fermentable lignocellulosic sugars, which serves as a sugar platform to produce an array of industrially important chemicals [29]. Earlier, even Gao et al. [26,28] reported the NaOH pretreatment of SCB at 16.67% solids, as it generated less effluent, reduced alkali consumption and further showed the competitive edge of using thermophilic *Kluyveromycesmarxianus* NCYC 587 over mesophilic *Saccharomyces* for cellulosic ethanol (EtOH) production in an SSF process.

Yet another featured study, which looks highly promising in terms of pretreatment and product yields, used dilute sulphuric acid followed by sodium hypochlorite. It only led to selective xylan hydrolysis of Chinese fir sawdust in stage I but delignified >90% biomass in stage II. Fed-batch SHF resulted in nearly complete glucan conversion, releasing 138.8 g L^{-1} glucose and attaining 93.2% EtOH yields and productivity of 5.4 g L^{-1} h^{-1} [37].

A decade ago, Zhu et al. investigated the performance of their newly developed SPORL (Sulphite Pretreatment to Overcome Recalcitrance of Lignocellulosics) pretreatment at highs-solids using aspen wood chips as starting material. Using the SSF approach, they demonstrated the superiority of SPORL over DA by attaining 65.7% more EtOH titres and confirmed that the net energy input of DA was 1.28-1.3 fold higher than SPORL [32].

Such state-of-the-art art has always inspired researchers to ameliorate existing pretreatments or explore newer pretreatment methods that are cost-competitive while aiming for efficient fractionation and upgradation of LCB components. In this aspect, recently, Kalyani et al. targeted holistic utilisation of birchwood pretreated via steam explosion (SE) [35]. During their study, cellulose was valorised to EtOH, while the pre-hydrolysate generated

after SE and the spent liquor with lignin-rich biomass produced after fermentation was used for bio-methane production via anaerobic digestion.

However, in the quest to attain high sugar or product titres, researchers tend to overlook the loss of carbohydrate fraction during pretreatment in many instances. For example, Ahmed et al. were able to attain 80.9% EtOH yields from paper bark tree pretreated with subcritical water, but 46.3% glucan loss cannot be ignored considering process economics [33]. Similarly, Molaverdi et al. gave credit to high EtOH titres from rice straw (RS) to their choice of pretreatment, which used Na_2CO_3 and later performing solid-state SSF with *Mucor indicus*. This pretreatment led to 87.8% deashing and improved the swelling characteristics of biomass by 3.5 fold, but simultaneously led to >55% loss in carbohydrate fraction [30]. Authors are of the view that high product yields at the cost of unrecoverable sugar fraction lost during pretreatment can be uneconomical and should be forbidden.

Very recently, Pratto et al. adopted a multi-criteria optimisation for obtaining enhanced EtOH yields from HT pretreated sugarcane straw. Their sensitivity analysis revealed that the OPEX of the process reduced by 23.3% by introducing pre-saccharification before SSF [34]. Such studies indicate that during LCB bioprocessing, tools like response surface methodology (RSM) can play a pivotal role. Such statistical techniques are time-saving as they facilitate optimisation of more than one process variables at a single instance, help in understanding the mutual interactions between the variables and their combined effect. The study further validates that pre-hydrolysis may be highly beneficial when SSF or SScF approach is adopted.

Emerging pretreatment strategies

Besides traditional pretreatment methods, researchers are developing innovative strategies that promote delignification, increase cellulase accessibility and benefit either in terms of cost or product yield. If some processes target complete utilisation of all the biomass components, other claim to be environmentally benign. Table 2 elucidate the emerging processes, where either existing technologies were significantly revamped or new chemical entities were used, understanding the urgency of HSES and further showcases their mechanistic action which make them distinctly unique. Table 3 depicts the efficacy of emerging pretreatment methods and their subsequent impact on product titres and conversion yields.

Unlike traditionally pretreated LCB's (Table1), most of the feedstock pretreated with emerging technologies displayed higher product conversion yields of $\geq 80\%$, highlighting visibly competitive advantages with the latter (Table 3).Pretreatments such formiline [49], organosolv alkali [50] and γ-valerolactone in the presence of mild acid [55] appear lucrative for attaining high glucan conversion while $\geq 85\%$ EtOH yields from

pretreatedLCB's using DryPB [40], PHP [42] and THF in presence of mild H_2SO_4 [53] motivate the researchers to explore newer avenues in this process module section. Performing pretreatment at high-solids (40-70%) in newer processes like DryPB, PHP, alkaline organosolv and ED showcases the commendable efforts to reduce the environmental burden and capital cost [40, 42, 50, 57]. Successful demonstration of using green organic solvents like THF and γ-valerolactone during pretreatment, attempts towards their recovery and reuse provides clear evidences for developing eco-friendly and resource-efficient processes [53, 55]. Yet another example of minimum chemical usage and attaining highly digestable corn stover (CS) was shown by Chen et al.[47]. They found that an extra step of Szego milling, after their newly developed process of deacetylation and disc refining (DDR), reduced the Cellic CTec3 loading by 50% (16 to 8 FPU g^{-1} glucan) compromising 8g L^{-1} lesser sugar release from 22.5% solids. Techno-economic analysis (TEA), revealed that the total sugar yield together with HSES had dominant effect over total plant investment, while calculating minimum sugar selling price (MSSP) and so an additional Szego milling step (DMR) was beneficial [47]. Later the group attained 157 and 114 g L^{-1} glucose and xylose respectively by increasing solid loading to 32% [62].

Advancements in saccharification module

Besides advancements in pretreatment, optimal process designing for biocatalytic cellulosic depolymerisation at high-solids is imperative. Among several interventions, use of additives, auxiliary enzymes, their combinations, efficient enzyme hydrolyser units, cellulase recycling are some proven methods to reduce overall enzyme loadings. Lately, it is observed that researchers are preferring integrated/hybrid hydrolysis-fermentation configurations to attain high-product titres, reduce the CAPEX and OPEX of the overall process.

Use of additives

Use of the additives has been one of the most attractive strategies to reduce cellulase loadings during LCB hydrolysis. These additives primarily belong to two major groups, namely surfactants and non-catalytic proteins. The mechanistic action of the former group of additives has been comprehensively discussed by Al-Azkawi et al. [63]. State of the art reveals that surfactants may be either of chemical or biological origin. They primarily enhance the saccharification by preventing the unproductive binding of cellulases to lignin or increasing the stability of cellulases or preventing shear deactivation of cellulases at the air-liquid interface [64,65]. Thus, their addition not only reduces the enzyme loading and hydrolysis time but also aid in enzyme recovery.

