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Trends in Biotechnology

Opinion

Making the biochemical conversion of lignocellulose more robust

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Lignocellulose is an alternative to fossil resources, but its biochemical conversion is not economically competitive. While decentralized processing can reduce logistical cost for this feedstock, sugar platforms need to be developed with energy-saving pretreatment technologies and cost-effective cellulases, and products must be selected correctly. Anaerobic fermentation with less energy consumption and lower contamination risk is preferred, particularly for producing biofuels. Great effort has been devoted to producing cellulosic ethanol, but CO_2 released with large quantities during ethanol fermentation must be utilized *in situ* for credit. Unless titer and yield are improved substantially, butanol cannot be produced as an advanced biofuel. Microbial lipids produced through aerobic fermentation with low yield and intensive energy consumption are not affordable as feedstocks for biodiesel production.

Urgency for alternatives to fossil resources

Fossil resources such as coal, crude oil, and natural gas have been exploited with a long history, predominately as fuels, but they are not renewable for sustainable supply. Meanwhile, CO_2 released with the consumption of fossil-derived products has created significant impact on climate, which is highlighted by global warming [1].

Lignocellulose (see Glossary) is renewable and abundantly available, and **life cycle analysis** indicates that CO_2 released with the consumption of lignocellulose-derived products can be fixed through plant photosynthesis, making it an alternative to fossil resources for **neutral or negative carbon emissions**, to mitigate global warming and climate change that have increasingly contributed to natural disasters (Figure 1, Key figure) [2]. In general, lignocellulose can be converted through chemical/thermochemical routes with intensive energy input, harsh reaction conditions, and more chemicals consumed [3,4]. Within the past decades, substantial progress has been made in life science fundamentals and biotechnological innovations to drive the biochemical conversion of lignocellulose under mild conditions with less energy and chemicals consumed, making such a route more promising for developing environmentally friendly processes, sometimes called lignocellulose biorefinery [5].

Both agricultural residues and forest wastes are lignocellulosic resources, but agricultural residues such as corn stover and wheat and rice straw are more suitable for biochemical conversion [6]. By contrast, forests are growing generally in mountainous areas with poor roads for transportation, and more and more forests are to be protected due to their irreplaceable role in capturing and fixing CO_2 to fight against global warming [7]. As a result, the supply of forest wastes is expected to be reduced in the future, and such a limited resource should be processed directly as materials for furniture-making, packing, and other applications with higher revenues.

Highlights

Agricultural residues are more suitable for biochemical conversion to produce bulk commodities preferentially through anaerobic fermentation with less energy consumption and lower contamination risk.

Steam explosion is applicable for industrial production, and biological pretreatment is not suitable for fermentation under pure culture conditions due to its slow rate and contamination risk.

Cellulases produced *in situ* and formulated with glucosidases are cost-effective for building up sugar platforms through simultaneous saccharification and co-fermentation (SSCF) and separate hydrolysis and co-fermentation (SHCF) to produce volatile and nonvolatile products, respectively.

In situ utilization of CO_2 can credit cellulosic ethanol, and both yield and titer must be improved substantially for butanol to be produced as an advanced biofuel, but microbial lipids produced aerobically with intensive energy consumption and low yield cannot be affordable as feedstock for biodiesel production.

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Key Figure

Biochemical conversion of agricultural residues to foster the circular economy integrated with the CO_2 life cycle

