Experimental and computational studies of cellulases as bioethanol enzymes

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ABSTRACT

Bioethanol industries and bioprocesses have many challenges that constantly impede commercialization of the end product. One of the bottlenecks in the bioethanol industry is the challenge of discovering highly efficient catalysts that can improve biomass conversion. The current promising bioethanol conversion catalysts are microorganism-based cellulolytic enzymes, but lack optimization for high bioethanol conversion, due to biological and other factors. A better understanding of molecular underpinnings of cellulolytic enzyme mechanisms and significant ways to improve them can accelerate the bioethanol commercial production process. In order to do this, experimental methods are the primary choice to evaluate and characterize cellulase’s properties, but they are time-consuming and expensive. A time-saving, complementary approach involves computational methods that evaluate the same properties and improves our atomistic-level understanding of enzymatic mechanism of action. Theoretical methods in many cases have proposed research routes for subsequent experimental testing and validation, reducing the overall research cost. Having a plethora of tools to evaluate cellulases and the yield of the enzymatic process will aid in planning more optimized experimental setups. Thus, there is a need to connect the computational evaluation methods with the experimental methods to overcome the bottlenecks in the bioethanol industry. This review discusses various experimental and computational methods and their use in evaluating the multiple properties of cellulases.
Research Highlights

- Methods and tools to evaluate cellulas can improve bioethanol production.
- Cellulases enzyme mechanisms are usually studied using experimental techniques.
- Computational evaluation of cellulas’ properties reduces cost and time.
- Combination of different evaluation methods can aid in optimization of cellulas and the glucose yield.

1. Introduction

The United Nations (https://www.un.org/en/sections/issues-depth/population/index.html) predicts the increase of the world population by 2 billion persons in the next 30 years, i.e., the current population of 7.7 billion may reach 9.7 billion by 2050. Energy sources are at the most significant threat because of their versatile use in human development. Necessary human activities like mobility, health, communication, irrigation, cooking, space travel, etc., are at the cost of depleting energy sources [1,2]. The answer to this critical question of the increasing energy demand is currently met, in large, by fossil fuels. It is a known fact that natural resources and renewable energy improve environmental quality in the distant future [3]. The transition from fossil fuels to renewable sources of energy and new advances in energy storage systems opens the possibility of clean fuel and an opportunity to tackle climate change [4–8]. Biofuels are one of the options for mitigating dependency on fossil fuels and reducing carbon emissions in the global energy system [9].

1.1. Generation of biofuels

There are three generations of biofuels: namely, first-, second-, and third-generation biofuels, where the categorization is based on 1) the biomass sources used, 2) the limitations of these biomass sources, and 3) their technological progress [10,11].

The first-generation biofuels come from edible biomass or food crops like corn, sugar beets, sugarcane, wheat, grains, industrial sweet potatoes, oilseeds, vegetable oils, and rendered animal fats. The second-generation biofuels come from non-edible biomass such as wood, sawdust, wheat straws, corn husks, seed waste, manure, paper waste, household waste, wastewater, etc. [12,13]. In the last decade, researchers have been focusing on second-generation biomass for biofuel. Research focus in the recent decade has been on, but not limited to, wood bark [14], olive stone [15], pine pellets [16], avocado stone [17], wheat straw, wood [18], walnut shell [19], peanut shell [20], mango stone [21], sunflower seed husk [22], corn cob waste [23], palm oil kernel shell [24], and others. The third-generation biomass for biofuels like algae [25] and woody biomass are considered a better alternative than second-generation because they do not compete with food/feed sources. However, they are limited by economic feasibility because of the high cost of production and treatments [26,27].

Compared to first- and third-generation biomass, the second-generation biomass is relatively more sustainable [28]. This is because they are the byproducts of agricultural industry and there is no additional requirement of land, water, and fertilizer use to derive these sources. The agricultural plant wastes are majorly lignocellulosic biomass, composed of lignin, cellulose, and hemicellulose that constitute the plant cell wall, where the recalcitrant polysaccharides and lignin are strongly cross-linked via ester and ether linkages [29–31].

1.2. Pretreatment of biomass

Regardless of first-, second-, or third-generation biomass, pretreatment is a required process for the biomass to be utilized to its full potential. Pretreatment is the process to weaken and break these strong cross-links, so that the recalcitrant polymers are amenable to hydrolysis with cellulas into simpler sugars [32]. The general biomass pretreatment process is shown in Figure 1. There are many types of pretreatments, and they are categorized into: 1) physical pretreatment processes that include milling, irradiation, extrusion, pyrolysis, etc.; 2) chemical pretreatment processes that include acid treatment, alkali treatment, use of ionic
liquids, organosolv, etc.; 3) physico-chemical pretreatment processes that include steam explosion, liquid hot water, ammonia fiber explosion, ammonia recycling percolation, wet oxidation, etc.; and 4) biological pretreatment processes using an enzyme cocktail. All these pretreatment methods loosen up the cellulose fibers and further degradation by the cocktail of enzymes leads to the release of glucose, which releases ethanol after fermentation. The pretreatment step is essential for removing some by-products that inhibit enzyme activity [33,34]. These by-products bind to the enzyme’s active site or cavity and prevent the turnover of the enzyme for subsequent reactions. There are multiple reports of hybrid pretreatment methods, where a combination of physical, chemical, and biological methods have been used. Table 1 lists the various types of pretreatment processes individually with their advantages and disadvantages.

1.3. Cellulases and their importance in biofuel production

The hydrolysis of cellulose is a complex process that involves the interaction of cellulase enzyme with multiple cellulose chains. The increased hydrolysis of the cellulose chains results in a higher yield of glucose from cellulose. Cellulases are potential modular enzymes (discrete units in a multi domain protein, where the functions are separable [57–60]) hydrolyzing insoluble cellulose to soluble oligosaccharides. Cellulases are important biofuel enzymes because of their ability to hydrolyze cellulose into glucose, a sugar that can be fermented to ethanol. Cellulases, similar to any enzyme, are affected by numerous external parameters that in turn cause changes in their activity. Parameters such as pH, temperature, substrate concentration, etc. affect the structural stability, enzymatic activity, and ultimately the glucose yield.

1.4. Latest advances of cellulases for biofuels and biorefinery

Extraction of ethanol from biomass is achieved by techniques of biorefinery, and there are several methods to do it [61], and over the course of time, enzymatic refining procedures have proven to be the most economic, and also give the best yield. In these practices, the usage of cellulases for ethanol production from lignocellulosic biomass is quite familiar, but this method faces issues: slower conversion rates due to biomass retention or recalcitrance [62], high cost and scale-up challenges [63] *. Various improvement strategies have been explored in this regard [64]. The simplest approach is to employ a synergistic cocktail of enzymes as accessory enzymes to complement cellulases, such as xylanases and lytic polysaccharide monooxygenases [64], and cellobiohydrolases and endoglucanases [65]. Co-expression of cellulase and xylanase enzyme genes in Saccharomyces cerevisiae.
| Pretreatment methods | Process conditions | Advantages | Disadvantages |
|----------------------|--------------------|------------|---------------|
| **Physical methods** |                    |            |               |
| Disk milling         | Milling (10–30 mm) and grinding, particle size (0.2–2 mm) | No need of chemical, it is scalable | It is highly energy intensive process, poor in sugar conversion |
| Extrusion            | Screw speed, 350 rpm, barrel temperature, 80 °C, 40 % moisture. | Low pretreatment temperature and degradation products not formed, no need of washing, can be used continuously. | High energy cost, needs more aberration of metal surface. |
| Microwave radiation | Microwave 680 W, irradiation time 24 min and substrate concentration 75 g/L | Less processing time, less energy input than conventional heating, and high uniformity and selectivity. | Reactor cost is high, needed additional safety, sugar conversion and substrate concentration are low. |
| Pyrolysis            | 1 N sulfuric acid, temperature at 97 °C for 2.5 hours. | More efficient when carried out in the presence of oxygen at low temperature. | Loge solid residence time. |
| **Chemical methods** |                    |            |               |
| (1) Acid pretreatment | Dilute sulfuric acid | Temperature 140–190 °C, 0.4–2 % sulfuric acid, resident time 1–40 min. | Used for wide range of biomass, and during pretreatment process produce hydrolyzed xylene. | Need to use costly hastelloy reactor, controlling reaction condition is not easy, produces toxic degradation and during recycling water removal of salt is costly. |
|                     | Organic acid       | Temperature 130–190 °C, 50–90 mM of organic acid. | Fractionation of biomass into soluble lignin rich hemicellulose stream, and low reaction pressure. | More water needed to clean substrate after pretreatment and acid recovery is very costly. |
|                     | Concentrated acid  | Shorter residence time. | In some case no need of enzyme for cellulose depolymerization, cellulose is converted to well reactive amorphous cellulose when phosphoric acid is used. It is very effective on softwood. | The step of acid recovery is energy exhaustive. |
|                     | Acidic organosolv | Acetone-water pretreatment (acetone : water molar ratio of 1 : 1) at temperature 195 °C, pH 2.0, and residence time 5 minutes. | It can separate pure lignin stream, removal of lignin enhance the digestibility of cellulose. | High-pressure operation has high risk and used solvents are flammable and volatile. |
|                     | SPORL              | Temperature 180 °C, residence time 25 minutes and ratio of liquor/wood = 3 : 1 v/w. | Removal of lignin is more effective and high sugar yields, recovered components of biomass in less chemical transformed forms. | The degradation of sugar at harsh conditions, post pretreatment process used large water and pretreatment chemical recovery is very costly. |
| (2) Neutral pretreatment | Ionic liquid | Temperature 100–150 °C and residence time few minutes to hour. | Carbohydrate losses are low and only at severe condition, degradation products are significant. | Solvent loading, solvent cost and cost of solvent regeneration are very high. |
|                     | Liquid hot water   | Temperature 160–220 °C, 15 minutes residence time. | No need of external chemical, and reactor system is simple. | Use of more water, loss of some hemicelluloses in water stream and loading of solids is low. |
|                     | Ozonolysis         | Room temperature, Ozone sparging. | Lignin removal is effective, the production of inhibitory products is very low and reaction can be performed at atmospheric conditions. | Large amount of ozone is required i.e., costly and some portion of lignin is lost during pretreatment process. |
| (3) Alkaline Pretreatment | Ammonia Fiber Explosion (AFEX) | Temperature 100–140 °C, 1 : 1 ammonia to biomass loading, residence time 30–60 minutes, 60–100 % moisture. | Volatile ammonia can be recovered and reused, degradation product form very less and lignin is relocated on the surface that help to densify the biomass. | Safety issues in use of ammonia, recovery of ammonia is costly and not proficient for hardwood biomass. |