The following paragraphs give a glance about the stimulatory effect of surfactant addition during HSES of various LCB's hydrolysed at low enzyme loadings.

Cannella and Jørgensen showed that PEG 3000 addition with 30% HT pretreated WS reduced the Cellic CTec2 loading by ~34% [66]. Despite of any process (SSF, SHF, PSSF) configuration employed, the EtOH yields improved between 18-24%. They further advocated preferential use of SHF over SSF for LPMO containing Cellic CTec2, as in the later case yeast cells competed with LPMO enzymes for molecular oxygen, suppressing their performance. Earlier, while performing SScF, Zhu et al. showed that Tween-20 addition reduced cellulase loadings from 15 to 7.5 FPU to obtain similar EtOH yields from 8.78% (w/v) aqueous NH_3 pretreated CS. Further, fed-batch SScF at 13.17 and 17.56% solids resulted in 52.9 and 68.8 g L^{-1} EtOH respectively in 96 h [67]. In yet another study, RS pretreated with novel strategy of combining $AlCl_3$ with glycerol, and hydrolysed with 3.3 FPU g^{-1} DM Cellic CTec2 achieved 12% enhanced glucan conversion by addition of Tween 80 (40 mg g^{-1} DM) in merely 48 h [51]. Likewise, Agrawal et al. in their recent investigation demonstrated that switching from batch to fed-batch mode and adding Ecosurf E6 enhanced the HSES of 20% (w/v) DA pretreated RS by ~10.2% in 30 h when hydrolysed with Sacchari SEB C-6 dosed at 5.66 FPU g^{-1} glucan [68].

Zhou et al. hypothesised that low molecular weight (LMW) lignosulfonates show hydrophilic surface characteristics and hence act as an anionic surfactant. They not only block the lignin moieties and prevent the non-productive binding of cellulases but stimulated biomass digestibility [69]. Liu et al. took the lead from this postulation [70]. When the performance of sulfonated lignin and alkali lignin was evaluated for HSES of alkali pretreated SCB, 5-8% higher glucose titres were obtained during fed-batch hydrolysis [70].

There is one instance where the efficacy of non-catalytic protein namely soy protein was tested for bench-scale fed-batch hydrolysis of 15% (w/v) SCB, pretreated by two different methods namely SE and LWH. Addition of 12% (w/w) soy protein improved glucose yields by 42% and 62% respectively in 24h from SE and LWH pretreated SCB [71]. However, in the present study the effect of pretreatment on altered biomass recalcitrance cannot be overlooked, as despite 15% higher enzyme loading SE pretreated SCB showed lower glucan conversion as compared to LHW owing to higher xylan, lignin and ash content.

Use of accessory enzymes

Besides additives, the role of accessory enzymes (AE) in enzymatic saccharification also cannot be ignored. Interplay between AE and cellulases can change the entire process dynamics of cellulose hydrolysis. Supplementation of β-glucosidase /cellobiase during hydrolysis not only alleviate the product inhibition but yielded higher glucose titres and enhanced saccharification yields as reviewed by Srivastava et al. [72]. Likewise, there are many instances where the addition of xylanase has improved cellulose accessibility and enhanced overall fermentable sugar yields [73,74]. However, the new generation enzyme cocktails exploit the

activity of unusual non-hydrolytic and copper containing enzyme namely lytic polysaccharide monooxygenases (LPMOs) for significantly boosting the industrial bioprocessing of 2G feedstocks. They catalyse the hydroxylation of C1 and/or C4 carbons (primarily involved in the formation of β-1,4 glycosidic linkage) in the presence of molecular oxygen, thereby destabilising the glycosidic bond and stimulating biomass hydrolysis [75]. This unique feature of LPMO's which belong to AA9 family of enzymes is being harnessed during HSES, both by commercially evolving cellulase cocktails and with newer promising enzymes either being an integral part or being added externally [76-77].The succeeding paragraphs depict the vital role of AE where they have acted synergistically with cellulase cocktails.

Bals et al. demonstrated the importance of Cellic HTec3 addition along with Cellic CTec3 when each of them dosed at 10 mg protein g^{-1} glucan. In a 72h fed- batch hydrolysis, ~100 and 30 g L^{-1} glucose and xylose was released from 30% (w/v) AFEX™ pretreated CS [78].

When fed-batch SScF studies were conducted with 20.7% (w/v) bagasse sulphite pulp (BSP) using thermophilic *Bacillus coagulans* CC17 (glucose, xylose and cellobiose assimilating) and hydrolysis was initiated by combination of Cellulclast 1.5 L (10 FPU g^{-1} glucan) and Pentopan Mono BG (120 IU xylanase g^{-1}), 110 g L^{-1} L(+) lactic acid was produced in 120 h [79]. Earlier, under batch SScF, addition of 120 IU g^{-1} Pentopan Mono BG increased lactic acid yields by 13.4% with 8.12% solids. Very recently, Liu et al. showed that xylanase significantly promoted cellulose hydrolysis [80]. Fortification of xylanase at 1200 IU/g substrate, enhanced Cellic CTec2 mediated glucose release by 19% from NaOH pretreated SCB.

Way back in 2011, the addition of PEG 6000 (70 mg g^{-1} glucan) was assessed for HSES of SO_2 catalysed and steam-exploded CS using Celluclast 1.5 L (10 FPU g^{-1} glucan) [81]. The saccharification efficiency of 20% (w/v) solids increased from 73.55 to 83.49% within 48 h. Fortification with Novozyme 188 at 30 CBU g^{-1} glucan, further enhanced the efficiency to 91.32%, glucose titres reaching 102 g L^{-1} [81].

Combinatorial approach of using additives and auxiliary enzymes

In the last three years, researchers have succeeded in harnessing the full potential of cellulase cocktails during HSES at ultra low loadings (<7.5 FPU or mg protein g^{-1} glucan) by adopting a combinatorial approach involving use of additives and AE to stimulate biomass liquefaction.