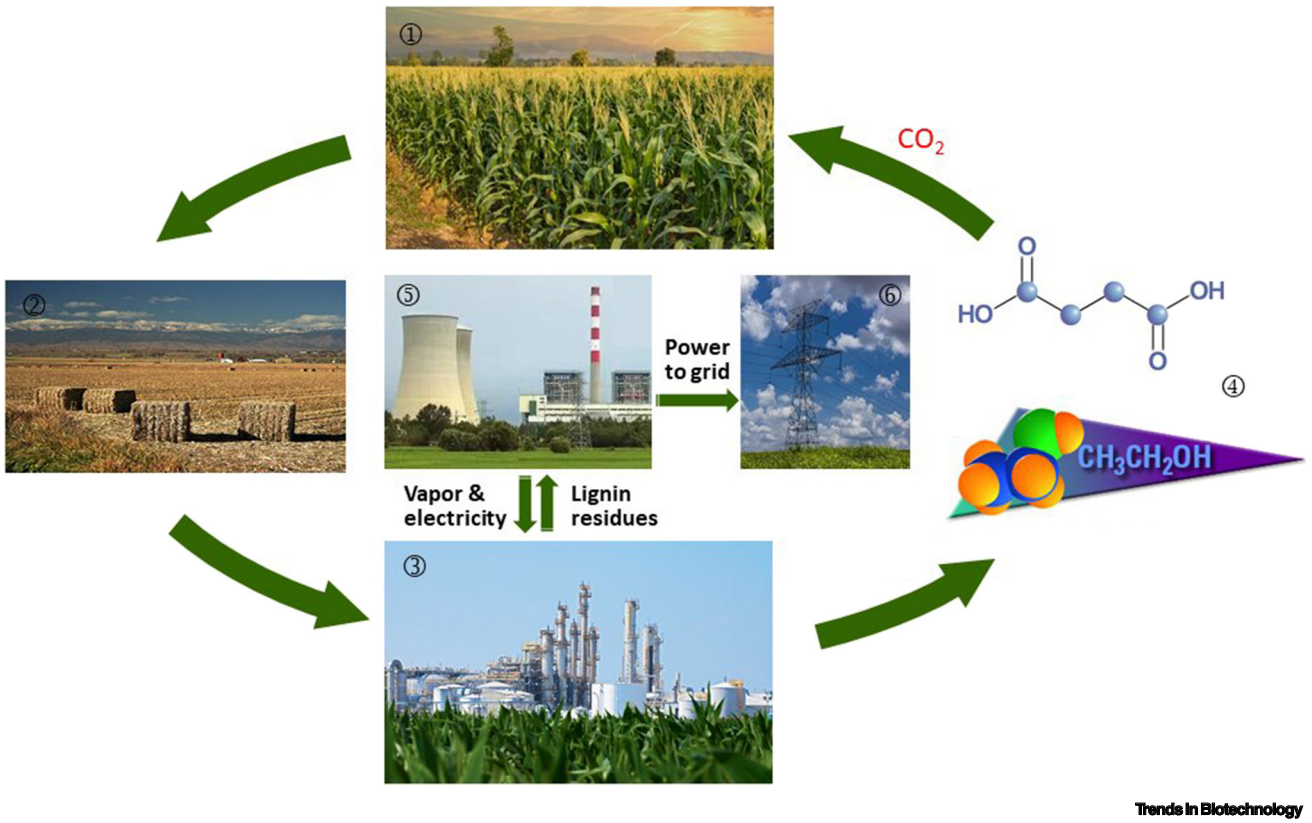

Figure 1. ① Agricultural residues such as corn stover are produced simultaneously with grain production. ② Logistics for harvesting, packing, and transportation is developed. ③ The feedstock is processed through biochemical conversion, in particular microbial fermentation to produce targeted products from sugars released through the enzymatic hydrolysis of cellulose and hemicelluloses. ④ Bio-based products are consumed, but released CO_2 can be fixed through plant photosynthesis. ⑤ Lignin residues with higher energy density are left for burning to generate steam and power, which can be used to drive the conversion and product recovery processes. ⑥ Power in surplus is exported to and transmitted through local grid for neutral, even negative, CO_2 emissions, compared with the production and consumption of fossil-derived products.

So far, almost no products derived from agricultural residues through biochemical conversion are economically competitive. Here, we analyze root reasons for this phenomenon, and propose strategies for guiding research correctly to save funding and labor as well as preventing risks with capital investment on production facilities.

Logistics and processing systems for agricultural residues

Agricultural residues are distributed in various farmlands and characterized by lower mass and **energy densities**, which not only result in a large volume for transportation with high cost [8], but also make their processing very sensitive to energy input, particularly for producing biofuels with a net energy gain. As a result, decentralized processing is needed for agricultural residues (Figure 2), and capacities for those plants can be optimized through balancing costs in the logistics and processing of the feedstock to maximize their revenues.

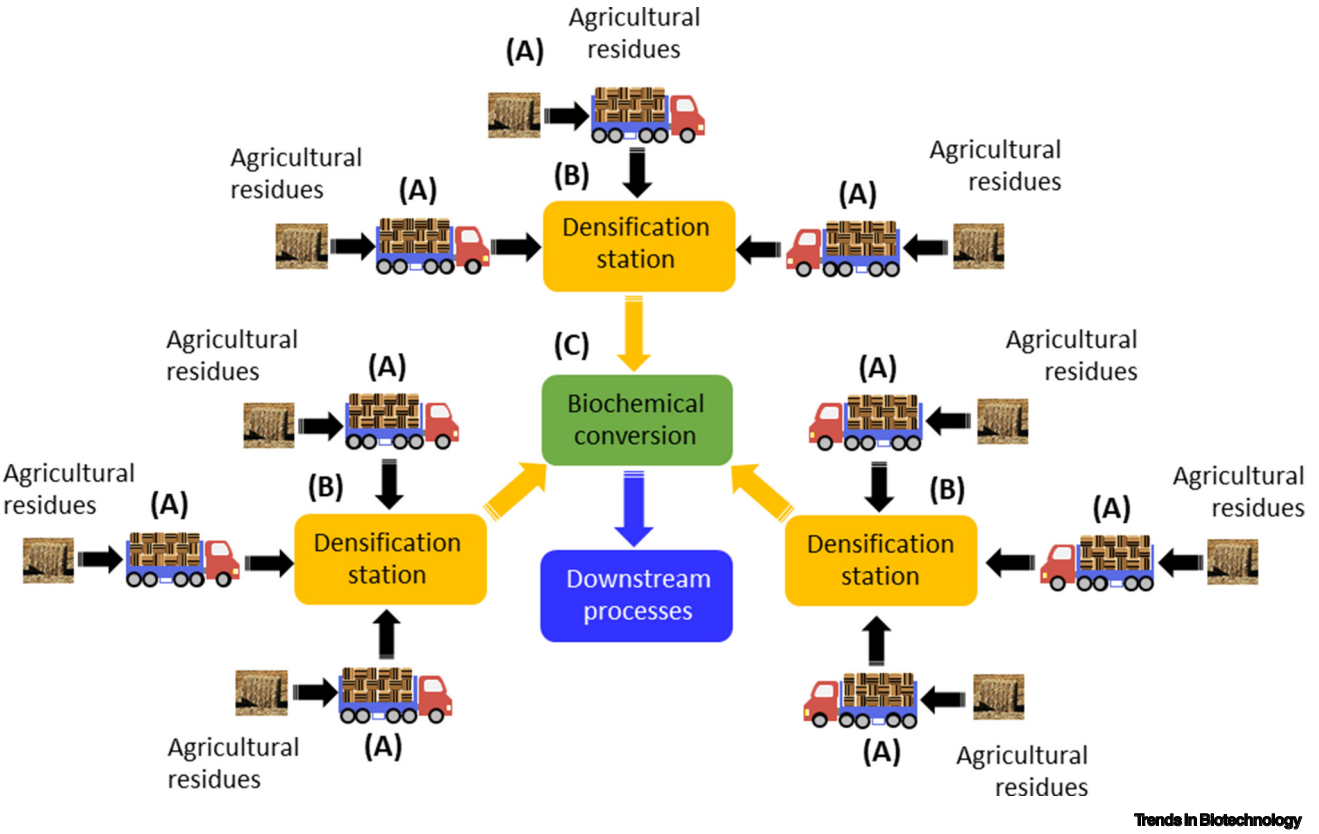

Figure 2. Decentralized processing systems for agricultural residues, in which agricultural residues are collected and packed for being transported with short distances (A), densified for being transported further with relatively long distances (B), and converted to targeted products through biochemical conversion (C).

