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Enzymatic Hydrolysis of Lignocellulosic Biomass for Second-Generation Bioethanol Production

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ABSTRACT

Background: Second-generation bioethanol converts non-food lignocellulosic residues into fermentable sugars, but native plant cell walls resist enzymatic attack, a property termed biomass recalcitrance. Objective: This review synthesises published evidence on pretreatment, enzymatic hydrolysis, and process integration strategies that overcome this recalcitrance. Methods: A narrative review of published studies on lignocellulosic biomass pretreatment, enzymatic hydrolysis, and saccharification strategies for second-generation bioethanol production was conducted, with studies identified through PubMed, Google Scholar, and Crossref. Findings: Dilute-acid, steam explosion, ammonia fibre expansion, liquid hot water, ionic liquid, and organosolv pretreatments each raise enzymatic digestibility substantially, while synergistic cellulase-hemicellulase cocktails supplemented with lytic polysaccharide monooxygenases and reduced lignin binding further increase glucose release. Simultaneous saccharification and fermentation outperforms separate hydrolysis and fermentation in reported ethanol yields. Conclusions: Combining optimised pretreatment, engineered enzyme systems, and integrated processing offers the clearest path toward economically viable cellulosic ethanol, though enzyme cost and lignin-derived inhibition remain the central bottlenecks.

Keywords: *lignocellulose; enzymatic hydrolysis; cellulase; biomass pretreatment; bioethanol*

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Abstract

Background: Second-generation bioethanol converts non-food lignocellulosic residues into fermentable sugars, but native plant cell walls resist enzymatic attack, a property termed biomass recalcitrance [1]. **Objective:** This review synthesises published evidence on pretreatment, enzymatic hydrolysis, and process integration strategies that overcome this recalcitrance. **Methods:** A narrative review of published studies on lignocellulosic biomass pretreatment, enzymatic hydrolysis, and saccharification strategies for second-generation bioethanol production was conducted, with studies identified through PubMed, Google Scholar, and Crossref. **Findings:** Dilute-acid, steam explosion, ammonia fibre expansion, liquid hot water, ionic liquid, and organosolv pretreatments each raise enzymatic digestibility substantially, while synergistic cellulase-hemicellulase cocktails supplemented with lytic polysaccharide monooxygenases and reduced lignin binding further increase glucose release. Simultaneous saccharification and fermentation outperforms separate hydrolysis and fermentation in reported ethanol yields. **Conclusions:** Combining optimised pretreatment, engineered enzyme systems, and integrated processing offers the clearest path toward economically viable cellulosic ethanol, though enzyme cost and lignin-derived inhibition remain the central bottlenecks.

Keywords: lignocellulose; enzymatic hydrolysis; cellulase; biomass pretreatment; bioethanol

Introduction

Lignocellulosic biomass — agricultural residues, energy crops, and forestry by-products — is the most abundant renewable carbon source on Earth and a feedstock for second-generation bioethanol that avoids competition with food crops and arable land. Global interest in cellulosic ethanol stems from its potential to reduce greenhouse-gas emissions relative to fossil fuels while valorising residues that would otherwise be burned, landfilled, or left to decompose. Its three principal polymers, cellulose, hemicellulose, and lignin, are tightly interwoven into a composite structure that evolved to resist microbial and enzymatic degradation, a property termed biomass recalcitrance [1,2]. Converting this structure into fermentable sugars therefore

requires a pretreatment step to disrupt the cell wall and increase enzyme accessibility, followed by enzymatic hydrolysis using cellulase and hemicellulase cocktails, and finally fermentation of the released sugars to ethanol [3]. Each of these steps has been the subject of intense research: dozens of candidate pretreatment chemistries have been benchmarked against one another, enzyme manufacturers have progressively reduced the protein loading needed per tonne of biomass, and process engineers have explored configurations that combine hydrolysis and fermentation to reduce capital cost. Despite decades of research and several pilot and commercial deployments, enzyme cost and incomplete saccharification of pretreated material continue to limit the economics of the process, and a number of flagship commercial plants have struggled to remain in continuous operation [4,5]. This review synthesises the published literature on pretreatment technologies, cellulolytic and hemicellulolytic enzyme systems and their engineering, the physicochemical factors that limit hydrolysis efficiency, and process configurations that integrate these steps, with the goal of clarifying where the field has made durable progress and where bottlenecks persist.

