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Production of butanol from lignocellulosic biomass: recent advances, challenges, and prospects

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Due to energy and environmental concerns, biobutanol is gaining increasing attention as an alternative renewable fuel owing to its desirable fuel properties. Biobutanol production from lignocellulosic biomass through acetone–butanol–ethanol (ABE) fermentation has gained much interest globally due to its sustainable supply and non-competitiveness with food, but large-scale fermentative production suffers from low product titres and poor selectivity. This review presents recent developments in lignocellulosic butanol production, including pretreatment and hydrolysis of hemicellulose and cellulose during ABE fermentation. Challenges are discussed, including low concentrations of fermentation sugars, inhibitors, detoxification, and carbon catabolite repression. Some key process improvements are also summarised to guide further research and development towards more profitable and commercially viable butanol fermentation.

1. Introduction

The dual pressures of international energy security and climate change are encouraging the development of biofuels.[1] Butanol, a useful biofuel and important platform chemical, has attracted great interest due to its excellent fuel properties (high energy density, excellent mixability, low volatility and corrosiveness).[2,3] However, high substrate cost, solvent toxicity, low butanol titre and poor productivity make butanol fermentation expensive for large-scale practical applications.[4] Traditionally, butanol has been produced *via* acetone–butanol–ethanol (ABE) fermentation from sugars or starch as substrates, but the cost of substrates (~60% of the total cost of ABE fermentation) significantly decreased the competitiveness of ABE fermentation, so much so that biobutanol produced by Clostridium cannot compete with petrochemical butanol.[5,6]

Selection of feasible substrates plays an important role in butanol production. At present, four generations of feedstocks have contributed to produce butanol, and their benefits and limitations are listed in Table 1.[7] Among them, lignocellulosic biomass has been evaluated as cheap feedstock for biobutanol production, including rice straw,[8] corncobs,[9] wheat straw,[10] barley straw,[11] sorghum bagasse,[12] and different types of wood such as pine[13] and elm.[14,15] Lignocellulosic biomass is the most abundant, sustainable, and cost-effective form of biomass, hence valorisation to biofuel could provide notable benefits, including (i) CO_2 fixation from the atmosphere, (ii) reduced air pollution from incineration, and (iii) enhanced energy security for oil-importing countries.[16,17] More importantly, utilising lignocellulosic materials could also significantly reduce environmental pollution caused by accumulation of agricultural forestry wastes,[18–20] hence it represents an alternative strategy for large-scale biobutanol production.

Recently, several articles have been published that summarise general aspects of lignocellulosic butanol fermentation and focus on the selection of cheap feedstocks, optimisation of cost-efficient processing methods, and the development of improved microbial strains.[21–24] Herein, we review advances in the pretreatment and hydrolysis of lignocellulosic biomass into fermentable substrates, focusing on recent research progress in overcoming the factors limiting lignocellulosic butanol fermentation. Additionally, improvement of lignocellulosic butanol fermentation by metabolic engineering and process integration strategies are also discusses. This review aims to provide guidance for improving the overall performance of lignocellulosic butanol fermentation.

2. Overall process for biobutanol production from lignocellulosic biomass

2.1 Microorganisms and metabolic pathways related to lignocellulosic butanol fermentation

Clostridia are obligate anaerobes and spore-forming bacteria with a complex life cycle, among which *C. acetobutylicum*, *C.*

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Table 1 Benefits and limitations of biomass[21,22,25]

Feedstock	Benefits	Limitations
First-generation feedstock		
Starch and sucrose feedstocks	High butanol yield	Occupies potential crop land
	Sufficient fermentable substrates through simple pretreatment processes	Competes with food supply
		Significantly increases the overall cost of fermentation
Second-generation feedstock		
Lignocellulose biomass	Cost-effective, huge carbon resources	Difficult to achieve sufficient fermentable sugars from complex and recalcitrant biomass
	No competition with food supply	Requires complex pretreatment and detoxification processes
Third-generation feedstock		
Algal biomass	No competition with cultivation land and food supply	Significantly increases the cost of downstream processes due to low production and productivity of butanol
	Fewer or no lignin and fermentation inhibitors	Difficult to obtain sufficient fermentable sugars
Fourth-generation feedstock		
Syngas	Increased CO_2 capture ability	Still at its infancy as a technology
	No complex pretreatment process needed	Several unknown key parameters that limit butanol production
	Directly utilises clostridia with a high production rate	Poor mass transfer from gas to liquid