The concept of biorefinery has been derived from crude oil refineries with processing capacities as large as tens of millions of tons per year to co-produce multiple products, since crude oil has been deposited centrally with large reserves for extraction at low cost, which can be transported conveniently through pipelines or tankers over long distances. However, decentralized systems for agricultural residues with maximal processing capacities at only tens of thousands of tons per year are too small to produce multiple products, particularly for producing bulk commodities. Therefore, we argue that biorefining is not suitable for lignocellulosic biomass, and lignocellulose biorefinery is conceptually wrong.

Applicable pretreatment technologies

The major components of agricultural residues are cellulose, hemicelluloses, and lignin, and evolution has developed these components into the lignin-carbohydrate complex (LCC) [9,10], which not only provides support for the growth and development of plants, but also endows them with recalcitrance to degradation in natural environments (Box 1) [11]. Therefore, pretreatment is needed to disrupt LCC to expose cellulose and hemicelluloses for enzymatic hydrolysis to release sugars.

Various technologies have been developed for lignocellulose pretreatment, but thermochemical processes are more practical for industrial applications [12,13]. A typical thermochemical process is steam explosion, using saturated steam to pressurize the feedstock for a few minutes, followed

by explosive decompression. Under high temperature and pressure conditions, acetyl groups in hemicelluloses are released to form acetic acid for autocatalysis to further enhance their hydrolysis, creating more pores with the LCC for the explosive decompression to disrupt its structure more effectively [14]. Studies indicate that dilute acid can be supplemented for steam explosion to be operated at relatively low temperature, reducing byproduct formation and sugar loss as well [15].

It should be noted that biological pretreatment is not suitable for the biochemical conversion of agricultural residues, although it has been intensively studied [16]. First, the rate of biological pretreatment is too slow to match with other unit operations. Second, various microorganisms used for biological pretreatment are contaminants in the subsequent fermentation operated under pure culture conditions, since sterilization is not feasible for lignocellulosic hydrolysate in industrial production due to intensive energy consumption and sugar loss, and the only process without this problem is producing biogas through anaerobic digestion. Third, although ligninolytic enzymes that are secreted by wood-rotting fungi for biological pretreatment can selectively attack lignin to disrupt the LCC [17], the lignin as a byproduct is ultimately lost. Last but not least, sugars released during the hydrolysis of hemicelluloses are consumed preferentially by microorganisms that are involved with biological pretreatment, which consequently compromises product yield from total sugars in the feedstock.

As emerging technologies, ionic liquids (ILs) and deep eutectic solvents (DESs) have been developed to selectively extract either cellulose or lignin [18,19], but they are too costly for pretreating agricultural residues at large scales. Agricultural residues contain more cellulose and less lignin, and lignin contains more energy for burning to generate steam and power to drive production. Thus, ILs or DESs might be specifically designed to extract a small amount of lignin in surplus from the energy balance perspective for valorization to credit the biochemical conversion of agricultural residues.

Cost-effective cellulases

Cellulases have been studied for more than 70 years, but their production cost is still too high for the biochemical conversion of agricultural residues [20]. Cellulases are a mixture of hydrolytic enzymes that hydrolyze cellulose synergistically [21]. While endo-β-glucanases randomly attack the β-(1,4)-glycosidic bond of cellulose to produce cellodextrin, exo-β-glucanases or cellobiohydrolases further hydrolyze the cellodextrin from reducing and nonreducing ends to release cellobiose for β-glucosidases to finally hydrolyze to glucose [22]. In addition, cellulase cocktails also contain accessory enzymes including hemicellulases with xylanases as major components and noncatalytic proteins such as swollenin to enhance the enzymatic hydrolysis of cellulose in lignocellulose [21].

Both bacteria and fungi can synthesize cellulases, but fungi, particularly *Trichoderma reesei*, are more preferred for cellulase production due to their excellent properties of protein synthesis at large quantities and extracellular secretion. So far, strains for cellulase production have been developed predominately from *T. reesei*, which are cultured aerobically and synthesize cellulases through induction [23–25].

Cellulose has been regarded as a natural inducer, but it is not a true inducer, since the polymer is insoluble and thus cannot be assimilated directly. Only when cellulose is hydrolyzed to oligosaccharides, particularly the disaccharide cellobiose, by basal cellulases that are secreted by *T. reesei* at extremely low levels, can cellulases be synthesized substantially [26]. Such an inducing mechanism significantly prolongs fermentation time for cellulase production. Moreover,