(Continued)
led to efficient hydrolysis of LCB, better than wild-type S. cerevisiae [66]. Wang et al. proved the involvement of extracellular products of white-rot fungus in enhancing cellulase function [67].

Hot water pre-treatments have been tested for promoting autohydrolysis before complete hydrolysis of biomass [68]. However, the effects of this step on cellulases are inconclusive, and more research in this area is required. An integrated process employed by Lian et al. [69], where autohydrolysis, nanofiltration and xylanase hydrolysis are combined to give a prebiotic that is processed better than traditional multi-process techniques is an attractive novel
approach. An alternative is to engineer proteins by inducing deliberate mutations, seeking structure-function relationships, to give suitable results [70].

1.5. Enzyme mimicking nanomaterials

Current technologies focusing on lignin degradation are expensive, leave undesirable and wasteful residues (whose disposal incurs additional costs), and sometimes can cause formation of unwanted compounds. To address these setbacks, greener methods of lignin depolymerization are being approached. Specifically, nanomaterial-based enzymes have been approached for their inherent enzyme-like properties and increased surface area to volume ratio, improving reaction rates. Deng et al., explored the usage of palladium nanoparticles supported by cerium oxide [71]. Another study employed Nickel nanoparticles to get a better yield of saturated hydrocarbons after performing a special type of chemical extraction called the organosolv process [72]. Molybdenum oxide supported by carbon nanotubes were deemed as an economic alternative to reduce lignin to phenolic derivatives which prove useful for further processes of biofuel production [73]. A Fenton-like process utilizing iron oxide nanoparticles by mimicking their peroxidase activity to reduce lignin was successful in the process while also not detrimentally impacting the carbohydrate content of the biomass [74]. This approach is gaining research limelight, and many versions and derivatives are under investigation. Several advantages provided by nanomaterial-based enzymes are enhanced reaction kinetics, low mass transfer resistance, better flexibility of reactor design, assured recovery which prompts reuse, thereby becoming more economic, and stability in various reaction conditions [75]. These advantages have promoted nanozyme-based biofuel cell research in recent times.

1.6. Effect of substrates produced in pretreatment

Pretreatment of lignocellulosic biomass has become a pre-requisite during the process of biofuel production, which helps in superior cellulase-mediated catalysis [76]. While physical and chemical procedures for the same have their places, they tend to have harsh impacts on reactor walls and/or reactor constituents. Hydrothermal methods are a suitable alternative in this regard. Currently, their application has spread to many operations in the lignocellullosic biomass biorefinery set of procedures. It gives the liberty of enabling flexible temperature and pressure setting based on the intention of the process, with two main kinds of methodologies: subcritical and supercritical operations, with reference to the critical point of water. Many types of reactors (batch, semi-continuous, continuous, and integrated) have been employed for the hydrothermal treatment of various types of biomass, but full-fledged commercial scale operations are yet to be implemented. More interest in this area is underway, and their results will help in finding a feasible approach for lignocellulosic biomass pretreatment by hydrothermal techniques like steam explosion.

The use of improved strain of *Trichoderma reesei* RUT-C30, which has β-glucosidase gene from *Talaromyces emersonii* and invertase gene from *Aspergillus niger* heterologously expressed, has improved the yield of glucose by 50 % [77]. In contrast, the ionic liquid method yields 81.5 % ethanol conversion, but the downside is the high ionic liquid cost [78]. Recent reviews support the novel and multiple pretreatments optimization of lignocellulose biomass, including greener pretreatment technologies [79–81].

1.7. Reactor design

Enzymatic degradation of lignocellulosic biomass in a large-scale bioreactor is the rate-limiting step for biofuel production because it incurs a higher cost, and the prospects of enzyme inhibitors and undesirable intermediates are significant. Therefore, the design of the bioreactor plays a pivotal role in addressing these issues [82]. The problem of the enzyme being expensive is approached by the recycling of cellulases in the reactor. This is accomplished by various methods such as recycling in the liquid or solid medium, adsorption into fresh medium, whole slurry recycling technique, membrane retention followed by concentration, and enzyme immobilization [83]. Processing higher amount of biomass may seem like a tempting option to
consider, but slurries above 20% (w/w) become too viscous to breakdown. But exploitation of horizontal bioreactors has proven effective in degrading pre-treated corn stover [84].

Many studies have also confronted the mass transfer issues, although more research is expected in this area, especially in pilot- and large-scale reactors. Studies suggest that utilizing a pre-mix in a fed-batch reactor with horizontal rotation can help combat this problem [85]. A prospective reactor can be developed with lower energy consumption and better mass transfer coefficients. In this regard, gas lift bioreactors and bubble column bioreactors have been put forth for consideration [82]. Researchers have explored the influence of varying pH [86] and alkali concentration [87] levels on the yield of reducing sugars in enzymatic hydrolysis and fermentation process. Besides cellulases, lignin degrading enzymes are also becoming a vital part of biofuel producing industries [88].

The unique standpoint of this study is the combination of experimental and computational methods of evaluation of cellulases for the production of bioethanol. Experimental validation of cellulase activity has been conducted extensively throughout literature. Their enzyme activity under different conditions is studied to optimize reaction conditions for biofuel production on a large scale. While wetlab techniques provide a realistic outlook towards functional aspects of cellulase bioconversion, they consume a lot of time, energy, capital and resources. In this regard, computational evaluation methods are a favorable alternative. Computational analyses of reaction parameters and conversion dynamics significantly shorten the time span necessary to study these in a reactor. They also provide a molecular-level understanding of the chemistry behind bioethanol production. As a novel strategy, a hybrid method has emerged, that combines the rapid screening of computational techniques and the conventional validation of laboratory procedures. This review will highlight both sides of the coin – experimental and computational study of cellulase activity for biofuel production from various sources.

2. Evaluation of cellulases

There are multiple ways to evaluate cellulase properties. Figure 2 provides an overview of the current evaluation methods reported in the scientific literature. Unlike the experimental methods, computational methods such as sequence- and structure-based analyses use the scientific literatures information to extrapolate and predict cellulases’ various properties. This

Figure 2. Experimental and computational evaluation of cellulase properties. There are multiple methods that can be used to evaluate various properties of cellulases.
review is divided into two significant aspects of evaluation for cellulases: experimental and computational evaluations.

2.1. Experimental evaluation of cellulases

Physicochemical characteristics for cellulases’ characterization have been an active research area for decades [89]. Enzyme stability and activity at varying pH and increasing temperature are essential properties that need to be studied [90].

Researchers have explored three bacterial strains of Cellulomonas sp., Bacillus sp., and Micrococcus sp., for endoglucanase activity against coir fiber at different pH (ranging from 5 to 9) and temperature (ranging from 20 °C to 50 °C). Here, Cellulomonas sp., showed the highest activity at neutral pH and at 40 °C [91]. Fungal cellulase study of strain Aspergillus niger MS82 shows optimum enzyme activity at pH 4.0 at 35 °C [92].

A high-throughput method for evaluating temperature and pH dependence, simultaneously, of various enzymes using 96-well plate and a gradient PCR cycler has garnered attention because of its combined study criteria. The study demonstrated its applicability in the single enzyme (endoglucanase Cel8A from Clostridium thermocellum) and the commercially available complex enzyme mixture Celluclast® [93]. The above-discussed examples of studies providing detailed optimization criteria provide a starting step in designing laboratory experiments for yield enhancement.