beijerincki, *C. saccharoperbutylacetonicum* and *C. saccharobutylicum* are the most well-known ABE fermenters, sharing a typical life cycle and metabolic pathways for butanol production.[26,27] Following the publication of the genomes of several solventogenic clostridia, such as *C. acetobutylicum* ATCC 824,[28] *C. acetobutylicum* DSM 1731[29] and *C. acetobutylicum* EA2018.[30] Clostridia are among the most important industrial production strains for biobutanol, and the main advantage is the availability of a wide range of sugar sources, including pentose (xylose and arabinose), hexose (glucose, fructose, mannose and galactose), disaccharides (lactose, sucrose, maltose and cellobiose) and polysaccharides (starch). Lignocellulosic biomass is composed of various monosaccharides, and is the most abundant renewable resource on earth. Producing biobutanol from wood cellulosic biomass with fermentable sugars an economical and effective strategy.

In ABE industrial fermentation processes, bacterial strains use sugar substrates to fuel their rapid growth, and produce acetic acid, butyric acid and other organic acids, while generating ATP (*e.g.*, the ATP yield of butyrate fermentation is 3 ATP per glucose; this is more than has been reported for fermentations to date[31]). ATP is the essential energy source needed for bacterial growth and fermentation, and this stage is the acid production stage. During acid production, organic acids such as acetic acid and butyric acid rapidly accumulate, and dissociated acids can adhere to the surface of the cell membrane as an unpaired ion, triggering a collapse of the pH gradient of the cell membrane.[32] However, solvent production increases only when butyric acid accumulates and pH decreases, due to the essential role of undissociated butyric acid on the induction of solvent production in *C. acetobutylicum*.[33,34] Subsequently, bacteria consume ATP to maintain the pH gradient across the membrane, and synthesise acetone, ethanol and butanol, during the alcohol production stage. This stage is characterised by solvent toxicity, the most serious of which is butanol toxicity,[35] since the fermentation concentration is in the range of the inhibitory concentration for this solvent. To produce butanol efficiently, microbes must possess strong physiological robustness and fitness, coupled with strong metabolic capabilities, to enable them to work efficiently during bioprocesses. In some bacteria, the intracellular pH declines as a function of extracellular pH, and these are more resistant to the toxic effects of fermentation acids.[34] In addition, higher ATP levels can divert carbon flux toward butanol formation.[36]

2.2 Substrates for lignocellulosic butanol

Lignocellulosic biomass, mainly comprising of cellulose, hemicellulose and lignin, are the most abundant, sustainable, and cost-effective types of biomass for butanol production.[39,40] Among them, cellulose is an unbranched homopolysaccharide consisting of D-glucopyranosyl units that can be broken down to hexoses (C-6 sugars), and hemicellulose are branched heteropolysaccharides consisting of both hexose and pentose sugar residues.[41,42] Lignin consists of phenylpropane units linked through different types of interunit linkages, playing a cementing role to connect cells and increasing the mechanical strength properties, which makes lignocellulosic resistant against biodegradation by microorganisms.[43] Therefore, removal of lignin from lignocellulose biomass before hydrolysis of cellulose and hemicellulose is highly desirable for successful lignocellulosic ABE fermentation.[44] Due to the physiological properties of solventogenic clostridia, a typical lignocellulosic butanol fermentation process can be summarised in four major steps: pretreatment (breaking down the complex structure of

lignocellulosic biomass), hydrolysis (providing fermentable monomers), fermentation and distillation[8,45] (Fig. 1).