Methods

This is a narrative review of published studies on lignocellulosic biomass pretreatment, enzymatic hydrolysis, and saccharification strategies for second-generation bioethanol production. Relevant peer-reviewed articles, technical reports, and reviews published between 1985 and 2026 were identified through PubMed, Google Scholar, and Crossref using combinations of the terms "lignocellulose," "pretreatment," "cellulase," "hemicellulase," "enzymatic hydrolysis," and "bioethanol." Priority was given to studies reporting quantitative saccharification or ethanol yields, mechanistic studies of enzyme-substrate interactions, and techno-economic assessments, including the widely cited NREL process-design reports that anchor much of the field's cost modelling. Reference lists of retrieved reviews were screened for additional primary sources, and preference was given, where available, to studies reporting numerical glucan or glucose conversion so that comparable figures could be extracted across pretreatment classes. Findings were organised thematically around pretreatment strategies, enzyme systems and engineering, recalcitrance factors, and process integration; no new laboratory experiments were performed for this article.

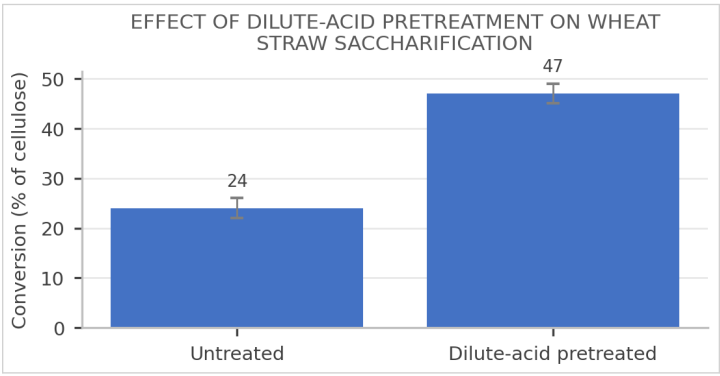
Biomass Pretreatment Strategies

Pretreatment aims to increase cellulose accessibility by removing or redistributing lignin, solubilising hemicellulose, and reducing cellulose crystallinity [6,7]. Dilute-acid pretreatment of corn stover, optimised in NREL's benchmark process design, hydrolyses most hemicellulose and enables enzymatic conversion of the remaining cellulose to glucose at high conversion after enzymatic hydrolysis [4]. Steam explosion combines high-temperature autohydrolysis with explosive decompression to fragment fibre structure and has been applied across solid, liquid, and gaseous bioenergy pathways [8]. Ammonia fibre expansion (AFEX) treats biomass with liquid ammonia under moderate temperature and pressure, swelling and decrystallising cellulose while retaining nearly all lignocellulosic carbohydrates; AFEX-treated switchgrass reached high glucan and xylan conversions across diverse harvests and locations [9]. Liquid hot water and organosolv processes solubilise hemicellulose and lignin respectively using water or organic solvents without added acid catalysts, and organosolv fractionation additionally recovers a relatively pure lignin stream valuable for co-product valorisation [10]. Ionic liquids dissolve cellulose directly, disrupting its hydrogen-bond network and yielding highly amorphous, more digestible regenerated material [11]. Dilute-acid pretreatment of wheat straw approximately doubled enzymatic cellulose-to-glucose conversion relative to untreated straw (from 24% to 47%), illustrating the substantial accessibility gain a single acid-catalysed cook can deliver even before further enzyme or process optimisation is applied (Figure 1) [12]. Comparative reviews conclude that no single pretreatment is universally optimal; feedstock composition, downstream enzyme cost, capital intensity, and inhibitor formation all influence the choice of technology, and woody feedstocks in particular demand more severe or energy-intensive pretreatment than herbaceous residues because of their higher lignin content and tighter fibre structure [13,14]. Reported performance for representative feedstock-pretreatment pairs is summarised in Table 1.

Table 1: Representative pretreatment technologies and reported effects on enzymatic digestibility.

Pretreatment	Feedstock	Reported effect	Reference
Dilute acid	Corn stover	High cellulose conversion at commercial-scale process design conditions	[4]
Ammonia fibre expansion (AFEX)	Switchgrass	High combined glucose/xylose release across harvests and locations	[9]
Steam explosion	Various residues	Fibre disruption enabling multiple downstream bioenergy pathways	[8]
Ionic liquid	Lignocellulose	Decrystallisation of cellulose, increased digestibility	[11]
Organosolv	Lignocellulose	Lignin fractionation and improved saccharification	[10]

Figure 1: Enzymatic cellulose-to-glucose conversion of wheat straw before and after dilute-acid pretreatment [12].