The natural biomass complex components are cellulose, hemicelluloses (xyloglucan, xylan, and/or glucomannan), lignin, pectin, oil, fats, waxes, proteins, and various extractives. The synergy of the cocktail of enzymes acting on different components at the same time on the biomass is anticipated to give a high yield. For example, in 1999, a report talked about using a cocktail of pure cellulose-, xylan-, and mannan-degrading enzymes in birch and pine kraft pulp [94]. Later in 2008, a cocktail of xylanase and esterase on pretreated corn stover was suggested [95]. Saddler et al.’s extensive cocktail study with cellulases concluded that a good synergistic interaction of endo-xylanases and xyloglucanases with cellulase improved biomass hydrolysis [96]. Similarly, xylanase and cellulase enzymes’ synergistic effect [97] and addition of accessory enzymes and cellulases are reported to enhance hydrolytic performance [98]. Østby et al. reported the interplay of enzymes and the relationship between enzymes used in a cocktail, their appropriate ratio, the impact of physicochemical conditions on enzyme activity, etc. [99].

Incubation time has also been reported as a factor for optimization. For example, extraction of cellulase from A. niger in varied carbon sources showed that the incubation period affects cellulase activity. The study reported a holding time of 10 min as an optimum time for expression of cellulase when wheat straw is used as a substrate [100]. It is also reported that the rate of product formation is not a linear reaction, and an increase in incubation time will not always increase activity and product formation. The optimum incubation time was identified as 24 hrs for bacterial cellulase in molasses [101].

The innovation in genome tailoring provides an opportunity to recreate desired improved potential strains with high enzymatic activity. Traditional chemical or physical mutagenesis approaches produced some improved strains; Aspergillus sp. XTG-4 [102], T. viride N879 [103], Cellulomonas sp. TSU-03 mutant M23 [104], and Bacillus sp. C1 mutant C1M26 [105]. However, mutants were incompetent in terms of cellulase production and activity, and were time-consuming; hence utilization of rational strategies to alter cellulase production is worth seeking. Thermotoga maritima Cel5A is an example of site-directed mutagenesis and CBM modification of endoglucanase, resulting in obtaining hyperthermostable enzymes [106].

Carbohydrate-binding modules (CBM) are essential components and increase the enzyme’s proximity to its substrate. Designing chimeric enzymes by modifying cellulase CBM to enhance their hydrolysis activity is a promising genetic engineering approach. Chimeric enzymes are synthesized by the fusion of the catalytic domain from one enzyme (of one species or organism) with that of the CBM from another species or organism’s enzyme. Recent studies have shown encouraging results of creating thermostable and thermotolerant chimeric enzymes [107] and increased substrate specificity [108–111].
Binding kinetics is another parameter that needs to be evaluated in cellulases. The study on Cel6B and Cel9A cellulases showed that while using the photobleaching method, an increase in temperature decreases the binding affinity while exhibiting partial reversibility in the presence of CBM [112]. Simultaneously, 45 °C temperature was not high enough to be detrimental to substrate binding for Cel5A; it may relate to the thermal stability of the protein also, determined by the protein fold, specifically, whether it is an \((\alpha/\alpha)_8\), \((\alpha/\beta)_8\), or \(\beta\)-jelly roll fold [113]. According to a recent study, increasing the ratio of productive to non-productive binding sites promotes hydrolysis. To prevent hydrolysis slowdown during conversion, it is essential to maintain a high productive binding capacity [114].

Significance of enzymatic cocktails have been investigated in cellulase production and improvement [115,116]. Usage of enzymatic cocktails raised additional questions and challenges about interactions and interplay between enzymes that would be beneficial or detrimental, missing information on optimal enzyme ratios, and design of optimal genome tailoring routes to be deployed focusing on facilitated production. For instance, a recent study reported success in generating a “trigenic recombinant strain” of *Penicillium oxalicum* with improved cellulosylytic activity through a combinatorial manipulation of three regulators, *chRB*, *bgl2*, and *creA*, in its regulatory pathway [115].

Another study focused on a systems biology approach and studied *T. reesei*’s 28 regulatory genes overexpression, to identify optimum conditions for enhanced cellulase production [117]. Interestingly, deletion of *ace3* gene was detrimental for cellulase production, which also significantly reduced xylanase production in the widely used cellulosylytic organism *T. reesei* [117].

Experimental evaluation provides a qualitative view of reaction kinetics in real-time. Different organisms, enzymes and enzyme cocktails can be tested for LCB hydrolysis, at different physical and chemical conditions. Reaction parameters can be modified at any point in the process to observe changes in the system. It is also possible to detect, quantify, and characterize any inhibitors and/or toxic intermediates in the mixture. This is an especially useful step to perform in laboratory scale and pilot scale studies to avoid heavy losses in large-scale operations.

While experimental techniques have their place in the analyses of LCB breakdown, they come with their own set of downfalls. The main disadvantages of these techniques are the longer periods of time required to conduct the tests and the cost incurred thereof. Each reaction in the lineup of processes requires at least a few hours and culturing of microbes for microbial treatment of biomass demands anywhere between a few days to a few weeks time to grow to the required stage. It also compels extended periods of time for any mutation studies to lead to observable changes which prolongs the evaluation stage. Identifying high-yielding strains is a challenge in itself, and finding the right media, and optimum conditions for cellulase production are bigger obstacles.

The cost of running the machinery add up to a significant amount and also, cellulase enzymes are exorbitantly expensive. The cost is exponentially high for enzyme cocktails, modified and recombinant enzymes. Meeting these expenses in laboratories is difficult without adequate funding.

### 2.2. Future directions

Currently, enzyme cocktails, fungal cellulase production, and high-throughput screening variants seem to be the direction that researchers might want to take to discover close-to-ideal enzyme systems for biofuel production, particularly, biobutanol. Biobutanol is said to have better fuel properties than ethanol, and it is produced at higher efficiency by fungal systems. Moreover, utilizing fungal cellulases with other enzymes for enzyme cocktails is a smart choice to employ for faster, and economical fuel production. Lastly, high-throughput screening provides a means to select for higher yielding strains and enzymes in a fraction of the time, which is a bonus in these expeditious times.
2.3. Computational evaluation of cellulases

Yan and Wu reported predictors to identify optimum pH of cellulases in *Pyrococcus horikoshii* using a 20–1 feedforward backpropagation neural network [118] and also the prediction of optimal pH and temperature of cellulases using 20–2 feedforward backpropagation neural network [119]. BRENDA database provided the relevant properties of 20 amino acids used in the study for the cellulase enzyme class EC 3.2.1.4 [120–123].

Advances in computational methods can help predict cellulases’ physical and chemical properties, and information such as optimal pH ranges for the highest enzymatic activity. Piecing together this kind of essential information can guide future experimental studies. The sequence mutations and tertiary (i.e., three-dimensional) structure analyses of glycoside hydrolase 6 (GH6) family were performed to find the optimal pH for enzyme activity. The analyses showed that altering the properties of surface charge in GH6 family cellulases enhanced their activity by 62% with respect to that of the wild type [124]. Another study conducted by Lugani et al. is the best example of utilization of *in silico* tools for the characterization of cellulase enzymes from different *Bacillus* species for their physicochemical characteristics, ancestral relationship, and structure determination at various levels [125].

The computational approach involves the usage of a repository of tools such as homology modeling [126], binding site identification [127], and molecular docking [128]. A study mainly consisted of 3D models (Modeler 9v9) of cellulase from *Acinetobacter* sp., prediction of substrates’ binding sites, and active site characterization based on the substrates’ docking studies [129]. Information of binding efficacy of enzyme with substrate might provide prospective substrates of choice for carbon and nitrogen sources. These docking studies revealed that cellulase has better affinity towards cellulotetraose as a substrate for higher yield of ethanol among the selected substrates [129]. Tang et al. focused on the construction of mutants of 1,4-β-glucosidase with enhanced activity based on homology modeling, molecular docking, and the site-directed mutagenesis of target residues to modify spatial positions, steric hindrances, or hydrophilicity/hydrophobicity. The mutants created by site-directed mutagenesis were successfully expressed in the *Pichia pastoris* expression system and enhanced activity for the same mutants (pPICZaA-G235 M and pPICZaA-N347S) was verified. These type of findings guide alternative ways for improving the properties of 1,4-β-glucosidase [130].