For instance Xu et al. used a diverse combination of AE and additives for fed-batch hydrolysis of 22% (w/v) alkali pretreated SCB. After 48h, they reported release of 122 g L^{-1} glucose when Cellic CTec2 (6.35 FPU g^{-1} glucan) with productivity being 2.54 g L^{-1} h^{-1}. They used AE like hemicellulase, β-glucosidase and additives such as whey protein, Tween 80, sophorolipid and calcium lignosulphonate to boost enzymatic

saccharification[82].Table 4 highlights some of the worth mentioning studies where cellulase loadings as low as 6 mg protein or FPU g^{-1} glucan yielded industrially relevant product titres from different 2G feedstocks. These processes clearly reveal that interplay between altered substrate characteristics after pretreatment, enzyme performance, combinatorial stimulation using AE and additives together with process configurations can change the entire dynamics of LCB bioprocessing (Table 4).

Activating LPMO activity present in new generation enzymes by co-substrate addition

The pioneering work of Bissaro et al., where they deciphered that H_2O_2 was preferred co-substrate over molecular oxygen for LPMO containing cellulase cocktails, has opened newer channels for exploiting the efficacy of these enzymes [86]. Costa et al. successfully demonstrated the use of H_2O_2 (fed at 200 µM h^{-1}) to trigger the LPMO activity of Cellic CTec3 during hydrolysis of spruce pretreated by a proprietary sulphite mediated process BALI™ [87]. Hydrolysis was carried out in a demonstration-scale module which could process ~4000 kg of biomass and had a three reactor system, where the first reactor was a screw feeder which could hold 30% dry matter. Later the substrate was bifurcated in two parallel reactor systems with final loading being 12% in each. With 4 mg enzyme addition g^{-1}DM, they validated that H_2O_2 addition resulted in 82 ± 3% glucan conversion of sulphite pulp spruce in 96 ± 2 hours. However, trials where no H_2O_2 addition was done, 13.5% reduced glucose yields were obtained after ~169h of saccharification [87].

Authors are of the view that such innovative approaches for harnessing the potential of AA9 enzymes during industrial LCB bioprocessing can turn incremental changes to disruptive, especially in terms of product titres. However, fundamental understanding into the functionalities of LPMO's, their mechanistic action, primary physicochemical parameters conferring them stability is a pre-requisite to utilise their capabilities completely.

Novel hydrolysis reactor configurations

To promote efficient mixing of enzymes with the substrate during HSES and maximise production of monomeric sugars researchers are working on its mechanical aspect as well.

For instance the performance of two reactors namely horizontal rotating reactor (HRR) and vertical stirred tank reactor (VSTR) was compared during fed-batch liquefaction of 25% (w/v) steam-exploded CS dosed with Cellic CTec2 [88]. After 87h hydrolysis, 14.3% enhanced glucose yields were obtained in HRR as compared to VSTR, indicating that the former configuration was able to mitigate the high-solid effect more efficiently, compared to the latter. In yet another study, Jung et al. conducted Cellic CTec2 (15 FPU g^{-1} glucan) mediated fed-batch hydrolysis of 30% (w/v) maleic acid pretreated RS in a 250 ml working volume enzyme reactor fitted with

double-helical impellor. When the agitation speed of the impellor was increased from 30 to 80 rpm, the glucose release was enhanced from 115 to 132 g L^{-1} within 60 h [89].

However, Katsimpouras et al., [90] accredited attaining high glucose titres to both optimised pretreatment strategy and use of free-falling mixer during hydrolysis. During the said study, CS was subjected to acetic acid-catalysed HT pretreatment (231.2 °C; 15.8 min). When Cellic CTec2 (9 mg g^{-1} DM) was used for liquefaction of 24% solids, 119.5 and 130.9 g L^{-1} of total reducing sugars was released after 12 and 24h, respectively, of which 69-71% accounted for glucose.

Liu and Chen recently introduced the unique concept of "periodic peristalsis enzymatic hydrolysis" or PPEH to intensify the HSES of steam-exploded CS [91]. In this study, the hydrolysis reactor had four peristalsis arms with several peristalsis balls which were motor-driven. Using this hydrolysis reactor, glucan and xylan conversion efficiencies with 20% (w/v) solids, improved by 25-36% and 3-9%, when compared to static enzymatic hydrolysis and incubator shaker enzymatic hydrolysis respectively where Cellic CTec2 was dosed at 10 FPU g^{-1} glucan. Detailed process investigation revealed that this method was not only curtailed down the transition time of biomass from solid to slurry phase leading to early onset of viscosity reduction but also successfully prevented enzyme deactivation during hydrolysis.

Enzyme Recycling

Several enzyme recycling strategies are explored in the quest to reduce the overall enzyme loadings during hydrolysis with uncompromised cellulase performance and product yields [92]. Some standard options involve contacting liquids or solids displaying high cellulase activity with fresh substrates, using molecular cut off membranes to concentrate cellulase from liquid stream and reuse it or using immobilised enzymes. However, these strategies have been successfully proven to work at only low solid loadings [93-95].

However, only one report exists where enzyme recycling has been successfully depicted during HSES. Recently a new process was developed by Jin et al. [96] wherein they claimed that it reduced the cellulase loadings by ~40% when compared to SHF. This technology referred to as RaBIT (Rapid Bioconversion with Integrated recycle Technology) exploited the fact the ~70-75% carbohydrate hydrolysis occurs in the first 24h, which was removed and subjected to high cell density fermentation. Meanwhile, the recalcitrant residual biomass with adsorbed enzyme is fed to a second reactor containing fresh substrate and topped up with lower enzyme loadings. This group demonstrated the production of ~90 g L^{-1} fermentable sugars (60 g L^{-1} glucose; 30 g L^{-1} xylose) in first five cycles from EA pretreated CS. However, the last step yielded ~75 g L^{-1} fermentable sugars from the said biomass. The process started with enzyme loading at 12 mg g^{-1} glucan and ended with

anloading ~8.4 mg g^{-1} glucan. SHF process liberated 77.4 and 38.5 g L^{-1} glucose and xylose respectively in 96 h from 17.3% (w/v) solids. For the said study, they used 8 mg Cellic CTec2, 2 mg Cellic HTec2 and 2 mg Multifect pectinase accounting for total enzyme loading of 12 mg protein g^{-1} glucan [96].

Upcoming trends in fermentation

In the recent past, researchers are boldly experimenting with hybrid/integrated hydrolysis and fermentation processes by introducing unique concepts to intensify and improve their efficiencies.