Glossary

Carbon atom economy: a terminology derived from the atom economy of a chemical reaction to specifically highlight the conversion efficiency of carbon atoms in the biochemical conversion of agricultural residues. However, due to the intrinsic nature of microbial fermentation with CO_2 released as a byproduct at mass quantity, it is further defined as the percentage of carbon atoms that is stored in targeted products compared with total carbon atoms in the feedstock. How to improve the carbon atom economy is a challenge for microbial fermentation under mild conditions with a substantial carbon loss.
Energy density: the amount of energy that is stored in raw materials and products based on per unit mass or volume, which is a key parameter for assessing their economic performance, particularly for producing energy products such as cellulosic ethanol as a biofuel. Compared with fossil resources, the energy density of agricultural residues is much lower, making them very sensitive to energy input associated with the feedstock logistics and processing for a net energy gain with targeted products.
Life cycle analysis (LCA): a methodology for assessing environmental impact associated with all stages for the production and consumption of designated products, from feedstock supply and processing to the delivery and consumption of products. In this article, LCA is specifically referred to tracing the carbon footprint of products that are derived through the biochemical conversion of agricultural residues with an objective to judge CO_2 emissions, the biggest culprit for global warming and climate change, with each production unit, which can be performed through developing models based on mass and energy balance for all operation units and the whole processing system as well.
Lignocellulose: the biomass synthesized by plants through photosynthesis as a major component of the cell wall, which is composed predominately of cellulose, hemicelluloses, and lignin. Natural evolution has made these components entangled together delicately and tightly, with crystalline cellulose as the core, not only providing strength to support the growth and development of plants, but also protecting them from degradation in natural environments. Such a structure has made the cellulose component not easily to be accessed and hydrolyzed

Box 1. Recalcitrance of lignocellulose to enzymatic degradation

Cellulose, hemicelluloses, and lignin are synthesized by plants through photosynthesis, and deposited within the cell wall, predominately within the secondary cell wall, where they are assembled as LCC (Figure I) [30].

As a linear polymer, cellulose is synthesized from glucose through β-1,4-glycosidic bonds and characterized by a crystalline morphology: microfibers are bound together through strong intra- and intermolecular hydrogen bonds as well as van der Waals forces for strength, which act as a scaffold for LCC. Hemicelluloses are heterogenous polysaccharides composed of xyloglucan, xylan, mannan, glucomannan, and glucan. Unlike cellulose, hemicelluloses are amorphous, and thus can act as glue to further enhance the strength of LCC. Amorphous hemicelluloses can be hydrolyzed easily during pretreatment to release both hexose and pentose sugars. Lignin is a phenolic polymer that also imparts strength and rigidity for LCC through covalent links with hemicelluloses and cellulose, particularly for woody plants such as trees that in general can grow much higher with large canopies.

With such a complex structure, LCC is recalcitrant in nature to enzymatic degradation. Therefore, the first step for the biochemical conversion of agricultural residues is to disrupt the LCC structure through pretreatment so that cellulose, hemicelluloses, and lignin can be separated effectively for cellulases to access and hydrolyze the cellulose component more efficiently. Pretreatment is energy intensive, particularly when a thermochemical process is employed. Moreover, various byproducts such as acetic acid, furfural, hydroxymethylfurfural, and even phenolic compounds are generated during the pretreatment, which are inhibitory for microbial fermentation thereafter [42].

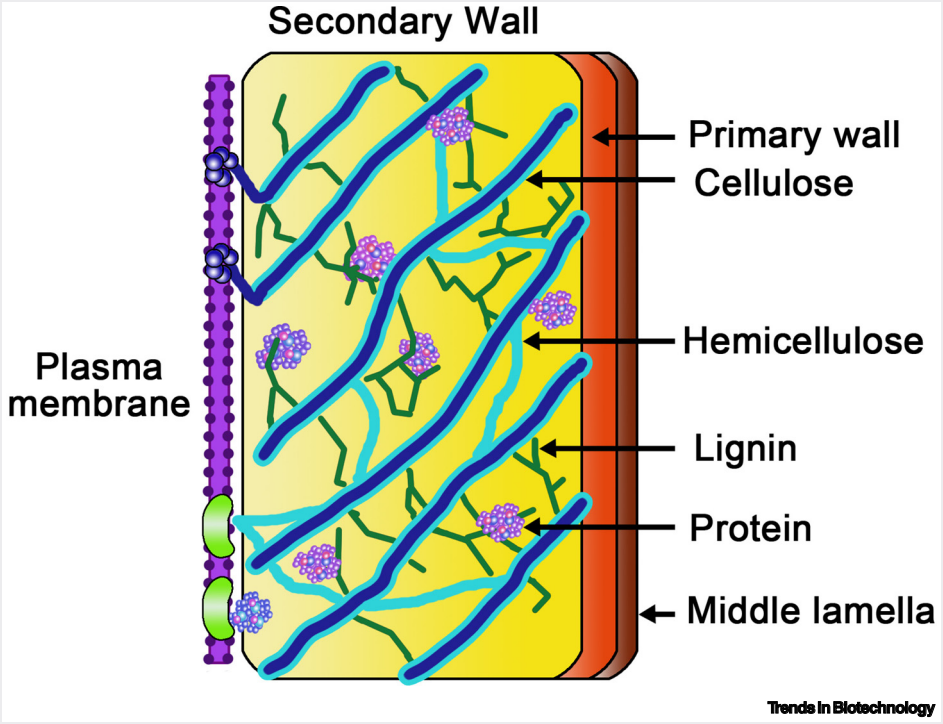

Figure I. Schematic diagram for LCC in lignocellulose, adapted from [30].

by cellulases to release glucose as feedstock for microbial fermentation to produce targeted products.

Neutral or negative carbon emissions: the root reason for global warming is the emissions of greenhouse gases into the atmosphere, with CO_2 as the major component due to burning fossil fuels at mass quantities. Thus, for any processes, when all CO_2 released is captured completely without net emissions into the atmosphere, they are carbon-neutral. Furthermore, when CO_2 already accumulated in the atmosphere can be captured and fixed by a process, it is carbon-negative. Models are needed for tracing the footprint of CO_2 to assess whether a process is carbon-neutral or carbon-negative.

microcrystalline cellulose is most effective in inducing cellulase production among cellulose-based materials, but it is too costly for producing cellulases to hydrolyze the cellulose component in agricultural residues completely to glucose as feedstock for microbial fermentation.