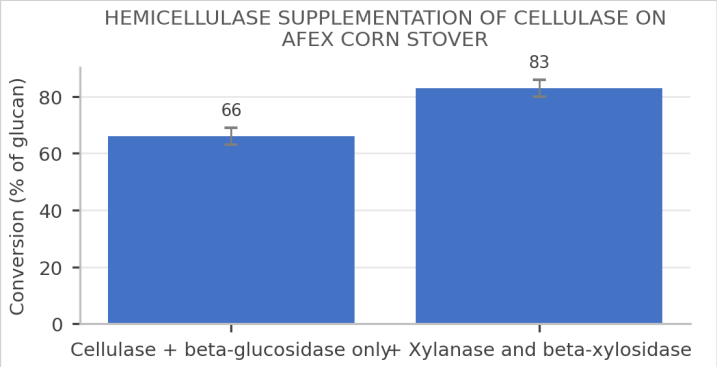


Cellulase and Hemicellulase Enzyme Systems and Engineering

Complete cellulose depolymerisation requires the synergistic action of endoglucanases, which create new chain ends within

amorphous regions, cellobiohydrolases, which processively cleave cellobiose units from chain ends, and beta-glucosidases, which hydrolyse cellobiose to glucose and relieve end-product inhibition of the upstream enzymes; this synergistic system was historically characterised in the *Trichoderma reesei* secretome and remains the structural template for most commercial cellulase cocktails [15]. Hemicellulose removal by xylanases and beta-xylosidases further increases cellulose accessibility and relieves product inhibition from accumulating xylo-oligomers: supplementing a base cellulase/beta-glucosidase mixture with xylanase and beta-xylosidase raised glucan-to-glucose conversion of AFEX-pretreated corn stover from about 66% to 83% (Figure 2) [16]. Screening of enzyme mixtures shows that the optimal ratio of core glycosyl hydrolases depends strongly on the pretreatment and feedstock combination, underscoring the need for tailored cocktails rather than fixed formulations [17]. The discovery of glycoside hydrolase family 61 (now classified as auxiliary activity family 9 lytic polysaccharide monooxygenases) revealed an oxidative, copper-dependent mechanism that cleaves crystalline cellulose and substantially boosts hydrolytic enzyme performance when added to classical cellulase cocktails [18,19]. Protein engineering and directed evolution have since been used to improve cellulase thermostability, specific activity, and tolerance to lignin-derived inhibitors, supporting reduced enzyme loadings in industrial hydrolysis [20,21,22]. High-throughput screening and rational mutagenesis of catalytic and carbohydrate-binding domains have both contributed to these gains, and industrial enzyme suppliers have progressively lowered the recommended protein dose per gram of cellulose over successive commercial cocktail generations, narrowing but not closing the gap between enzyme cost and a competitive minimum ethanol selling price [22,20].

Figure 2: Glucan-to-glucose conversion of AFEX-pretreated corn stover with a base cellulase cocktail versus the same cocktail supplemented with xylanase and beta-xylosidase [16].



Factors Limiting Hydrolysis Efficiency

Cellulose crystallinity is a strong predictor of initial hydrolysis rate, with less-ordered amorphous regions hydrolysed far faster than crystalline microfibrils, so that pretreatments capable of lowering crystallinity index tend to accelerate the early, rate-limiting phase of saccharification even before overall sugar release is considered [23]. Residual and redeposited lignin after pretreatment binds cellulase non-productively, reducing the enzyme fraction available for catalysis and constituting one of the principal economic roadblocks in biomass conversion [24]. Lignin structure, molecular weight, and the presence of phenolic and hydrophobic surfaces all modulate the strength of this non-productive binding, and pretreatments that relocate or remove lignin generally improve downstream digestibility [25,26]. Feedstock particle size, degree of polymerisation, and porosity add further layers of recalcitrance that interact with all of the above factors, so that effective pretreatment strategies typically target several of these variables simultaneously rather than a single structural feature [1,26].

Process Integration and Economics

Simultaneous saccharification and fermentation (SSF), in which enzymatic hydrolysis and fermentation occur in the same vessel, reduces product inhibition of cellulase by glucose and cellobiose relative to separate hydrolysis and fermentation (SHF); on steam-pretreated corn stover, SSF achieved a substantially higher overall ethanol yield (72.4% of theoretical) than SHF (59.1%) [27]. Techno-economic analysis of the full biochemical conversion pathway identifies enzyme cost, pretreatment severity, and feedstock handling as dominant contributors to minimum ethanol selling price [4]. Commercial-scale deployment has proven difficult: several flagship cellulosic ethanol facilities, including Clariant's sunliquid plant in Romania, have been idled or closed, reflecting the gap between demonstrated laboratory yields and sustained commercial profitability, even as global tracking of operating and planned facilities continues [5]. Enzyme recycling, consolidated feedstock logistics, and co-production of lignin-derived materials or chemicals have each been proposed as levers to improve overall plant economics, since enzyme cost and feedstock supply chains together represent a large share of total production cost in NREL-style techno-economic models [4]. Representative yield and economic metrics from the literature are summarised in Table 2.