For instance, Wang et al., coupled an innovative concept of non-isothermal fed-batch SSF with newly developed H_3PO_4/H_2O_2 pretreatment for their feedstock [43]. After conducting a pre-hydrolysis at 50°C for 24h, *S. cerevisiae* was added and the temperature was reduced to 30°C and switched to 43°C constantly after every 6 h. Temperature switching not only promoted the rapid utilisation sugars at 30°C by yeast cells, relieving cellulases from product inhibition but later elevation at 43°C restored the enzyme activity boosting substrate hydrolysis and subduing the yeast metabolism during that period. After 48h, this novel approach of SSF yielded 63.9 ± 1.5 g L^{-1} EtOH from 40% (w/v) solids and was far superior to SHF performed at 43°C where the titre was 48.2 ± 2.2 g L^{-1} [43].

Use of eukaryotic systems like *Kluveromycesmarxianus*NCYC 587, *Mucor indicus*, dry active yeast, *S. cerevisiae* 424A, *S. cerevisiae* D_5A, *S. cerevisiae* SyBE005, co-culturing temperature resistant *S. cerevisiae* and xylose utilising *S. cerevisiae* and prokaryotic *Bacillus coagulans* CC17 for attaining high product titres are few examples that reaffirm that use of temperature resistant, product tolerant and multiple sugar assimilating microbes for high solids hydrolysis and fermentation is booming [26, 28, 30, 43, 45, 53, 57, 58, 79].

Further the demand of exploiting robust microbes capable of metabolising wide range of carbon sources and display adaptability to low pH, high temperatures, soluble and insoluble inhibitors, high substrates and fermentative metabolites thereby catalysing efficient bio-transformations will remain the centre-stage.

The state of the art clearly indicates that pre-hydrolysis is an inevitable and first step of any high-solids SSF or SScF process. However, depending on the sugar-uptake rate of each fermenting microbe, this duration varies from 6-24h. Besides pre-hydrolysis, the authors are of the opinion that enzyme dosing and microbial inoculum both critically govern high-solids SSF or SScF. The success of these processes relies on fine balance between enzyme and microbial inoculum loadings that promote continuous and steady consumption of hydrolysedsugars facilitating efficient fermentation in an uninterrupted fashion.

Exceptional cases which involved no chemical pretreatment

Zheng et al. proved that the pretreatment step could completely be eliminated in case of CC if the right choice of bio-surfactants is made [97]. For their study, unwashed CC's devoid of xylan and having traces of xylose, obtained from Chunlie Furfural Corporation, China were used. After 120 h SSF of 20% (w/v) CC fortified with 0.2 g L^{-1} rhamnolipid resulted in 82.38 % glucan conversion with Cellic CTec2 dosed at 10 FPU g^{-1}glucan and EtOH concentration being 30.76 g L^{-1}. However, when no rhamnolipid was added the same conversion yields (82.18%) were achieved with enzyme load being 15 FPU g^{-1} glucan with 23.87 g L^{-1} EtOH titres. Thus, rhamnolipid addition not only aided in enzymatic hydrolysis but had a positive impact during fermentation. Later, they improvised the process by replacing rhamnolipid with tea cake which contained nearly 10% tea saponins. At 15% (w/v) solids, co-feeding with tea cake led to EtOH yields of 86.5% with only 3.12 g L^{-1} residual glucose detected after 120h. However, when no tea cake was added, Cellic CTec2 mediated SSF at the same FPU dosage (10 FPU g^{-1} glucan)resulted in 74.55% EtOH yields [98].

Lu et al. [99] adopted an entirely different approach to obtain industrially relevant sugar titres from CS. They chose ball milling to reduce the CrI and DP of biomass. This optimised process was able to disrupt the LCC linkages successfully, thereby boosting the release of sugars from the biomass at high-solids. At 10 FPU g^{-1} DM, Cellic CTec2 was able to liberate nearly ~130.5 g L^{-1} of fermentable sugars from 30% (w/v) solids in 48h.

Key insights and learning's

Better product conversion yields and attempts towards chemical recycling using emerging pretreatment strategies indicate that new chemical entities, their chemistry would predominate the future and will play a decisive role for the cleaner biomass fractionation. However, in depth elucidation of the functional group changes within the biomass after pretreatment and molecular interactions of biomass with enzymes at nano-scale is highly recommended. Similarly, recent incorporation of low-cost tea saponins and low molecular weights lignin derivatives as additives to stimulate HSES gives a lead to researchers on using compounds which are presently treated as waste but are abundant, cheap and can prove to be a game-changer.

The researchers are already witnessing the synergistic role of AA9 enzymes in boosting the efficacy of biofuel cellulases. Thus, the authors anticipate that in the coming decade, bioprospecting potent AA9 enzyme producers and validating their potential towards depolymerisation of industrially pretreated LCB's would be emphasised. Likewise, the novel reactor designs for efficient enzymatic bioprocessing would predominate to turn "sugar-based platform" into commercial reality.

Smart choices of waste products such as corn-cob residues obtained from furfural industries may give an insight on exploring those industries that generate similar type of by-products [97,98]. It would partially reduce the pretreatment processing costs of the biomass and environmental burden as well.

However, it is essential that researchers recognise that in a spur to attain higher sugar titres at low enzyme loadings, but neglecting carbohydrate conversion yields and productivities can be deleterious. Sustainability and profitability of sugar-based platform relies on extracting at least 80% sugar monomers from LCB and their successful biotransformation within stipulated time. Moreover, it is a general observation that enzyme loadings are done on dry matter basis as compositional analysis is not essential for kick-starting the experiments. But it often leads to results which cannot be reproduced and wrong data interpretations. Especially, while benchmarking two pretreatment methods [77] or while evaluating same pretreatment method for two different LCB's [24] the researchers should consider cellulase loading based on glucan content rather than DM of biomass. Different pretreatments with same biomass or different biomasses with same pretreatment are likely to have different cellulose recoveries. Enzyme dosed based on glucan content would eliminate the problem of inconsistent glucan: enzyme ratio and will give consistent and reliable results.

Similarly, pretreated LCB's especially having high water-holding capacities, tend to entrap significant fractions of sugars as shown in our earlier study [100]. A washing step, post-saccharification will ensure valuable sugar recoveries and help in recognising the real saccharification yields.