2.2.1 Pretreatment processes for lignocellulosic biomass. Due to the recalcitrance of lignocellulosic biomass,[46] pretreatment is a crucial step that breaks down the complex structure of lignocellulosic materials and increases the surface area and porosity, thereby enhancing saccharification to release fermentable sugars (Fig. 2).[47,48] Primarily, pretreatment processes can be classified into four general categories (reviewed in detail by:[21,49] (1) physical or mechanical treatment, including milling/grinding, chipping, shredding, extrusion, microwave and ultra-sonication, which effectively breaks down the physical structure of lignocellulosic biomass;[50] (2) chemical treatment, including application of concentrated/dilute acid, alkali, ozonolysis, organic solvents and ionic liquids;[51,52] (3) physicochemical treatment, involving the use of steam explosion, ammonium fiber explosion, CO_2 explosion and hot water, to effectively release monosaccharides and oligosaccharides;[53,54] (4) biological treatments, using microorganisms (such as fungi, bacteria, or consortia of fungi and bacteria) to degrade lignin, hemicellulose and cellulose, potentially high selectivity of enzymatic hydrolysis,[55] but the effectiveness is influenced by substrate concentration, enzyme loading, pH and temperature.[56]

There are several advantages and disadvantages for different pretreatment processes, limiting the economics and feasibility of lignocellulosic butanol. For example, dilute acid and alkali pretreatments are widely used to prepare digestible substrates, but a neutralization step is required to maintain pH before enzymatic hydrolysis. More importantly, salts formed in the neutralisation process may also influence the efficiency of enzymatic hydrolysis and ABE fermentation.[57] Recently, deep eutectic solvents have been applied for biomass pre-treatment due to their facile preparation with 100% atom efficiency, low melting point, easy biocompatibility, cost-effectiveness, low viscosity, and environmental friendliness.[58,59] In a short time (90 min) and with low energy consumption (120 °C) and a deep eutectic solvent (DES) pretreatment process, total sugars were increased to 37.94 g L^{-1} (30.59 g L^{-1} glucose, 7.35 g L^{-1} xylose) at a low enzyme loading of 7.5 g of filter paper units (FPU) per L of pretreated corn stover (CS), and 13.65 g L^{-1} total ABE was achieved with high productivity (0.68 g L^{-1} h^{-1}) and yield (0.38 g g^{-1}) from DES-pretreated CS hydrolysate without any detoxification and sterilisation.[60] In addition, combinations of pretreatment method have been also employed and shown to exert synergic effects on the quality of resulting hydrolysate, including a high yield of monomeric sugars and low levels of inhibitors.[61,62] For example, after pretreatment with optimised microwave-assisted alkali pretreatment followed by acid hydrolysis, 76.3% lignin removal, 21.1% hemicellulose and 71.9% cellulose were obtained at 640 W microwave power, 2.8% NaOH, and 19 min treatment time, producing 46.2 g L^{-1} of reducing sugar and 18.7 g L^{-1} ABE.[63]

Unfortunately, such pretreatment processes are costly and time-consuming, and pretreating lignocellulosic biomass can also produce several undesirable compounds, in which the