Computational evolutionary and structural analyses of GH48 (classification according to the CAZy database) [131] enzymes encoded by horizontally transferred genes were performed to distinguish cellulase from non-cellulase proteins to reduce sample protein space upstream of a computational predictive pipeline. The essential structural element ω-loop on the surface of the GH48 enzyme significantly differentiates between cellulase and non-cellulase proteins [132,133]. The search for putative cellulases in metagenomic data was done using the highly conserved and rare amino acids of the ω-loop [134]. In another study, mutation and enzyme fusion analyses were used to improve the activity of hyperthermophilic β-1,4-endoglucanase (EGPh) from *Pyrococcus horikoshii*. Cysteines were mutated to disrupt the disulfide bonds, which increased the activity of mutated enzyme without the loss of thermostability. In the same study, fusion enzyme of EGPh with a chitin binding domain enhanced activity compared to wild type EGPh [135].

SCHEMA structure guided-recombination of three fungal class II cellulohydrolases (CBH II cellulases) was used to construct a collection of highly thermostable CBH II chimeras. A sample set of 48 chimeric sequences out of a total of possible 6,561 sequences was chosen. Among 48, 23 were from a heterologous host, *Saccharomyces cerevisiae*, in their catalytically active form. Five chimeras showed a greater half-life thermal inactivation at 63 °C in comparison to the most stable parent. Twenty-five new CBH II sequences from thermophilic fungus *Humicola insolens* were designed based on theoretical modeling of thermostabilities. Ten catalytically active chimeras out of 25 were more stable and active than those in the stable wild-type parent thermophilic strain *H. insolens*. A set of 15 sequences validated as CBH II thermostable enzymes showed high
sequence diversity and hydrolyzed more cellulose than the parent enzyme [136].

Computational methods also identify the N-linked and O-linked glycosylated residues in the cellulase enzyme [137]. These residues affect stability, binding affinity, and catalytic efficiency. The N-linked glycosylated residues are primarily found in the glycoside hydrolases (GH) domains, whereas the O-linked glycosylated residues are mostly found in the linker regions between GH and CBM domains. Highly O-linked linker regions are protected from proteolytic degradation [137], and their identification is a part of high-precision protein engineering efforts.

Four broad methods of protein engineering have emerged over the decades. They are site-directed mutagenesis, directed evolution, computer-guided rational method, and semi-rational methods [138].

Site-directed mutagenesis involves targeting the active site of cellulases and hemicellulases by side chain modification [139,140]. In this strategy, enzymes can be modified to produce longer-chain alcohols, such as 3-Methyl-1-butanol, for their better conversion rates into biodiesel [141]. Alternatively, some enzymes have been shown to have preference for certain co-enzymes. But site-directed mutagenesis can reverse this preference to give better yields of ethanol [142]. Additional studies have been performed focusing on different components of the bioethanol production pathway to improve fuel yield.

In directed evolution methods, there is induction of random mutations, followed by extensive screening procedures to select for mutants with high bioethanol conversion rates [143]. These mutations lead to the generation of a large library of mutants, which are selected by high-throughput methods [144]. Computer-guided rational methods involve usage of computational techniques such as simulations, Quantum Mechanics calculations, Molecular Mechanics calculations, and docking studies [145]. These methods reduce the time required for analyzing enzyme properties, and screening thousands of compounds simultaneously. On the other hand, semi-rational methods are a combination of directed evolution and computational methods. Here, data from mutation studies is analyzed for designing enzyme active sites and scaffolds [146,147]. Combination of these methods provides a method to evaluate changes observed after directed evolution, and this information, along with structure–function relationship knowledge, is a smart way to formulate cellulase enzyme design.

The other protein engineering studies to improve cellulases toward enhanced activity include cellulose degradation, thermostability, pH stability, enhanced performance in non-conventional media, etc. They are well explained by Contreras et al. in a recently published article of 2020 [139]. The altering of transcription units on the genome by switching promoters or increasing copy numbers of cellulase genes, or creating fusion proteins are some of the approaches used in genetic tailoring [139,148–150].

Mathematical modeling and agent-based modeling/cellular automata have been used to model the kinetics of cellulose catalysis. An excellent review by Payne et al. describes these methods in detail [151]. There are additional methods available, such as molecular dynamics, constant-pH molecular dynamics, thermodynamic integration, quantum and molecular mechanics, and others that can successfully evaluate cellulases’ various properties. Among these methods, the sensitivity of the results depends on whether the cellulase is being analyzed at a fine-grained level (atomistic calculations) or a coarse-grained level (residue-level calculations). In some cases, it could be a mix of both. Detailed descriptions of these methods’ applications to evaluate cellulases are reviewed by Arora et al. [152].

Some of the computational methods used in evaluating cellulases described elaborately in literature include Constant pH Molecular Dynamics, Thermodynamic integration, Metadynamics, Continuum Molecular Dynamics, Monte Carlo methods, and Simulated Annealing. A brief description of each method is provided here as a guide for the readers. Since this short section does not do justice for these commonly used computational methods, we highly encourage the readers to refer to a large body of literature to learn more about these methods [153–156].
2.3.1. Constant-pH molecular dynamics (CpHMD)
The method identifies the protonation states of titratable sites in a protein at a given pH. This method is helpful to understand the pH-dependent conformational changes that take place in a protein. Using this method, one can predict experimental pKa values and the dynamics induced at various pH values [159].

2.3.2. Continuum-molecular dynamics
Multi-domain proteins such as cellulases are connected via a flexible linker region can leading inter-domain conformational changes. Longer MD simulations can identify nano to microsecond-time-scale changes, where the gradual macro-scale dynamical motions or continuum mechanics are ignored. The continuum-molecular dynamics method is an excellent alternative to generalize simulated tempering over a continuous temperature range to understand macroscale dynamics of the coupled dynamics of the catalytic subunit and CBM in cellulases [158].

2.3.3. Simulated annealing
Simulated annealing (also known as generalized simulated annealing) is used to identify the most stable conformations of a protein, for example, in cases where the protein undergoes engineered mutations. When applied to a cellulase, the system is computationally heated to a high temperature then it is gradually cooled to reach the lowest energy functional states of the enzyme [159].

2.3.4. Quantum and molecular mechanics
Quantum mechanical (QM) approaches can model accurate electronic rearrangements of active site atoms. However, they are computationally expensive [160]. Alternatively, molecular mechanics (MM) methods use more approximated force fields that are less accurate than QM, but they are faster and therefore computationally cheaper. The hybrid QM/MM methods are an option to overcome the limitations of a full quantum mechanical or a full molecular mechanics modeling, where the system is treated in part at the level of quantum chemistry (QM), retaining the computationally cheaper force field (MM) for the larger part.

2.4. Evaluation of cellulases for glucose yield in a hybrid production process.
Although it is slightly beyond our review scope, given that pretreatment during the biofuel production process is one of the most critical steps that can influence cellulase enzyme efficiency during industrial production (Table 1). Ishiguro and

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**Figure 3. Methods to engineer proteins with favorable or desired qualities/characteristics.** A schematic representation of futuristic engineering proteins with favorable qualities using machine learning and/or artificial intelligence approaches.
Endo [161] considered the possibility of a hybrid processing approach to increase glucose yield. Two well-known pretreatment methods, the alkali method, popular in bioethanol production and the hydrothermal method frequently used in paper and pulp industries, were combined.

Figure 3 recapitulates the approach proposed by Ishiguro and Endo, where the first prerequisite step was reducing the size of hardwood biomass of Eucalyptus to 3 mm to decrease the tenacious nature of the wood, followed by the applications of different concentrations of sodium hydroxide (NaOH) and at various high temperatures in a reactor. The samples are then wet ball-milled for four hours. The required alkali fraction, for dissolving the lignin content, is removed by thorough washing before the next step of lyophilization, which was performed over a week’s time. The enzymatic saccharification step was performed for 48 hours, and the glucose yield was measured. The findings indicated increase in glucose yield by 55 \% at 20 \% sodium hydroxide solution at 170 °C. The hydrothermal process makes the recalcitrant cellulose microfibrils amenable for further digestion by reducing the particle size and converting it into carbonaceous materials, thereby providing a promising proof-of-concept for translating the process at an industrial scale.

3. Conclusion

Non-judicious consumption of conventional fossil fuel mandates a shift to renewable and sustainable sources of energy. The drive for biofuels primarily originates from the desire to reduce greenhouse emission against the deleterious effects of climate change. Still, economic assessment of biofuel supply chain and production as well as the trade-off of using traditional fuels facilitate its adoption as a fuel alternative, and possibly even future replacement of conventional fuels.

Evaluation of enzymes is an essential step in any biochemical process. The activity of the catalyst and its turnover rate determine the cost, time, and yield of the valuable end-product. In bioethanol, the yield of glucose at the end of the pretreatment and fermentation/saccharification process indicates the ease with which industrial and commercial demands can be met. Over the years, ample experimental and computational methods have been standardized for the physico-chemical analysis and exploration of biological properties of cellulases, the bioethanol industry’s potential catalyst.