Some researchers passionately work towards enzyme reduction and tend to increase the duration of SHF/SSF or SScF by extending intermittent substrate feeding till 48h. Earlier studies and our own experience during HSES of alkali pretreated SCB has shown that between 8-10 hours, the hydrolysis escalates rapidly [101]. Hence feeding fresh substrate beyond this time limit can adversely affect saccharification owing to feedback inhibition by monomeric sugars released expeditiously. Further, intervention of TEA at the right stage can help in evaluating the trade off between bioprocessing time and enzyme cost. Hence, it should be an integral part of any study where the process has been established and validated, but is pending scale-up, as displayed by Baral et al [29]. Recent attempts by Nwamba et al. [102] to minimize enzyme loadings to 2 mg protein g^{-1} DM show their committed efforts to maximize sugar release (158 g/L total sugars and 83% glucose yield) from 20% al-AGO pretreated SCB within 72 h using LT4 enzyme in combination with AE and additives. The group has been aggressively working towards improving the techno-economics of the process by incremental changes in sugar yields [77, 84,102].

Prospects

In the current scenario, most of the research is oriented towards attaining industrially relevant titres of glucose in particular and fermentation products like LA and EtOH. Nevertheless, trends for other industrially important platform chemicals such as succinic acid, fumaric acid, 2,3 butanediol, glutamic acid are also emerging steadily. Presently, researchers are conducting high-solids hydrolysis at relatively high enzyme loadings to achieve commercially desirable product yields of these chemicals [103-107]. The authors anticipate the biomass processing with low cellulases would soon gain momentum for the biotransformation of these products as well. Progressive innovations in industrially deployable pretreatment strategies are changing the entire perspective of 2G biorefinery as they are targeting holistic utilisation of LCB in a sustainable manner. Further, pretreatment at highs-solids and recycling of chemicals used during pretreatment are gradually trending to reduce both CAPEX and OPEX of this crucial module.

Similarly, an upsurge in the commercial exploitation of LPMO containing tailor-made enzyme cocktails during industrial bioprocessing of LCB's cannot be denied. Besides genetic interventions, the use of next-generation sequencing methods, metagenomic bioprospecting, molecular docking, secretome and transcriptome analysis will dominate the sector of "carbohydrate processing enzymes" to improvise their hydrolytic potential [108,109]. Likewise, cutting-edge, cost competitive and sustainable technologies involving whole-cell biocatalysts proficient in either consolidated bio-saccharification or bioprocessing may predominate during prospective LCB biorefining [110,111].On-site enzyme production is perceived as most recommended strategies to overall reduce the enzyme-costs for future refineries as reviewed extensively by Dragone et al. [112] and Siqueira et al. [113]. It offers some remarkable advantages by eliminating the fear of supply chain management, enhances chances of alternately using processed/unprocessed lignocellulosic biomass as carbon source and waives off the necessity for performing enzyme downstream processing & stabilization thereby impacting the environment favourably and fostering circular biorefining.In this scenario, industrial giants' prefer either partnership with strong enzyme manufacturers or acquire enzyme companies to facilitate consistent supply of tailor-made/ customised cellulase cocktailsand diversify their product portfolio [114].Parallely, theresearchers are making serious attempts to overcome the monopoly of enzyme manufacturers and become self-reliant by developing in-house enzyme producing capabilities at commercial scale. However,for its successful implementation, development of efficient, highly processive and robust cellulase cocktails is pre-requisite that can release reproducible and high gravity sugar solutions overcoming their inherent problem of end-product inhibition[112].

TEA and energy assessment are vital tools that identify the key cost drivers and energy-intensive steps in the 2G biorefinery or its independent process modules and help to prioritise and improvise in those areas. Significant progress has been made in the last five years towards developing techno-economic models to calculate the production cost of industrially relevant sugars and valorised products from cellulosic route [115-119]. Likewise, life cycle assessment (LCA) is yet another analysis technique to spot those process lacunas which impose environmental burdens and help the researchers to mitigate and overcome those loopholes taking an eco-friendly approach.

Concluding remarks

HSES at low enzyme loadings is one of the ways towards process intensification for production of industrially relevant sugars and products exploiting the biotechnological platform. Bio-catalytic depolymerisation of LCB can never be forbidden as it is a greener approach with high substrate specificity and production of no side products. Integration of TEA and LCA approaches indicate that presently the enzyme cost along with feedstock logistics and its price are the key economic barriers for the commercial realisation of the 2G biorefinery using the biochemical approach. However, the new pretreatment strategies, use of tailor-made enzyme cocktails, novel bioreactor designs, hydrid processes, robust microbes for biotransformation, diversifing product portfolio and their rational selection can bring transformational improvements in process economics of the sugar-platform. Authors predict a paradigm shift in the energy sector through profitable and integrated management of all the fractions of LCB. The authors are quite optimistic that the coming generations will witness secured future with renewable, sustainable and cost-competitive LCB-based biorefinery.

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Disclosure Statement

The authors declare that they have no competing interests.

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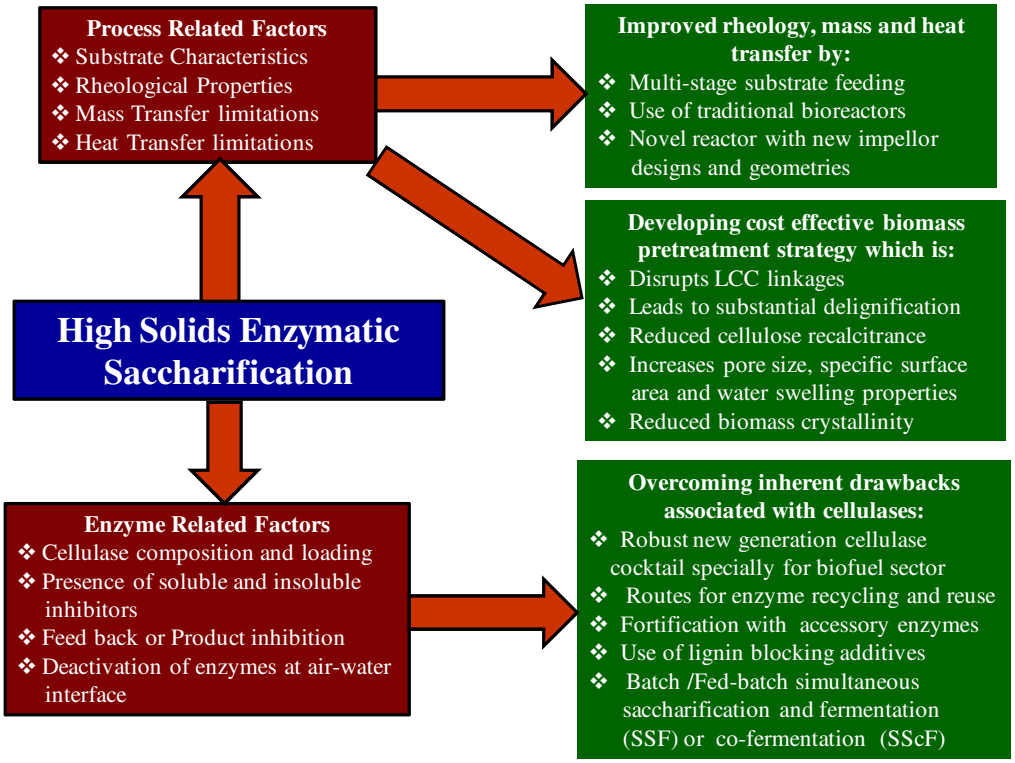