β-glucosidases hydrolyze cellobiose and thus compromise its ability to induce cellulase production. Natural evolution has made *T. reesei* synthesize insufficient β-glucosidases, a disadvantage for hydrolyzing cellulose completely to release glucose. Engineering *T. reesei* with the

overproduction of β-glucosidases has been studied to address this issue [27], but more β-glucosidases hydrolyze cellobiose more quickly, further compromising its induction of cellulase production. At present, β-glucosidases are produced at low cost in industry, which can be supplemented into raw cellulases produced by *T. reesei* to enhance their effectiveness in hydrolyzing cellobiose to release more glucose [28]. We argue that this strategy is more economically competitive than producing complete cellulases by engineering *T. reesei* with the overexpression of β-glucosidases.

Cellulases have been produced at low cost through solid state fermentation for a long time [20]. However, they are not suitable for hydrolyzing cellulose to release glucose as feedstock for microbial fermentation to be operated under pure culture conditions due to poor sanitary environments with the process and their contamination risk to the enzymatic product, needless to say difficulties in scaling up such a process to produce cellulases at large quantities. As a result, submerged fermentation is required, and fed-batch operation can be further developed to improve cellulase production (Box 2).

Microcrystalline cellulose and other cellulose-based materials are not suitable for feeding to be controlled properly under sterilized conditions, so a soluble inducer with low cost is specifically needed for submerged fermentation to be operated in a fed-batch mode [29]. On the other hand, when cellulases are produced by *T. reesei* through submerged fermentation, non-Newtonian fluid properties can develop quickly with the fermentation broth as mycelia grow, which are characterized by high viscosity, making mixing and aeration very energy intensive. Therefore, improving cellulase titers and shortening the fermentation time to increase productivity remain a priority for saving energy consumption on cellulase production.

Box 2. Submerged fermentation for cellulase production

Submerged fermentation, also termed as liquid state or deep-tank fermentation, was developed to address challenges for aerobic culture performed at early stage through solid state fermentation using many shallow dishes, since this kind of operation is very labor intensive with low productivity, a bottleneck for producing bulk commodities such as penicillin when it was discovered with a big demand for treating bacterial infections.

Submerged fermentation employs large tanks, which are mixed by impellers and aerated properly through controlling dissolved oxygen that can be detected online very conveniently for oxygen to be transported more efficiently from air bubbles into the liquid medium. After it was successfully developed in the mass production of penicillin, submerged fermentation has been applied quickly to produce not only other antibiotics, but also many different fermentation products such as amino acids, vitamins, and enzymes. Compared with solid state fermentation, submerged fermentation is more productive and can be operated more reliably with lower risk of contamination to guarantee the quality of products, so-called quality control for industrial production.

When cellulases are produced through submerged fermentation, either batch or fed-batch process can be employed, but fed-batch is more productive. However, when microcrystalline cellulose and other cellulose-based materials are used as 'inducer', a batch process is usually employed, since they are not suitable for developing a fed-batch process with feeding rate to be controlled properly under sterilized conditions. As a result, soluble inducer is a necessity for developing a fed-batch fermentation process to produce cellulases more efficiently.

Soluble inducer can be synthesized from glucose or syrup through transglycosylation reaction, which can be catalyzed either by acids or enzymes to synthesize β-disaccharides such as sophorose and cellobiose to induce cellulase production. Since the catalytic activity of transglycosylation is predominated by the hydrolysis of glycosidic bonds, much more glucose is needed for the synthetic reaction to be carried out forward, and thus a substantial amount of unconverted glucose is left, which can be used as carbon source for mycelial growth, but glucose concentration must be maintained at relatively lower levels through controlling the feeding rate to prevent its feedback inhibition of the production of β-glucosidases, one of the major components in the cellulases [29].

Furthermore, *in situ* production of cellulases allows raw enzymes to be used directly without the need for downstream processing, including concentration of the raw enzyme and supplementation of preservatives to extend the shelf life for the enzyme product to be transported over long distances and stored properly. This strategy can save cellulase production cost, making the enzyme more economically competitive.

Sugar platforms

As a glucan, cellulose can be hydrolyzed completely to glucose, but hemicelluloses are heterogeneous polysaccharides that are hydrolyzed to a mixture of pentose (C5) and hexose (C6) sugars [30]. Thus, lignocellulosic hydrolysate contains both C5 and C6 sugars. Most strains with good production performance cannot assimilate C5 sugars efficiently, but they can be engineered with pentose metabolism for co-fermentation of C5 and C6 sugars [31]. Depending on arrangements for enzymatic hydrolysis of the cellulose component and co-fermentation of the C5 and C6 sugars, simultaneous saccharification and co-fermentation (SSCF) and separate hydrolysis and co-fermentation (SHCF) have been developed, particularly for cellulosic ethanol production.

For SSCF, pretreated feedstock is pre-saccharified properly under optimal conditions to release sugar for inoculation, and then extensive cellulose hydrolysis is performed under fermentation conditions. Because sugar released during enzymatic hydrolysis is fermented immediately without accumulation, its end product inhibition of cellulase (β-glucosidase) activity is alleviated, and microbial contamination can also be prevented. However, it is not possible to operate both the enzymatic hydrolysis and fermentation under optimal conditions. For example, optimal temperature for cellulases to hydrolyze cellulose is around 50°C, but ethanol fermentation with the brewing yeast *Saccharomyces cerevisiae* is operated below 35°C, so the enzymatic hydrolysis must be carried out at the lower temperature, which substantially compromises its reaction rate [30].