Recently, a new chemocatalyst approach reported cellulose’s direct conversion to ethanol using a chemocatalyst consisting of molybdenum and platinum [162]. It involves a one-step route of the tandem reaction, cellulose conversion to ethylene glycol and then to ethanol in the same reaction setup, aptly called ‘one-pot production’. The advantages of the chemocatalytic process make it a promising sustainable alternative to the current bioprocess; translating this approach into large-scale ethanol production in a real-world scenario can be a new research area for investigators. The interdisciplinary research and global trends coupled with heterogeneity of supply and demand systems, and economic analyses create a highly complex set of challenges. The scientific and technological aspects need attention to give rise to developing potential methods, stable and efficient enzymes, minimizing the steps of processing, and ultimately cost-effectiveness. Researchers also need to find an answer to the economical challenges, such as the cost of corn production, trade-off of using corn as a biofuel precursor instead of food or feed, the ultimate cost of building and operating plants of biofuel production, and the relative overall cost of biofuel end-product against conventional fuels (e.g., oil).

In this review, we highlight the numerous methods used to evaluate various properties of cellulases, experimentally and computationally. While experimental evaluation is ideal, there are instances where computational evaluation has provided new biological insights and saved time, thus having an economic advantage. The active research area of using hybrid methods that combines more than one pretreatment process is gaining researchers’ attention [161]. There are areas yet to be explored, such as integrating computational and experimental outcomes, creating standard testing and validation guidelines, and using machine learning and artificial intelligence
methods to expand our understanding of biofuel enzymes to develop more optimized industrial processes.

The industry of biofuel production has picked up pace in the last two decades in view of the impending complete exhaustion of fossil fuels, and also the need for more sustainable, greener alternatives that deal with the enormous amount of biomass waste generated. With advancements in cellulase production technology, protein engineering to enhance cellulase activity, and methods to analyze production parameters and strategies, many milestones have been reached, and yet several more remain. Prospects in this arena are aplenty.

For example, a recent topic of interest has been the production of thermostable cellulolytic enzymes, which can be beneficial in many ways, such as higher rates of bioconversion, minimized contamination by microorganisms, and abated costs required for plant cooling [163]. Butanol seems to be the alcohol of choice as per research in the last two decades [164]. Although many Clostridia are known to be excellent producers of butanol, and several mutants have been created to maximize production, their full-fledged large-scale production is still underway since that necessitates additional studies and optimization.

This is where rational computational methods and hybrid techniques come into the picture: to estimate reaction conditions, predict unfavorable process parameters, and analyze potential properties by simulations and docking studies. According to predictions, systems biology studies are next in the pipeline to help conceptualize, design, and implement biofuel production strategies.

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**Consent for publication**

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**Authors’ contributions**

All authors contributed toward the writing of the manuscript. All authors have approved the final article.

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There was no human subjects or animal subjects used/involved in this study.

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**References**

[1] Edenhofer O, Pichs-Madruga R, Sokona Y, et al. Renewable energy sources and climate change mitigation. special report of the intergovernmental panel on climate change. Cambridge: Cambridge University Press; 2011. DOI:10.1017/CBO9781139151153

[2] Goldemberg J. Energy needs in developing countries and sustainability. Science. 1995;269(5227):1058–1059.

[3] Samset BH, Sand M, Smith CJ, et al. Climate impacts from a removal of anthropogenic aerosol emissions. Geophys Res Lett. 2018;45(2):1020–1029.

[4] Anam K, Naem A, Muhammad Shoaib S, et al. Role of energy storage systems in energy transition from fossil fuels to renewables. Energy Storage. 2020;3:1–27.

[5] Hansen K, Breyer C, Lund H. Status and perspectives on 100 % renewable energy systems. Energy. 2019;175:471-480.

[6] Vasić K, Knez Ž, Leitgeb M. Bioethanol production by enzymatic hydrolysis from different lignocellulosic sources. Molecules. 2021;26(3):753.
[7] Fatma S, Hameed A, Noman M, et al. Lignocellulosic biomass: a sustainable bioenergy source for the future. Protein Pept Lett. 2018;25(2):148–163.

[8] Antolini E. Lignocellulose, cellulose and lignin as renewable alternative fuels for direct biomass fuel cells. ChemSusChem. 2021;14(1):189–207.

[9] Wang Y, Van Le Q, Yang H, et al. Progress in microbial biomass conversion into green energy. Chemosphere. 2021;281:130835.

[10] Alalwan H, Aba A, Aljaafari H. Promising evolution of biofuel generations. Subject Review Renewable Energy Focus. 2019;28:127–139.

[11] Ho DP, Ngo HH, Guo W. A mini review on renewable sources for biofuel. Bioresour Technol. 2014;169:742–749.

[12] Robak K, Balcerek M. Review of second generation bioethanol production from residual biomass. Food Technol Biotechnol. 2018;56(2):174–187.

[13] Sims REH, Mabee W, Saddler JN, et al. An overview of second generation biofuel technologies. Bioresour Technol. 2010;101(6):1570–1580.

[14] Lee Y, Park J, Ryu C, et al. Comparison of biochar properties from biomass residues produced by slow pyrolysis at 500 C. Bioresour Technol. 2013;148:196–201.

[15] Mata-Sánchez J, Pérez-Jiménez JA, Díaz-Villanueva MJ, et al. Statistical evaluation of quality parameters of olive stone to predict its heating value. Fuel. 2013;113:750–756.

[16] Arranz JI, Miranda MT, Montero I, et al. Characterization and combustion behaviour of commercial and experimental wood pellets in southwest Europe. Fuel. 2015;142:199–207.

[17] Perea-Moreno AJ, Aguilera-Ureña MJ, Manzano-Agugliaro F. Fuel properties of avocado stone. Fuel. 2016;186:358–364.

[18] Dai J, Saayman J, Grace JR, et al. Gasification of woody biomass. Annu Rev Chem Biomol Eng. 2015;6(1):77–99.

[19] Şenol H. Effects of NaOH, thermal, and combined NaOH-thermal pretreatments on the biomethane yields from the anaerobic digestion of walnut shells. Environ Sci Pollut Res Int. 2021;28(17):21661–21673.

[20] Perea-Moreno MA, Manzano-Agugliaro F, Hernandez-Escobedo Q, et al. Peanut shell for energy: properties and its potential to respect the environment. Sustainability. 2018;10(9):3254.

[21] Perea-Moreno AJ, Perea-Moreno MA, Dorado MP, et al. Mango stone properties as biofuel and its potential for reducing CO2 emissions. J Clean Prod. 2018;190:53–62.

[22] Perea-Moreno MA, Manzano-Agugliaro F, Perea-Moreno AJ. Sustainable energy based on sunflower seed husk boiler for residential buildings. Sustainability. 2018;10(10):3407.

[23] Miranda MT, Sepúlveda FJ, Arranz JL, et al. Analysis of pelletizing from corn cob waste. J Environ Manage. 2018;228:303–311.

[24] Heredia Salgado MA, Tarelho LAC, Matos MAA, et al. Palm oil kernel shell as solid fuel for the commercial and industrial sector in Ecuador: tax incentive impact and performance of a prototype burner. J Clean Prod. 2019;213:104–113.

[25] Demirbas A. Progress and recent trends in biofuels. Prog Energy Combust Sci. 2007;33(1):1–18.

[26] da Costa Sousa L, Chundawat SP, Balan V, et al. “Cradle-to-grave” assessment of existing lignocellulose pretreatment technologies. Curr Opin Biotechnol. 2009;20(3):339–347.

[27] Himmel ME, Ding S-Y, Johnson DK et al. Biomass recalcitrance: engineering plants and enzymes for biofuels production. Science. 2007;315(5813):804–807.

[28] Dahman Y, Dignac N, Fiayaz A, et al. 13 - An introduction to biofuels, foods, livestock, and the environment. In: Verma D, Fortunati E, Jain S, et al, editors. Biomass, biopolymer-based materials, and bioenergy. Sawston, United Kingdom: Woodhead Publishing; 2019. p. 241–276. DOI:10.1016/B978-0-08-102426-3.00013-8.

[29] Zoghliami A, Paés G. Lignocellulosic biomass: understanding recalcitrance and predicting hydrolysis. Front Chem. 2019;7:874.

[30] Terrett OM, Dupree P. Covalent interactions between lignin and hemicelluloses in plant secondary cell walls. Curr Opin Biotechnol. 2019;56:97–104.

[31] de Vries L, Guevara-Rozo S, Cho M, et al. Tailoring renewable materials via plant biotechnology. Biochim Biophys Acta. 2021;14(1):167.

[32] Sankaran R, Parra Cruz RA, Pakalapati H, et al. Recent advances in the pretreatment of microalgal and lignocellulosic biomass: a comprehensive review. Bioresour Technol. 2020;298:122476.

[33] Lin Y, Tanaka S. Ethanol fermentation from biomass resources: current state and prospects. Appl Microbiol Biotechnol. 2006;69(6):627–642.

[34] Lynd L, Weimer P, Van Zyl W, et al. Microbial cellulose utilization: fundamentals and biotechnology. Microbiol Mol Biol Rev. 2002;66(3):506–777.