Figure 1: Technical hurdles associated with HSES at low enzyme loadings and broad approaches to overcome them

Table 1: Efficacy of traditional pretreatment strategies for cellulose enrichment, delignification of various lignocellulosic feedstocks and their impact of product titres and conversion yields

Biomass type	Pretreatment strategies	Removal (%) from pretreated biomass			Process type	Enzyme used and loading (g^{-1} glucan)	Product titre (g/L)	Cellulose hydrolysis (%)	EtOH yields (%)	Reference
		Gln	Xln	KL						
SCB	NaOH		<20	67.5	FBH (30%)`	Accellerase 1500 @ 15.5 FPU	Glucose: 82 Xylose: 20.33	50.85	-	[24]
WS		NA	<20	77.2		Accellerase 1500 @ 14.2 FPU	Glucose: 125.97 Xylose: 8.66	34.57	-	
SCB	NaOH		-NA-		FBH (36%)	Cellic CTec2 @ 14.12 FPU	Glucose: 134.9 Xylose: 60.3	59.8		[25]
SCB	NaOH	6.0	23.42	76.4	FBH (33%)	Cellic CTec2 @ 15.82 FPU	Glucose: 129.5 Xylose: 56.03 Cellobiose:9.37	59.88	-	[26]
SCB	NaOH	10.4	28.0	71.9	FBH (25%)	*Penicillium* derived cellulase @ 13.55 FPU	Glucose: 79.83 Xylose: 30.83 Cellobiose:25.02	60.73	-	[27]
SCB	NaOH		-NA-		FB-SSF (33%)	Cellic CTec2 @ 16.39 FPU	Ethanol:75.57	-	66.17	[28]
SCB	NaOH	6.9	34.6	65.5	FBH (20%)	Cellic CTec2 @ 15mg protein	Glucose: 126.8 Xylose: 51.95	84.3	-	[29]
RS	Na_2CO_3	21.2	37.5	55.9	SSSF (30%)	9:1 Cellic CTec2: Cellic HTec2 @8.72 FPU	Ethanol: 90.9	-	61.7	[30]
CS	DA		-NA-		BH (25%)	Spezyme CP @ 15 FPU	Glucose: 86.8	-	-	[31]
AWC	DA SPORL	12.5 15.5	87.8 93.1	5.9 24.8	SSF (18%)	Cellulclast 1.5L + N-188 @ 10 FPU	Ethanol: 35.1 Ethanol: 59.3	-	47.3 76	[32]
PBT	SCW	46.3	79.9	41.8	B-SSF (25%)	Cellulclast 1.5L @ 10 FPU	Ethanol: 54.6	-	80.92	[33]
SCS	HT	16.8	81.6	42.4	PSSSF* (19.3%)	Cellic CTec2 @ 14.5 FPU	Ethanol: 44.03	-	70.63	[34]
BW	SE		-NA-		FB-SSF (35%)	Cellic CTec2 @ 16.7 FPU	Ethanol: 83.2	76.8	68.7	[35]
MS	Aqueous NH_3	NIL	26.4	64.7	SHF (15%)	Cellic CTec2 @ 14.5 FPU	Glucose: 70.2 Xylose: 23.5 Cellobiose:6.6	64.34	60.9	[36]
CFS	DA followed by $NaClO_2$	9.5	96.1	93.1	FB- SHF (20%)	Cellic CTec2 @ 15 FPU	Glucose: 138.8 Ethanol: 64.6	~100	93.2	[37]
SCB	DA followed by NaOH		-NA- -NA-		BH (20%) FBH (24%)	Cellic CTec2 @ 13.96 mg protein	Glucose: 115.5 Glucose: 127	73 66.16	- -	[38]

SCB	SE followed by NaOH and then alkaline bleach	9.5	95.1	83	FBH (20%)	Cellic CTec2 @ 15.8 mg protein	Glucose: 125	67	-	[39]

Abbreviations: Gln- Glucan; Xln- Xylan; KL-Klason lignin; NA-not available; SE-steam explosion; WS-Wheat straw; SCB- Sugarcane bagasse; BW-Birchwood; SCS- Sugarcane straw; MS-Maize Stover; CS-Corn stover; PBT-Paper bark tree; AWC-Aspen Wood Chips; FBH-Fed-batch hydrolysis ; BH- Batch hydrolysis; PSSSF-pre-saccharification simultaneous saccharification and fermentation; PPEH-Periodic peristalsis enzymatic hydrolysis; SSSSF-solid-state simultaneous saccharification and fermentation; DA- Dilute acid; SCW- Subcritical water; HT-hydrothermal; SE- steam explosion; SPORL- Sulfite pretreatment to overcome recalcitrance of lignocellulose. ; CFS-Chinese fir saw dust; Data is ( ) indicate hydrolysis at various substrate loadings

Table 2: Emerging pretreatment strategies developed in recent past that promoted HSES with low enzyme loadings