Conclusion

Published evidence converges on a consistent picture: no single pretreatment or enzyme formulation is sufficient in isolation, but combinations of optimised physicochemical pretreatment, synergistic and oxidatively boosted enzyme cocktails, and integrated hydrolysis-fermentation configurations substantially narrow the gap between laboratory-scale saccharification and economically viable second-generation bioethanol. The recurring themes across the reviewed literature are that lignin removal or relocation and crystallinity reduction are prerequisites for high sugar release regardless of the pretreatment chemistry chosen, that hemicellulase and lytic polysaccharide monooxygenase supplementation meaningfully reduce the cellulase loading needed to reach a given conversion, and that process configurations such as simultaneous saccharification and fermentation capture some of these enzymatic gains at the plant scale. Continued reduction of enzyme cost, mitigation of lignin-mediated inhibition, and feedstock-specific process tuning remain the priorities identified across the reviewed literature, and closing these gaps will likely determine whether second-generation bioethanol can achieve durable commercial competitiveness.

Table 2: Process integration metrics reported for corn stover ethanol production.

Metric	Separate hydrolysis and fermentation (SHF)	Simultaneous saccharification and fermentation (SSF)
Feedstock	Steam-pretreated corn stover	Steam-pretreated corn stover
Ethanol yield (% of theoretical)	59.1	72.4
Reference	[27]	[27]

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Conflict of Interest

The authors declare no conflict of interest.

Author Contributions

Novotny K conceived the review, conducted the literature search, and drafted the manuscript. Rinaldi C contributed to the literature screening and technical framing and critically revised the manuscript for important intellectual content. Kazlauskas G contributed additional context and approved the final version for submission.

Ethics Statement

Not applicable. This is a narrative review of previously published literature and did not involve new experimental data, human participants, or animal subjects.

Data Availability

No new data were generated or analyzed for this review. All sources synthesized are cited in the reference list and are available through their original publishers.

References

1. Himmel ME, Ding SY, Johnson DK, Adney WS, Nimlos MR, Brady JW, Foust TD (2007) Biomass recalcitrance: engineering plants and enzymes for biofuels production. Science 315(5813):804-807.

2. Zhao X, Zhang L, Liu D (2012) Biomass recalcitrance. Part I: the chemical compositions and physical structures affecting the enzymatic hydrolysis of lignocellulose. Biofuels, Bioproducts and Biorefining 6(4):465-482.

3. Chundawat SPS, Beckham GT, Himmel ME, Dale BE (2011) Deconstruction of lignocellulosic biomass to fuels and chemicals. Annual Review of Chemical and Biomolecular Engineering 2:121-145.

4. Humbird D, Davis R, Tao L, Kinchin C, Hsu D, Aden A, Schoen P, Lukas J, Olthof B, Worley M, Sexton D, Dudgeon D (2011) Process Design and Economics for Biochemical Conversion of Lignocellulosic Biomass to Ethanol: Dilute-Acid Pretreatment and Enzymatic Hydrolysis of Corn Stover. NREL Technical Report NREL/TP-5100-47764.

5. Backovsky D, Sonnleiter M, Leal-Silva N, Souza S (2026) Tracking cellulosic ethanol: commercialization and regional insights. Biofuels, Bioproducts and Biorefining. doi:10.1002/bbb.70068.

6. Mosier N, Wyman C, Dale B, Elander R, Lee YY, Holtzapple M, Ladisch M (2005) Features of promising technologies for pretreatment of lignocellulosic biomass. Bioresource Technology 96(6):673-686.

7. Kumar P, Barrett DM, Delwiche MJ, Stroeve P (2009) Methods for pretreatment of lignocellulosic biomass for efficient hydrolysis and biofuel production. Industrial & Engineering Chemistry Research 48(8):3713-3729.

8. Yu Y, Wu J, Ren X, Lau A, Rezaei H, Takada M, Bi X, Sokhansanj S (2022) Steam explosion of lignocellulosic biomass for multiple advanced bioenergy processes: a review. Renewable and Sustainable Energy Reviews 154:111871.