[35] Hideno A, Inoue H, Tsukahara K, et al. Wet disk milling pretreatment without sulfuric acid for enzymatic hydrolysis of rice straw. Bioresour Technol. 2009;100(10):2706–2711.

[36] Yoo J, Alavi S, Vadlani P, et al. Thermo-mechanical extrusion pretreatment for conversion of soybean hulls to fermentable sugars. Bioresour Technol. 2011;102(16):7583–7590.

[37] Ma H, Liu WW, Chen X, et al. Enhanced enzymatic saccharification of rice straw by microwave pretreatment. Bioresour Technol. 2009;100(3):1279–1284.

[38] Xiao R, Chen X, Wang F, et al. Pyrolysis pretreatment of biomass for entrained-flow gasification. Appl Energy. 2010;87(1):149–155.

[39] Wyman CE, Dale BE, Balan V, et al. Comparative performance of leading pretreatment technologies for biological conversion of corn stover, poplar wood, and switchgrass to sugars. Aqueous Pretreatment of Plant Biomass for Biological and Chemical Conversion to
[40] Qin L, Liu ZH, Li BZ, et al. Mass balance and transformation of corn stover by pretreatment with different dilute organic acids. Bioresour Technol. 2012;112:319–326.

[41] Galbe M, Zacchi G. A review of the production of ethanol from softwood. Appl Microbiol Biotechnol. 2002;59(6):618–628.

[42] Sun F, Chen H. Organosolv pretreatment by crude glycerol from oleochemical industry for enzymatic hydrolysis of wheat straw. Bioresour Technol. 2008;99(13):5474–5479.

[43] Liu H, Zhu J. Eliminating inhibition of enzymatic hydrolysis by lignosulfonate in unwashed sulfite-pretreated aspen using metal salts. Bioresour Technol. 2010;101(23):9120–9127.

[44] Kim Y, Hendrickson R, Mosier NS, et al. Liquid hot water pretreatment of cellulosic biomass. In Jonathan R. Mielenz: Biofuels. Totowa NJ: Humana Press; 2009. p. 93–102.

[45] Garcia-Cubero MT, Gonzalez-Benito G, Indacoechea I, et al. Effect of ozonolysis pretreatment on enzymatic digestibility of wheat and rye straw. Bioresour Technol. 2009;100(4):1608–1613.

[46] Balan V, Bals B, Chundawat SP, et al. Lignocellulosic biomass pretreatment using AFEX. In Jonathan R. Mielenz: Biofuels. Totowa NJ: Humana Press; 2009. p. 61–77.

[47] Kim TH, Lee YY. Pretreatment and fractionation of corn stover by ammonia recycle percolation process. Bioresour Technol. 2005;96(18):2007–2013.

[48] Li X, Kim TH, Nghiem NP. Bioethanol production from corn stover using aqueous ammonia pretreatment and two-phase simultaneous saccharification and fermentation (TPSSF). Bioresour Technol. 2010;101(15):5910–5916.

[49] Taherzadeh MJ, Karimi K. Pretreatment of lignocellulosic wastes to improve ethanol and biogas production: a review. Int J Mol Sci. 2008;9(9):1621–1651.

[50] Banerjee G, Car S, Liu T, et al. Scale-up and integration of alkaline hydrogen peroxide pretreatment, enzymatic hydrolysis, and ethanolic fermentation. Bioelectron Bioeng. 2012;109(4):922–931.

[51] Xu J, Cheng JJ, Sharma-Shivappa RR, et al. Lime pretreatment of switchgrass at mild temperatures for ethanol production. Bioresour Technol. 2010;101(8):2900–2903.

[52] Banerjee S, Sen R, Pandey RA, et al. Evaluation of wet air oxidation as a pretreatment strategy for bioethanol production from rice husk and process optimization. Biomass Bioenergy. 2009;33(12):1680–1686.

[53] Oliveira FM, Pinheiro IO, Souto-Maior AM, et al. Industrial-scale steam explosion pretreatment of sugarcane straw for enzymatic hydrolysis of cellulose for production of second generation ethanol and value-added products. Bioresour Technol. 2013;130:168–173.

[54] Narayana Swamy N (2010). Supercritical carbon dioxide pretreatment of various lignocellulosic biomasses (Doctoral dissertation, Ohio University).

[55] Wan C, Li Y. Fungal pretreatment of lignocellulosic biomass. Biotechnol Adv. 2012;30(6):1447–1457.

[56] Sindhu R, Binod P, Pandey A. Biological pretreatment of lignocellulosic biomass—An overview. Bioresour Technol. 2016;199:76–82.

[57] Khosla C, Harbury PB. Modular enzymes. Nature. 2001 Jan;409(6817):247–252.

[58] Campbell ID. Modular proteins at the cell surface. Biochem Soc Trans. 2003 Dec;31(6):1107–1114.

[59] Trifonov EN, Frenkel ZM. Evolution of protein modularity. Curr Opin Struct Biol. 2009 Jun;19(3):335–340.

[60] Fong M, Berrin J-G, Paës G. Investigation of the binding properties of a multi-modular GH45 cellulase using bioinspired model assemblies. Biotechnol Biofuels. 2016 Jan;9(1):12.

[61] Singh A, Rodriguez Jasso RM, Gonzalez-Gloria KD, et al. The enzyme biorefinery platform for advanced biofuels production. Bioresour Technol Rep. 2019 Sep;7:100257. DOI: 10.1016/j.biorep.2019.100257

[62] Auxenfans T, Terryn C, Paës G. Seeing biomass recalcitrance through fluorescence. Sci Rep. 2017 Aug;7(1, Art. no. 1):DOI: 10.1038/s41598-017-08740-1

[63] Singhania RR, Ruiz HA, Awasthi MK, et al. Challenges in cellulosic bioprocess for biofuel applications. Renew Sus Energ Rev. 2021 Nov;151:111622. DOI: 10.1016/j.rser.2021.111622

[64] Hu J, Chandra R, Arantes V, et al. The addition of accessory enzymes enhances the hydrolytic performance of cellulase enzymes at high solid loadings. Bioresour Technol. 2015 Jan;161:149–153. DOI: 10.1016/j.biortech.2015.03.055

[65] Karnaouri A, Topakas E, Matsakas L, et al. Fine-tuned enzymatic hydrolysis of organosolv pretreated forest materials for the efficient production of cellulosio. Front Chem. 2018;6:Accessed: Feb. 19, 2022:[Online]. Available DOI: 10.3389/chem.2018.00128

[66] Xiao W, Li H, Xia W, et al. Co-expression of cellulase and xylanase genes in Saccharomyces cerevisiae toward enhanced bioethanol production from corn stover. Bioengineered. 2019 Jan;10(1):513–521.

[67] Wang Y, Shao Y, Zou X, et al. Synergistic action between extracellular products from white-rot fungus and cellulase significantly improves enzymatic hydrolysis. Bioengineered. 2018 Jan;9(1):178–185.

[68] Fang H, Kandhola G, Rajan K, et al. Effects of oligosaccharides isolated from pinewood hot water pre-hydrolyzates on recombinant cellulases. Front Bioeng Biotechnol. 2018 May;6:55. DOI: 10.3389/fbioe.2018.00055

[69] Lian Z, Wang Y, Luo J, et al. An integrated process to produce prebiotic xylooligosaccharides by autohydrolysis, nanofiltration and endo-xylanase from alkali-extracted xylan. Bioresour Technol. 2020 Oct;314:123685. DOI: 10.1016/j.biortech.2020.123685

[70] Singh A, Patel AK, Adsul M, et al. Genetic modification: a tool for enhancing cellulase secretion. Biofuel Res J. 2017 Jun;4(2):600–610.
[71] Deng W, Zhang H, Wu X, et al. Oxidative conversion of lignin and lignin model compounds catalyzed by CeO2-supported Pd nanoparticles. Green Chem. 2015 Nov;17(11):5009–5018.

[72] Kasakov S, Shi H, Camaioni DM, et al. Reductive deconstruction of organosolv lignin catalyzed by zeolite supported nickel nanoparticles. Green Chem. 2015 Nov;17(11):5079–5090.

[73] Xiao L-P, Wang S, Li H, et al. Catalytic hydrogenolysis of lignins into phenolic compounds over carbon nanotube supported molybdenum oxide. ACS Catal. 2017 Nov;7(11):7535–7542.

[74] Rajak RC, Saha P, Singhvi M, et al. An eco-friendly biomass pretreatment strategy utilizing reusable enzyme mimicking nanoparticles for lignin depolymerization and biofuel production. Green Chem. 2021 Aug;23(15):5584–5599.

[75] Verma ML, Puri M, Barrow CJ. Recent trends in nanomaterials immobilised enzymes for biofuel production. Crit Rev Biotechnol. 2016 Jan;36(1):108–119.