Type of pretreatment	Details	Mode of action and advantages	Reference
Dry acid pretreatment and Biodetoxification (DryPB)	Dry H_2SO_4 pretreatment followed by fungal detoxification	❖ High-solids pretreatment (50-70%) ❖ Practically no effluent discharge ❖ Minimum energy consumption (especially steam) ❖ Particle size reduction by intermittent disc milling ❖ After pretreatment, selective inhibitor removal by *Amorphothecaresinae*	[40,41]
PHP treatment	Phosphoric acid and H_2O_2	❖ High-solids pretreatment (40%) ❖ Acid addition hydrolysis xylan and H_2O_2 accelerates the process ❖ Combined treatment cleaves aryl ether bonds, opened up lignin rings, breaks ethylenic carbonyl groups and other linkages present in lignin ❖ Enhanced biomass porosity due to lignin solubilisation ❖ Cellulose with reduced DP and CrI obtained	[42,43]
Fenton's reagent followed by alkali	Fe^{2+} in presence of H_2O_2 and NaOH	❖ Fenton's reagent generates hydroxy free radicals. ❖ Alkali facilitates the penetration of hydroxy free radicals in the biomass ❖ Combined effect leads to better solubilisation of lignin and its removal ❖ FT followed by alkali prevents significant loss of xylan fraction ❖ Cellulose enrichment in the biomass is predominant	[44]
Extractive Ammonia Process	Next generation ammonia pretreatment	❖ NH_3 reacts with ferulate and coumarate associated ester linkages ❖ Causes selective extraction and solubilisation of lignin preserving its native state ❖ Converts recalcitrant cellulose CI_β allomorph to highly digestible CIII allomorph ❖ No requirement of detoxification after pretreatment	[45]
Deacetylation and disc refining (DDR)	Use of mild NaOH + disc refining	❖ Mild NaOH treatment at 80°C for 2h solubilises 80% acetyl groups and ash ❖ Removes 30% lignin ❖ Disc refining causes extensive defibrillation ❖ Highly digestible biomass with reduced particle size and high specific surface area	[46]
Deactylation and mechanical refining (DMR)	Use of mild NaOH + disc refining+ Szego milling	❖ Additional step of Szego milling after DDR ❖ Enhances biomass delamination and defibrillation ❖ Increases biomass digestibility	[47]
Formiline	Combination of HCOOH and $Ca(OH)_2$	❖ HCOOH treatment delignifies the biomass ❖ Inhibitory effect of formylated is nullified by $Ca(OH)_2$ via saponification ❖ Generates highly digestible cellulosic biomass ❖ Spent liquor can be used for several cycles.	[48, 49]
Alkali organosolv	NaOH with organic solvents like CH_3OH	❖ High-solids pretreatment (50%) ❖ Lesser effluent discharge ❖ Delignified biomass and preservation of holocellulose	[50]

Metal assisted glycerol pretreatment	Combination of $AlCl_3$ and glycerol	❖ Disrupts LCC linkages leading to significant removal of xylan and lignin ❖ Pretreated biomass with increased specific surface area, pore volume and pore size ❖ Enhanced enzyme adsorption rate observed with pretreated biomass	[51]
Co-solvent Enhanced lignocellulosic fractionation (CELF)	Combination of tetrahydrofuran (THF) with Dilute acid (DA)	❖ Use of THF, a unique water-miscible aprotic green solvent that delignifies the biomass. ❖ DA catalysed degradation of the hemicellulosic fraction ❖ THF can extract furfurals from aqueous fraction produced during DA treatment ❖ Furfural and THF can be flashed off via steam into the azeotropic distillation column. ❖ Furfural can be purified separately, and THF can be recovered at room temperature by vacuum distillation with dissolved lignin as precipitate/dry powder	[52, 53]
Acid assisted Organosolv	Use of mineral acids in combination with organic solvents	❖ Disrupts LCC linkages leading to significant delignification and removal of xylan ❖ Native form of lignin is preserved even after solubilisation ❖ Renders amorphous nature to cellulose specially if the acid is H_3PO_4 ❖ Partially defibrillated cellulose –rich biomass is generated	[54]
GVL pretreatment	Use of γ-valerolactone with mild sulphuric acid	❖ γ-valerolactone has excellent lignin dissolution ability ❖ Recovered by liquid–CO_2 extraction and recycled back a number of times ❖ Use of low acid milder pretreatment, preserves holocellulose ❖ Negligible formation of sugar degradation products detected	[55]
Deep Eutectic Solvents	Mixture of hydrogen bond acceptor and hydrogen bond donor	❖ Disrupts LCC linkages leading to removal of xylan and lignin ❖ High-solids pretreatment can be done ❖ Some combinations have the ability of being recycled and reused	[56]
ED pretreatment	Ethylenediamine	❖ High-solids pretreatment at ambient temperature and pressure ❖ Disrupts LCC linkages leading to significant delignification ❖ Converts recalcitrant cellulose CI_β allomorph to highly digestible CIII allomorph ❖ Preserves carbohydrate fraction of biomass	[57, 58]

Table 3: Efficacy of emerging pretreatment strategies for cellulose enrichment, delignification of various lignocellulosic feedstocks and their impact of product conversion yields