Cellulose is very hygroscopic, presenting a challenge for developing good fluidity in medium for mixing, which, together with its slow hydrolysis by cellulases under lower temperature conditions, makes SSCF difficult to be operated at a high loading of pretreated feedstock for more sugar to be released and fermented to produce products with high titers, a disadvantage for product recovery [30]. Moreover, lignin cannot be separated before fermentation, so only volatile products such as ethanol that can be recovered easily through distillation are suitable for production through SSCF. When nonvolatile products need to be recovered through crystallization and other unit operations, lignin residues must be removed after fermentation, which inevitably results in a significant loss of products, making SSCF not economically competitive.

For SHCF, the enzymatic hydrolysis and fermentation are performed separately so that both processes can be optimized. Once cellulose is hydrolyzed completely by cellulases under optimal conditions, lignin can be separated conveniently through filtration, and the hydrolysate without solid residues is suitable for fermentation to produce various products [30]. Lignin cake contains some sugar, which can be recovered partly by washing, but compared with the loss of nonvolatile products in SSCF, the sugar loss is less significant. Challenges with SHCF are sugar inhibition of cellulase activities (β-glucosidases) and the risk of microbial contamination of the hydrolysate with more sugar as well as fermentation thereafter, since sterilization is too costly with energy consumption and also sugar loss due to byproduct formation.

Presently, SSCF is preferred for cellulosic ethanol production by most researchers and companies, but we believe that SHCF is more promising. Not only can ethanol fermentation be performed with less energy consumption on mixing and concentrated hydrolysate for higher

product titer, but also lignin residues can be separated before ethanol fermentation with high quality and purity for valorization to credit cellulosic ethanol production.

Utilization of sugars in the hydrolysate

Three strategies have been developed for utilizing sugars in lignocellulosic hydrolysate: (i) fermenting only C6 sugars, with C5 sugars left to be recovered as byproducts [32]; (ii) converting C6 and C5 sugars to different products such as ethanol and xylitol, respectively [33]; (iii) engineering strains with pentose metabolism to ferment both C6 and C5 sugars with same products produced [31]. Here, we argue that only strategy 3 is economically feasible, since the cost for recovering C5 sugars after C6 sugars are fermented with strategy 1 is too high, and converting C6 and C5 sugars to different products at low titers with strategy 2 makes all products economically uncompetitive.

Box 3. Engineering yeast for cellulosic ethanol production

Engineering microbial strains with pentose utilization has been performed predominately on *S. cerevisiae* for cellulosic ethanol production [30], with two pathways developed (Figure I): (i) xylose assimilation in fungi catalyzed by NADPH-linked xylose reductase and NAD-linked xylitol dehydrogenase; (ii) xylose utilization in bacteria catalyzed by xylose isomerase. When genes encoding these key enzymes are heterologous expressed in *S. cerevisiae*, xylose is converted into xylulose for phosphorylation by xylulose kinase (XK) to xylulose-5-phosphate, which can enter the nonoxidative pentose phosphate pathway for further metabolism. Therefore, the overexpression of XK is required for enhancing metabolic flux from xylulose to xylulose-5-phosphate for both strategies.

It is clear that the oxidoreductase pathway raises an issue with cofactor imbalance, which consequently results in xylitol accumulation. Although evolutionary engineering has been developed by the Nobel laureate Prof. Arnold for engineering the NAD/NADP cofactor preference, it is labor intensive with the screening of mutants, and also challenged with reverse mutation [38]. However, the heterologous expression of XI from bacteria in *S. cerevisiae* can substantially compromise its catalytic activity for xylose assimilation, although there is no concern on cofactor imbalance [39]. At present, significant progress has been reported for the two strategies in engineering *S. cerevisiae* with xylose metabolism to produce cellulosic ethanol, and engineered strains are being tested in the pilot- and demo-scale production.