[76] Ruiz HA, Conrad M, Sun S-N, et al. Engineering aspects of hydrothermal pretreatment: from batch to continuous operation, scale-up and pilot reactor under biorefinery concept. Bioresour Technol. 2020 Mar;299:122685. DOI:10.1016/j.biortech.2019.122685

[77] Fonseca LM, Parreira LS, Murakami MT. Rational engineering of the Trichoderma reesei RUT-C30 strain into an industrially relevant platform for cellulase production. Biotechnol Biofuels. 2020 May;13(1):93.

[78] Shafiee M, Hamid Z, Akram Z, et al. Enhancement of ethanol production from spruce wood chips by ionic liquid pretreatment. Appl Energy. 2013;102:163.

[79] Roy R, Md Rahman S, Raynie DE. Recent advances of greener pretreatment technologies of lignocellulose. Curr Res Green Sustainable Chem. 2020;3:100035.

[80] Galbe M, Wallberg O. Pretreatment for biofuineries: a review of common methods for efficient utilisation of lignocellulosic materials. Biotechnol Biofuels. 2019;12(1):294.

[81] Kucharska K, Rybarczyk P, Hołowacz I, et al. Pretreatment of lignocellulosic materials as substrates for fermentation processes. Molecules. 2018;23(11):2937.

[82] Pino MS, Rodriguez-Jasso RM, Michelin M, et al. Bioreactor design for enzymatic hydrolysis of biomass under the biorefinery concept. Chem Eng J. 2018 Sep;347:119–136. DOI:10.1016/j.cej.2018.04.057

[83] Jørgensen H, Pinelo M. Enzyme recycling in lignocellulosic biofuineries. Biofuel Bioprod Biorefin. 2017;11(1):150–167.

[84] Du J, Zhang F, Li Y, et al. Enzymatic liquefaction and saccharification of pretreated corn stover at high-solids concentrations in a horizontal rotating bioreactor. Bioprocess Biosyst Eng. 2014 Feb;37(2):173–181.

[85] Du J, Cao Y, Liu G, et al. Identifying and overcoming the effect of mass transfer limitation on decreased yield in enzymatic hydrolysis of lignocellulose at high solid concentrations. Bioreour Technol. 2017 Apr;229:88–95. DOI:10.1016/j.biortech.2017.01.011

[86] Immanuel G, Dhanusha R, Prema P, et al. Effect of different growth parameters on endoglucanase enzyme activity by bacteria isolated from coir retting effluents of estuarine environment. Int J Environ Sci Technol. 2006;3(1):25–34.

[87] Chen Y, Stevens M, Zhu Y, et al. Understanding of alkaline pretreatment parameters for corn stover enzymatic saccharification. Biotechnol Biofuels. 2013;6(1):8.

[88] Pollegioni L, Tonin F, Rosini E. Lignin-degrading enzymes. FEBS J. 2015;282(7):1190–1213.

[89] Shanmugapriya K, Saravana PS, Krishnapriya MM, et al. Isolation, screening and partial purification of cellulose from cellulose producing bacteria. Int J Adv Biotechnol Res. 2012;3:509–514.

[90] Abdelnasser SS, Ahmed IE. Isolation and identification of new cellulases producing thermophilic bacteria from an Egyptian hot spring and some properties of the crude enzyme. Aust J Basic Appl Sci. 2007;1:473–478.

[91] Immanuel G, Dhanusha R, Prema P, et al. Effect of different growth parameters on endoglucanase enzyme activity by bacteria isolated from coir retting effluents of estuarine environment. Int J Environ Sci Technol. 2006;3(1):25–34.

[92] Sohail M, Siddiqui R, Ahmad A, et al. Cellulase production from Aspergillus niger MS82: effect of temperature and pH. N Biotechnol. 2009;25(6):437–441.

[93] Herlet J, Kornberger P, Roessler B, et al. A new method to evaluate temperature vs. pH activity profiles for biotechnological relevant enzymes. Biotechnol Biofuels. 2017;10(1):234.

[94] Tenkanen M, Tamminen T, Hortling B. Investigation of lignin-carbohydrate complexes in kraft pulps by selective enzymatic treatments. Appl Microbiol Biotechnol. 1999;51(2):241–248.

[95] Selig MJ, Knoshaug EP, Adney WS, et al. Synergistic enhancement of cellobiohydrolase performance on pretreated corn stover by addition of xylanase and esterase activities. Bioreour Technol. 2008;99(11):4997–5005.

[96] Hu J, Arantes V, Pribowo A, et al. The synergistic action of accessory enzymes enhances the hydrolytic potential of a “cellulase mixture” but is highly substrate specific. Biotechnol Biofuels. 2013;6(1). DOI:10.1186/1754-6834-6-112

[97] Hu J, Arantes V, Saddler JN. The enhancement of enzymatic hydrolysis of lignocellulosic substrates by the addition of accessory enzymes such as xylanase: is it an additive or synergistic effect? Biotechnol Biofuels. 2011;4(1). DOI:10.1186/1754-6834-4-36

[98] Hu J, Chandra R, Arantes V, et al. The addition of accessory enzymes enhances the hydrolytic performance of cellulase enzymes at high solid loadings. Bioreour Technol. 2015;186:149–153.

[99] Østby H, Hansen LD, Horn SJ, et al. Enzymatic processing of lignocellulosic biomass: principles, recent advances and perspectives. J Ind Microbiol Biotechnol. 2020;47:623–657.
[100] Pedersen M, Johansen K, Meyer A. Low temperature lignocellulose pretreatment: effects and interactions of pretreatment pH are critical for maximizing enzymatic monosaccharide yields from wheat straw. Biotechnol Biofuels. 2011;4(1):11.

[101] Islam F, Roy N. Screening, purification and characterization of cellulase from cellulase producing bacteria in molasses. BMC Res Notes. 2018;11(1):445.

[102] Yu VH, Pham TA, Kim K. Fungal strain improvement for cellulase production using repeated and sequential mutagenesis. Mycobiology. 2009;37(4):267–271.

[103] Xu F, Wang J, Chen S, et al. Strain improvement for enhanced production of cellulase in Trichoderma viride. Applied Biochemistry and Microbiology. 2011;47(1):53–58.

[104] Sangkhara B, Vangirikul P, Janthachat S. Strain improvement and optimization for enhanced production of cellulase in Cellulomonas sp. TSU-03. Afr J Microbiol Res. 2012;6(5):1079–1084.

[105] Sadhu S, Ghosh PK, Aditya G, et al. Optimization and strain improvement by mutation for enhanced cellulase production by Bacillus sp. (MTCC10046) isolated from cow dung. Journal of King Saud University - Science. 2014;26(4):323–332.

[106] Mahadevan SA, Wi SG, Lee DS, et al. Site-directed mutagenesis and CBM engineering of Cel5A (Thermotoga maritima). FEMS Microbiol Lett. 2008;287(2):205–211.

[107] Gilmore SP, Lillingston SP, Haitjema CH, et al. Designing chimeric enzymes inspired by fungal celullosomes. Synth Syst Biotechnol. 2020;5(1):23–32.

[108] Chalak A, Villares A, Moreau C, et al. Influence of the carbohydrate-binding module on the activity of a fungal AA9 lytic polysaccharide monoxygenase on cellulose substrates. Biotechnol Biofuels. 2019 Sep;12(1):206.

[109] Koskela S, Wang S, Xu D, et al. Lytic polysaccharide monoxygenase (LPMO) mediated production of ultra-fine cellulose nanofibres from delignified softwood fibres. Green Chem. 2019;21(21):5924–5933.

[110] Crouch LI, Laboureul A, Walton PH, et al. The contribution of non-catalytic carbohydrate binding modules to the activity of lytic polysaccharide monoxygenases. J Biol Chem. 2016 Apr;291(14):7439–7449.

[111] Jagadeeswaran G, Gainey L, Mort AJ. An AA9-LPMO containing a CBM1 domain in Aspergillus nidulans is active on cellulose and cleaves cello-oligosaccharides. AMB Express. 2018 Oct;8(1):171.

[112] Lee H, Chang C, Teng K, et al. Construction and characterization of different fusion proteins between cellulases and beta-glucosidase to improve glucose production and thermostability. Bioresour Technol. 2011;102(4):3973–3976.

[113] Moran-Mirabal J, Bolewski J, Walker L. Reversibility and binding kinetics of Thermobifida fusca cellulases studied through fluorescence recovery after photobleaching microscopy. Biophys Chem. 2011;155(1):20–28.

[114] Yennamalli R, Rader A, Wolt J, et al. Thermostability in endoglucanases is fold-specific. BMC Struct Biol. 2011;11(1):10.

[115] Nill J, Jeoh T. The role of evolving interfacial substrate properties on heterogeneous cellulose hydrolysis kinetics. ACS Sustain Chem Eng. 2020;8(17):6722–6733.

[116] Yao G, Li Z, Gao L, et al. Redesigning the regulatory pathway to enhance cellulase production in Penicillium oxalicum. Biotechnol Biofuels. 2015;8(1):71.