LCB	Pretreatment strategies	Percentage Removal			Process type	Enzyme used and loading (g^{-1} glucan)	Product titre ($g\ L^{-1}$)	Glucan hydrolysis (%)	Ethanol yield (%)	Reference
		Gln	Xln	KL						
CS WS	DryPB	2.5	34.5 -NA-	NIL	SScF (30%)	Cellic CTec2@ 10 mg protein	Ethanol: 101.4 Ethanol: 90.3		84.7 82.8	[40]
WS	H_3PO_4 + H_2O_2	2.2	100	70.8	SSF (15.3%)	Cellic CTec2@ 13.2 mg protein	Ethanol: 69.9		88.2	[42]
SCB	Fenton's reagent and then NaOH	NA	32.2	20.6	FB-SScF (30%)	Cellic CTec2@ 15.48 FPU	Ethanol: 80		~69	[44]
CS	DDR DMR		-NA- -NA-		BH (25%)	Cellic CTec3@ 12 FPU + Cellic HTec3 @ 4 mg	Glucose:102.7 Xylose: 79.9 Glucose: 114.9 Xylose: 89.1	- -		[47]
SCB	Formiline	NA	86.9	84.8	FBH (30%)	Novozyme Cellulase@ 11.6 FPU	Glucose: 247.3	86.1		[49]
CS	NaOH +CH_3OH	2.6	10.6	70.7	FBH (40%)	Cellic CTec2@ 15 mg protein	Glucose: 146.7 Xylose: 58.7	89.5		[50]
RS	$AlCl_3$ + glycerol	7.8	94.2	83.1	BH (15%)	Cellic CTec2@ 4.36 FPU	-	65.7		[51]
CS	THF + 0.5% H_2SO_4	NA	34.2	23.7	FB-SSF (20%)	Accellerase 1500@ 15 mg protein	Ethanol: 86.5		90.5	[53]
BEW	Acetone + H_2SO_4 Ethanol + H_3PO_4	9.3 6.4	86.4 76.7	92.4 92.4	B-SSF (20%)	Cellic CTec2 @ 8.4 mg g^{-1} dry matter	Ethanol: 76.3 Ethanol: 80		75 83	[54]
BE WC	γ-valerolactone + 75mM H_2SO_4	5	77	78.5	BH (30%)	Cellic CTec2 @ 15 FPU	Sugars: 182	90		[55]
CS	ED		-NA-		SScF (19.9%)	Cellic CTec2 @ 6 FPU	Ethanol: 59.8		63.44*	[57]
WS	Formiline and then H_3PO_4	26.3	98.1	92.5	sSSF$^{\$}$ (15%)	Cellic CTec2@ 6.22 FPU	Glucose:93.8 Ethanol: 50.4	62	73.6	[59]
CCR	THF -H_2O	11.8		71.9	BH (20%)	Cellic CTec2 @ 9.14FPU	Glucose: 128.6	73.3		[60]
SG	Complex of EG + ChCl + PTSA	NIL	66.8	65.4	BH (20%)	Cellic CTec2 and HTec2 (10:1) @ 7.86 mg protein	Glucose: 128	78.4		[61]

Abbreviations: Gln- Glucan; Xln- Xylan; KL-Klason lignin; NA-not available; FBH-Fed-batch hydrolysis; BH- Batch hydrolysis; FB-SScF- Fed-batch simultaneous saccharification and co-fermentation; FB-SSF- Fed-batch simultaneous saccharification and fermentation; FB-SHF- Fed batch separate hydrolysis and fermentation; sSSF$^{\$}$-semi-simultaneous saccharification and fermentation; WS-Wheat straw; SCB-Sugarcane bagasse; BEW-Beechwood; BEWC-Beechwood chips; RS- Rice Straw; CS- Corn Stover; SG-Switch grass; CFS-Chinese fir saw dust; CCR- Corn Cob residues; ED- Ethylenediamine; THF- Tetrahydrofuran; Dry PB- Dry acid pretreatment and Biodetoxification ; DDR- Deacetylation and disc refining; DMR- Deacetylation and mechanical refining; ChCl- Choline chloride; EG- ethylene glycol;PTSA-*p*-toluenesulfonic acid. *refers to addition of BSA additive during SScF ;Data is ( ) indicate hydrolysis at various substrate loadings

Table 4: Processes featuring HSES with cellulase loading ≤ 6 mg protein or FPU g^{-1} glucan for achieving industrially relevant sugars and bio-based products

Pretreated Biomass	Cellulase type	Process novelty which facilitated cellulase loading ≤ 6 mg protein or FPU g^{-1} glucan	Process Type	Product	Reference
8% glucan loading of corn stover pretreated with extractive NH_3	Cellic CTec2 @ 3.75 mg protein g^{-1} glucan	• Extractive ammonia converted native crystalline cellulose Iβ (CI) to a highly digestible cellulose CIII allomorph. • Cellic HTec2 and Multifect Pectinase dosed at 1.875 g^{-1} glucan each, facilitated cellulose hydrolysis • Use of xylose utilising*S.cerevisiae* 424A	SHF using *S.cerevisiae* 424A	56.8 g L^{-1} ethanol	[45]
19.9% corn stover pretreated with ethylenediamine	Cellic CTec2 @ 6.0 FPU g^{-1} glucan	• Preserving carbohydrate fraction and significant lignin removal • Exploited the fact that Cellic CTec2 at 42°C favoured higher xylan hydrolysis than 50°C. • Judicious selection of two *Saccharomyces* strains one being temp resistant and other being xylose assimilating. • Performing temperature profiled SScF at 42°C • Used BSA as additive @ 50mg/g DM	SScF by co-culturing two strains of *S.cerevisiae*	59.8 g L^{-1} ethanol	[58]
20% sugarcane bagasse pretreated with al-AGO	Cellic CTec2 @ 3 FPU/g DM (5.26 FPU g^{-1} glucan)	• Synergism of accessory enzyme and additives reduced cellulase loading in partially delignified biomass • Endoxylanase @ 2.4mg/g DM; AA9 @ 1mg/g DM • Tween 80, BSA and tea saponin added at 40,20,10 mg/g DM	Fed-batch hydrolysis	105 g L^{-1} glucose	[77]
12% corn cobs pretreated with NH_3-H_2O_2	Cellulase derived from *T. viride* R16 @ 2 FPU/g DM (~4.5 FPU g^{-1} glucan)	• The protein pool of *T. viride* R16 was constituted of 41% cellulases and 20% xylanases • Its secretome exhibited aromatic dioxygenase activity which detoxified sugar-rich broth during fermentation	Fed-batch SSF using *B. coagulans* LA204	64.95 g L^{-1} lactic acid	[83]
30% sugarcane bagasse pretreated with al-AGO	Cellic CTec2 @ 3 FPU/g DM (5.26 FPU g^{-1} glucan)	• Improved process economics by reducing AA9 and BSA loading by 60% as mentioned in reference [77] • Increased loading of tea saponin by 3 fold	Fed-batch hydrolysis	125 & 56 g L^{-1} glucose and xylose	[84]
20% sugarcane bagasse pretreated with al-AGO	Cellic CTec2 @ 3 FPU g^{-1} DM (5.54 FPU g^{-1} glucan)	• Replaced the earlier AA9 with recombinant *Podospora anserina* PaAA9B • Used all other additives and AE same as in reference [77]	Fed-batch hydrolysis	105 g L^{-1} glucose	[85]

Note: al-AGO- alkaline-catalysed atmospheric glycerol organosolv process; BSA- Bovine serum albumin; DM-dry matter; AE- accessory enzymes; SHF- Separate hydrolysis and fermentation; SSF- Simultaneous saccharification and fermentation; SScF- Simultaneous saccharification and co-fermentation

Emerging trends in high-solids enzymatic saccharification of lignocellulosic feedstocks for developing an efficient and industrially deployable sugar platform

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