9. Bals B, Rogers C, Jin M, Balan V, Dale B (2010) Evaluation of ammonia fibre expansion (AFEX) pretreatment for enzymatic hydrolysis of switchgrass harvested in different seasons and locations. Biotechnology for Biofuels 3:1.

10. Nair LG, Agrawal K, Verma P (2023) Organosolv pretreatment: an in-depth purview of mechanics of the system. Bioresources and Bioprocessing 10:50.

11. Hou Q, Ju M, Li W, Liu L, Chen Y, Yang Q (2017) Pretreatment of lignocellulosic biomass with ionic liquids and ionic liquid-based solvent systems. Molecules 22(3):490.

12. Saha BC, Iten LB, Cotta MA, Wu YV (2005) Dilute acid pretreatment, enzymatic saccharification and fermentation of wheat straw to ethanol. Process Biochemistry 40(12):3693-3700.

13. Alvira P, Tomas-Pejo E, Ballesteros M, Negro MJ (2010) Pretreatment technologies for an efficient bioethanol production process based on enzymatic hydrolysis: a review. Bioresource Technology 101(13):4851-4861.

14. Zhu JY, Pan XJ (2010) Woody biomass pretreatment for cellulosic ethanol production: technology and energy consumption evaluation. Bioresource Technology 101(13):4992-5002.

15. Lynd LR, Weimer PJ, van Zyl WH, Pretorius IS (2002) Microbial cellulose utilization: fundamentals and biotechnology. Microbiology and Molecular Biology Reviews 66(3):506-577.

16. Qing Q, Wyman CE (2011) Supplementation with xylanase and beta-xylosidase to reduce xylo-oligomer and xylan inhibition of enzymatic hydrolysis of cellulose and pretreated corn stover. Biotechnology for Biofuels 4:18.

17. Banerjee G, Car S, Scott-Craig JS, Borrusch MS, Walton JD (2010) Rapid optimization of enzyme mixtures for deconstruction of diverse pretreatment/biomass feedstock combinations. Biotechnology for Biofuels 3:22.

18. Harris PV, Welner D, McFarland KC, Re E, Navarro Poulsen JC, Brown K, Salbo R, Ding H, Vlasenko E, Merino S, Xu F, Cherry J, Larsen S, Lo Leggio L (2010) Stimulation of lignocellulosic biomass hydrolysis by proteins of glycoside hydrolase family 61: structure and function of a large, enigmatic family. Biochemistry 49(15):3305-3316.

19. Vaaje-Kolstad G, Westereng B, Horn SJ, Liu Z, Zhai H, Sorlie M, Eijsink VGH (2010) An oxidative enzyme boosting the enzymatic conversion of recalcitrant polysaccharides. Science 330(6001):219-222.

20. Zhang YHP, Himmel ME, Mielenz JR (2006) Outlook for

cellulase improvement: screening and selection strategies. Biotechnology Advances 24(5):452-481.

21. Akram F, Haq IU, Aqeel A, Ahmed Z, Shah FI (2021) Thermostable cellulases: structure, catalytic mechanisms, directed evolution and industrial implementations. Renewable and Sustainable Energy Reviews 151:111597.

22. Merino ST, Cherry J (2007) Progress and challenges in enzyme development for biomass utilization. Advances in Biochemical Engineering/Biotechnology 108:95-120.

23. Hall M, Bansal P, Lee JH, Realff MJ, Bommarius AS (2010) Cellulose crystallinity - a key predictor of the enzymatic hydrolysis rate. FEBS Journal 277(6):1571-1582.

24. Huang C, Jiang X, Shen X, Hu J, Tang W, Wu X, Ragauskas A, Jameel H, Meng X, Yong Q (2022) Lignin-enzyme interaction: a roadblock for efficient enzymatic hydrolysis of lignocellulosics. Renewable and Sustainable Energy Reviews 154:111822.

25. Yoo CG, Meng X, Pu Y, Ragauskas AJ (2020) The critical role of lignin in lignocellulosic biomass conversion and recent pretreatment strategies: a comprehensive review. Bioresource Technology 301:122784.

26. Zhao X, Zhang L, Liu D (2012) Biomass recalcitrance. Part II: fundamentals of different pre-treatments to increase the enzymatic digestibility of lignocellulose. Biofuels, Bioproducts and Biorefining 6(5):561-579.

27. Ohgren K, Bura R, Lesnicki G, Saddler J (2007) A comparison between simultaneous saccharification and fermentation and separate hydrolysis and fermentation using steam-pretreated corn stover. Process Biochemistry 42(5):834-839.