[117] Hakkinen M, Valkonen MJ, Westerholm-Parvinen A, et al. Screening of candidate regulators for cellulase and hemicellulase production in Trichoderma reesei and identification of a factor essential for cellulase production. Biotechnol Biofuels. 2014;7(1):14.

[118] Yan S, Wu G. Searching of predictors to predict pH optimum of cellulases. Appl Biochem Biotechnol. 2011;165(3–4):856–869.

[119] Yan S, Wu G. Prediction of optimal pH and temperature of cellulases using neural network. Protein Pept Lett. 2012;19(1):29–39.

[120] Schomburg I. BRENDA, enzyme data and metabolic information. Nucleic Acids Res. 2002;30(1):47–49.

[121] Placzek S, Schomburg I, Chang A, et al. BRENDA in 2017: new perspectives and new tools in BRENDA. Nucleic Acids Res. 2017;45(D1):D380–D388.

[122] Schomburg I, Jeske L, Ulbrich M, et al. The BRENDA enzyme information system–From a database to an expert system. J Biotechnol. 2017;261:194–206.

[123] Jeske L, Placzek S, Schomburg I, et al. BRENDA in 2019: a European ELIXIR core data resource. Nucleic Acids Res. 2019;47(D1):D542–D549.

[124] Cockburn DW, Clarke AJ. Modulating the pH-activity profile of cellulase A from Cellulomonas fimi by replacement of surface residues. Protein Engineering Design and Selection. 2011;24(5):429–437.

[125] Lugani Y, Sooch BS. In silico characterization of cellulases from genus bacillus. Int J Curr Res Rev. 2017;9:30–37.

[126] Marti-Renom MA, Stuart AC, Fiser A, et al. Comparative protein structure modeling of genes and genomes. Annu Rev Biophys Biomol Struct. 2000;29(1):291–325.

[127] Ding Y, Tang J, Guo F. Identification of protein–ligand binding sites by sequence information and ensemble classifier. J Chem Inf Model. 2017 Dec;57(12):3149–3161.

[128] Meng X-Y, Zhang H-X, Mezei M, et al. Molecular Docking: a powerful approach for structure-based drug discovery. Curr Comput Aided Drug Des. 2011 Jun;7(2):146–157.

[129] Selvam K, Senbagam D, Selvakumar T, et al. Cellulase enzyme: homology modeling, binding site identification and molecular docking. J Mol Struct. 2017;1150:61–67.

[130] Tang Z, Jin W, Tang Y, et al. Research on homology modeling, molecular docking of the cellulase and highly expression of the key enzyme (Bgl) in Pichia pastoris. Int J Biol Macromol. 2018;115:1079–1087.

[131] Cantarel BL, Coutinho PM, Rancurel C, et al. The carbohydrate-active EnZymes database (CAZY). an
expert resource for Glycogenomics. Nucleic Acids Res. 2009;37(Database issue):D233–D238.

[132] Sukharnikov LO, Cantwell BJ, Podar M, et al. Cellulases: ambiguous nonhomologous enzymes in a genomic perspective. Trends Biotechnol. 2011;29 (10):473–479.

[133] Sukharnikov L, Alahuhta M, Brunecky R, et al. Sequence, structure, and evolution of cellulases in glycoside hydrolase family 48. J Biol Chem. 2012;287 (49):41068–41077.

[134] Cherry JR, Fidantsef AL. Directed evolution of industrial enzymes: an update. Curr Opin Biotechnol. 2003;14(4):438–443.

[135] Schubert C. Can biofuels finally take center stage? Nat Biotechnol. 2006;24(7):777–784.

[136] Heinzelman P, Komor R, Kanaan A, et al. Efficient screening of fungal cellulohydrolase class I enzymes for thermostabilizing sequence blocks by SCHEMA structure-guided recombination. Protein Eng Des Sel. 2010;23(11):871–880.

[137] Zhou F, Olman V, Xu Y. Large-scale analyses of glycosylation in cellulases. Genomics Proteomics Bioinformatics. 2009;7(4):194–199.

[138] Contreras F, Pramanik S, Rozhkova A, et al. Engineering robust cellulases for tailored lignocellulosic degradation cocktails. Int J Mol Sci. 2020 Jan;21 (5):1589.

[139] Schülein M. Protein engineering of cellulases. Biochim Biophys Acta Bioenerg. 2000 Dec;1543(2):239–252.

[140] Wen F, Nair NU, Zhao H. Protein engineering in designing tailored enzymes and microorganisms for biofuels production. Curr Opin Biotechnol. 2009 Aug;20(4):412–419.

[141] Connor MR, Liao JC. Engineering of an Escherichia coli strain for the production of 3-Methyl-1-butanol. Appl Environ Microbiol. 2008 Sep;74(18):5769–5775.

[142] Watanabe S, Kodaki T, Makino K. Complete reversal of coenzyme specificity of xylitol dehydrogenase and increase of thermostability by the introduction of structural zinc * . J Biol Chem. 2005 Mar;280(11):10340–10349.

[143] Packer MS, Liu DR. Methods for the directed evolution of proteins. Nat Rev Genet. 2015 Jul;16(7):379–394.

[144] Markel U, Essani KD, Besirlioglu V, et al. Advances in ultrahigh-throughput screening for directed enzyme evolution. Chem Soc Rev. 2020 Jan;49(1):233–262.

[145] Romero-Rivera A, Garcia-Borrías M, Osuna S. Computational tools for the evaluation of laboratory-engineered biocatalysts. Chem Commun. 2016 Dec;52(2):284–297.

[146] Chica RA, Doucet N, Pelletier JN. Semi-rational approaches to engineering enzyme activity: combining the benefits of directed evolution and rational design. Curr Opin Biotechnol. 2005 Aug;16(4):378–384.

[147] Contreras F, Pramanik S, Rozhkova AM, et al. Engineering robust cellulases for tailored lignocellulosic degradation cocktails. Int J Mol Sci. 2020;21 (5):1589.

[148] Liu Z, Ho S, Sasaki K, et al. Engineering of a novel cellulose-adherent cellulosytic Saccharomyces cerevisiae for cellulosic biofuel production. Sci Rep. 2016;6 (1):24550.

[149] Yao G, Wu R, Kan Q, et al. Production of a high-efficiency cellulase complex via β-glucosidase engineering in Penicillium oxalicum. Biotechnol Biofuels. 2016;9(1):78.

[150] Egelkroft E, McGaughy K, Keener T, et al. Enhanced expression levels of cellulase enzymes using multiple transcription units. BioEnergy Res. 2012;6(2):699–710.

[151] Payne C, Knott B, Mayes H, et al. Fungal Cellulases. Chem Rev. 2015;115(3):1308–1448.

[152] Arora M, Yennamalli R, Sen T. Application of molecular simulations toward understanding cellulase mechanisms. BioEnergy Res. 2018;11(4):850–867.

[153] Hollingsworth S, Dror R. Molecular dynamics simulation for all. Neuron. 2018;99(6):1129–1143.

[154] Radak B, Chipot C, Süh D, et al. Constant-pH molecular dynamics simulations for large biomolecular systems. J Chem Theory Comput. 2017;13(12):5933–5944.

[155] Laio A, Gervasio F. Metadynamics: a method to simulate rare events and reconstruct the free energy in biophysics, chemistry and material science. Rep Prog Phys. 2008;71(12):126601.

[156] Andricioaei I, Straub J. Simulated annealing methods in protein folding. In: Floudas CA, Pardalos PM, editors. Encyclopedia of Optimization. Boston MA: Springer; 2001:2393.

[157] Chen W, Morrow BH, Shi C. Shen J.K. Recent development and application of constant pH molecular dynamics. Mol Simulat. 2014;40(10–11):830–838.

[158] Lenner N, Mathias G. Continuous tempering molecular dynamics: a deterministic approach to simulated tempering. J Chem Theory Comput. 2016;12(2):486–498.

[159] Tsallis C, Stariolo DA. Generalized simulated annealing. Phys A Stat Mech Appl. 1996;233(1–2):395–406.

[160] van der Kamp MW, Mulholland AJ. Combined quantum mechanics/molecular mechanics (QM/MM) methods in Computational Enzymology. Biochemistry. 2013;52(16):2708–2728.

[161] Ishiguro M, Endo T. Addition of alkali to the hydrothermal-mechanochemical treatment of Eucalyptus enhances its enzymatic saccharification. Bioreasour Technol. 2014;153:322–326.

[162] Yang M, Qi H, Liu F, et al. One-pot production of cellulosic ethanol via tandem catalysis over a multifunctional Mo/Pt/WOx catalyst. Joule. 2019;3(8):1937–1948.

[163] Srivastava N, Srivastava M, Mishra PK, et al. Applications of fungal cellulases in biofuel production: advances and limitations. Renew Sust Energ Rev. 2018Feb;82:2379–2386. DOI:10.1016/j.rser.2017.08.074

[164] Dürr E. Fermentative production of butanol—the academic perspective. Curr Opin Biotechnol. 2011 Jun;22 (3):331–336.