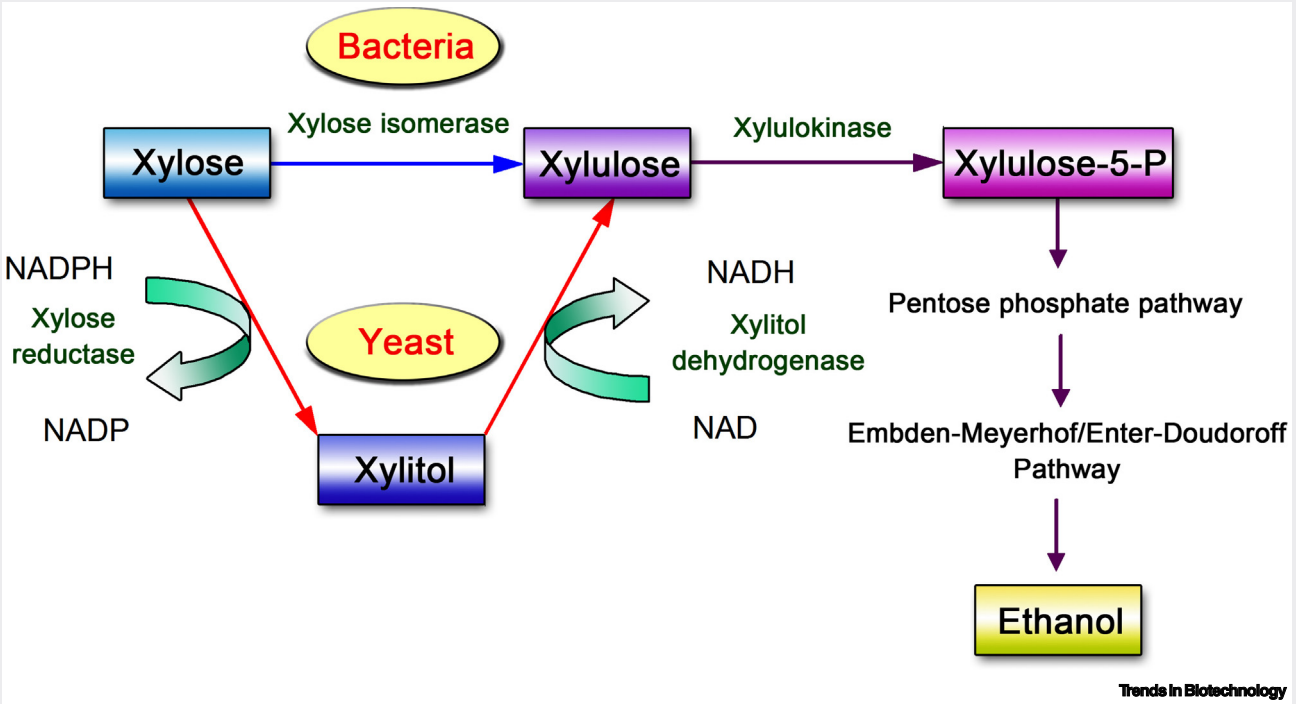

Figure I. Strategies for engineering *Saccharomyces cerevisiae* with xylose metabolism to produce cellulosic ethanol, adapted from [30].

Co-fermentation of C5 and C6 sugars

Both C5 and C6 sugars are present in lignocellulosic hydrolysate, but almost all microbial strains currently used for industrial production utilize C6 sugars only.

Engineering microbial strains with pentose metabolism has been focused predominately on *S. cerevisiae* to produce cellulosic ethanol [30], although research has also been done in engineering microbial cell factories to produce other products [34–37]. Two strategies have been developed so far: engineering host strains with the expression of either oxidoreductases or isomerases (Box 3) [38,39]. Yeast strains engineered with xylose metabolism have been tested to produce cellulosic ethanol at a pilot plant by COFCO in China and in demo production by Clariant in Romania [40,41].

Enhancement of stress tolerance to inhibitors

During pretreatment, particularly with thermochemical pretreatment such as steam explosion, toxic byproducts are inevitably generated, which consequently inhibit fermentation with more sugars left, leading to a so-called stuck fermentation [42]. Although various technologies have been developed for detoxification [43–45], none of them is suitable for industrial production due to high cost.

Great effort has been dedicated to engineering microbial strains with tolerance to inhibitors in lignocellulosic hydrolysate through random approach and rational design as well [46,47], but most work has targeted individual inhibitors such as acetic acid, furfural, hydroxymethylfurfural, and phenolic compound [48–50]. However, these inhibitors co-exist in the hydrolysate, so strains need be engineered with general stress response to tolerate all inhibitors effectively, but progress in this regard is very slow. A promising strategy is to perform in-depth bioinformatics analysis on mutants with tolerance to individual inhibitors for data mining to identify new pathways so that strains can be engineered with tolerance to multiple inhibitors through rational design [51].

Targeted products

While cellulosic ethanol is produced as the major product, xylose has been converted to produce xylitol and xylooligosaccharides as a sweetener and prebiotics through two-stage fermentation processes [32,52]. Terpenes have also been produced from lignocellulosic hydrolysate [53]. However, lignocellulosic hydrolysate contains various impurities, even toxic byproducts, which are not suitable for producing food ingredients, fine chemicals, and drug intermediates due to concerns with safety and high cost for product purification. On the other hand, agricultural residues are available at large quantities, and thus bulk commodities for non-food use, particularly biofuels and bio-based chemicals, can make sense. Furthermore, biofuels and bio-based chemicals that can be produced through anaerobic fermentation with less energy consumption are preferred for being produced from agricultural residues. Another advantage with anaerobic fermentation is lower risk for contamination, since most contaminants grow under aerobic conditions, so anaerobic fermentation can be operated without the necessity for sterilization, a prerequisite for producing bulk commodities at large quantities with marginal revenues.

Cellulosic ethanol is one of the most important biofuels, which has been intensively studied [30]. Although seed culture in small tanks is aerated properly for yeast propagation, ethanol fermentation in large tanks is operated under anaerobic conditions for high ethanol yield and controlling microbial contamination effectively. Currently, Clariant is testing its demo plant for cellulosic ethanol production [41], but it is still challenged by profitable operation, and DuPont's tragedy may

be waiting ahead for it: DuPont's cellulosic ethanol plant suffered a major loss and had to be refitted for producing biogas.

The reason for such a poor economic performance of cellulosic ethanol production is twofold. On the one hand, cellulases are more expensive than amylase and glucoamylase for fuel ethanol production from grains. On the other hand, when ethanol is produced from grains such as corn, protein-enriched residues are co-produced at large quantities as animal feed for credit, but no such an advantage is available for cellulosic ethanol production.

From the viewpoint of bioreaction stoichiometry, one glucose is fermented into two ethanol with two CO_2 released as a major byproduct, for a theoretical ethanol yield of 0.511 only. An observed ethanol yield of 0.459-0.470, equivalent to 90-92% of the theoretical maximum, can be achieved in both laboratory research and industrial production due to sugar consumption for yeast growth and the formation of other byproducts. This intrinsic disadvantage affects cellulosic ethanol more than fuel ethanol produced from grains with animal feed as a major byproduct.

While intensive studies have been devoted to developing energy-saving pretreatment technologies and robust cellulases as well as microbial strains for co-fermentation of C5 and C6 sugars, CO_2 released during ethanol fermentation needs to be utilized for credit. Succinic acid is a platform chemical [54], and glucose (C6) is split into two pyruvate (C3) through glycolysis with two CO_2 (C1) incorporated to produce such a C4 compound. Therefore, coupling cellulosic ethanol fermentation with succinic acid production can utilize CO_2 *in situ* to improve the **carbon atom economy** for the biochemical conversion of agricultural residues [55,56].

A major concern with such a strategy is the much larger volume for cellulosic ethanol as a biofuel and relatively smaller market for succinic acid as a bio-based chemical, but decentralized systems for the biochemical conversion of agricultural residues present an advantage for cellulosic ethanol production to be coupled with the fermentative production of succinic acid based on *in situ* utilization of CO_2, since the production capacity of a single cellulosic ethanol plant is small. Moreover, other processes with CO_2 as feedstock such as microalgae culture can also be coupled with cellulosic ethanol production for the same purpose.

However, the equilibrium solubility of CO_2 (C^*) in fermentation broth is too low for developing a concentration difference with dissolved CO_2 (C) to drive mass transfer, which is the first step for CO_2 to be utilized. Moreover, microbes have evolved intrinsic pathways for CO_2 production through decarboxylization, which must be deactivated to save carbon loss from pyruvate so that more sugar can be directed to product formation. Furthermore, key enzymes with intracellular CO_2 fixation need to be overexpressed functionally so that dissolved CO_2 can be assimilated timely to maintain the driving force (C^*-C) for the mass transfer. Further progress in bioprocess engineering principles and biotechnological innovations should address these challenges.

Compared with fuel ethanol, butanol presents many advantages, such as higher energy density, lower volatility, less hygroscopy, and better compatibility with existing infrastructure for fuel transport and storage. Therefore, it has been described as an advanced biofuel [57]. Butanol is produced traditionally through acetone-butanol-ethanol (ABE) fermentation with *Clostridium* spp. under obligate anaerobic conditions, and historically it was the second largest fermentation product after ethanol, but all ABE fermentation plants were closed in the 1990s, due to their poor competitiveness with petrochemical synthesis routes [58]. Motivated by its advantages as a biofuel,

fermentative production of butanol has garnered global interest again, but several challenges must be addressed adequately [59].

Theoretically, one glucose is metabolized into one butanol with two CO_2 released, making the theoretical yield for butanol production as low as 0.411 g/g. For ABE fermentation, acetone and ethanol are produced as major co-products, which further compromises butanol yield [60]. In addition, butanol is extremely toxic to cells, which consequently limits its titer for fermentative production. At present, upper limit for butanol titer is around 20 g/l [61], which not only makes its recovery through distillation very energy intensive but also discharges a large amount of stillage to be treated with even more energy consumption. Only when both yield and titer are improved substantially through engineering strains can the dream of butanol as an advanced biofuel come true.

Some biofuels are not suitable for being produced from agricultural residues through biochemical conversion, and microbial lipids are among them [62]. The theoretical yield of microbial lipids from sugar is only 0.32 g/g [63], so much more sugar is needed for their production. In addition, a high C/N ratio is required for limiting cell growth to direct more sugar to the intracellular accumulation of lipids [64], which substantially prolongs fermentation time. Moreover, lipid fermentation is aerobic with intensive energy consumption, and the long fermentation time further worsens the scenario: more energy is input for producing lipids than energy output with the product. Contamination control for lipid fermentation under aerobic conditions is also energy intensive, since sterilization is required for medium and facilities as well. Therefore, microbial lipids cannot be affordable as feedstocks for biodiesel production.

Concluding remarks

Decentralized systems are needed for the biochemical conversion of agricultural residues to balance costs with their logistics and processing. Among different pretreatment technologies, steam explosion with dilute acid is more practical than others for industrial applications. Compared with SSCF, SSHF presents more advantages for hydrolyzing pretreated agricultural residues to build up sugar platforms, but both pentose and hexose sugars need to be co-fermented to produce same products for developing economically viable processes.

Food-related products, fine chemicals, and drug intermediates are not suitable for being produced from lignocellulosic hydrolysate with various impurities, even toxic byproducts, due to safety concerns, the small market, and demand for high purity. Bulk commodities, in particular biofuels that can be produced through anaerobic fermentation with less energy consumption and lower contamination risk, are preferred.

Cellulosic ethanol is the best choice for being produced from agricultural residues. To make this biofuel product economically competitive, CO_2 released during ethanol fermentation needs to be utilized *in situ* through coupling with other processes such as the fermentative production of succinic acid to improve the carbon atom economy. Unless both titer and yield are improved substantially, butanol cannot be produced as an advanced biofuel from agricultural residues (see Outstanding questions). Microbial lipids produced from lignocellulosic hydrolysate through aerobic fermentation with low yield and intensive energy consumption cannot be affordable as feedstock for biodiesel production.

Outstanding questions

How mass transfer for CO_2 can be enhanced from the viewpoint of bioprocess engineering, since its low equilibrium solubility in fermentation broth substantially compromises the development of an effective driving force for such a process?

Will the intracellular generation of CO_2 through the complete oxidation of pyruvate be deactivated without a significant impact on the metabolic network such as redox balance for more sugar to be directed to the formation of targeted products?

How key enzymes for the intracellular assimilation of CO_2 can be overexpressed effectively for dissolved CO_2 to be assimilated timely so that the driving force can be maximized to facilitate the mass transfer process?

How pathways for producing acetone and ethanol can be knocked out from solvent-producing strains without disrupting their metabolic network so that more sugar can be directed to butanol production with improved yield?

Will nonsolvent producing species be engineered with heterologous pathways to produce butanol substantially for industrial applications rather than at a token amount to highlight the progress in synthetic biology?

How butanol-producing strains can be further engineered with tolerance to produce butanol at high titers for industrial applications, taking advantage of an in-depth understanding on their intracellular response to butanol toxicity?

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Declaration of interests

The authors declare no conflicts of interest.

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