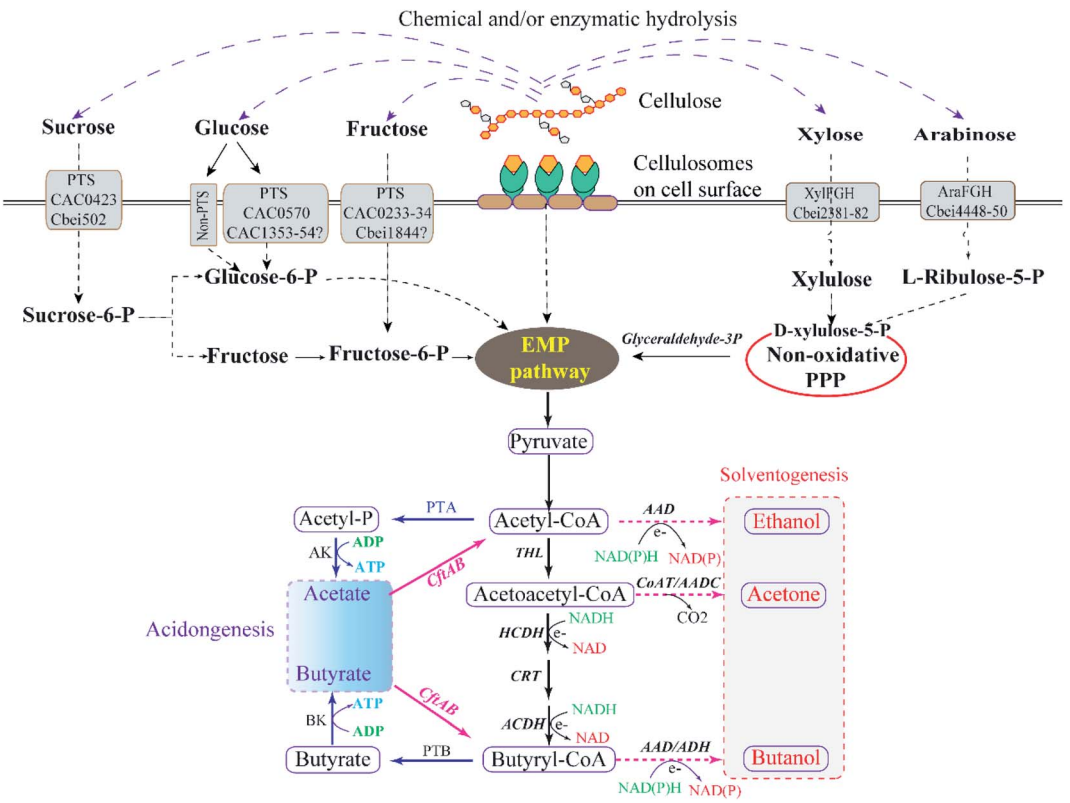

Fig. 1 Schematic diagram of butanol synthesis from lignocellulosic biomass in clostridia.[37,38]

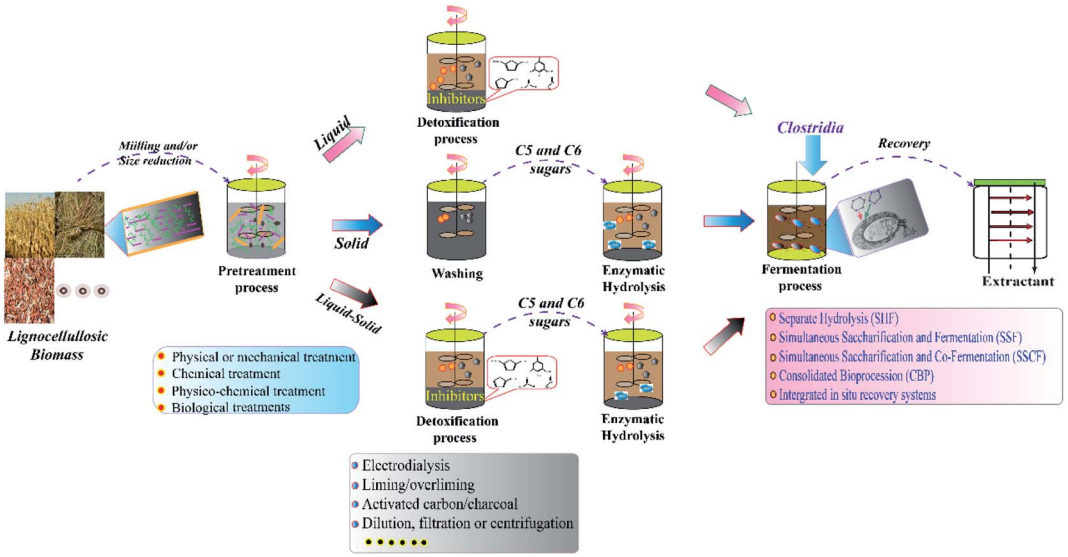

Fig. 2 Schematic diagram of fermentative butanol production from lignocellulosic biomass.[8,44]

concentrations of inhibitors depends on the lignocellulosic biomass and pretreatment methods.[17] Therefore, it is desirable to develop suitable pretreatment methods to avoid process inhibition, and thereby decrease costs and energy demands for butanol production from lignocellulosic materials.[64] Efforts could be made on the following directions: improving the conversion of lignocellulosic biomass with higher solid loading and low enzyme dosage under mild treat conditions; maximising hemicellulose and cellulose recovery with minimal degradation of lignin; or combining with microbial fermentation process optimization to obtain a more suitable substrate for butanol fermentation without increasing costs and processes. For example, lignocellulosic material wheat straw as a source of fermentable saccharides, and chicken feather as a source of amino acids and peptides, hydrolysis of both materials was carried out simultaneously, resulting in a cultivation medium that was suitable for direct use in biobutanol production.[65]

2.2.2 Hydrolysis of cellulose and hemicellulose. Cellulose, accounting for ~40% of lignocellulosic materials, is a long-chain polymer formed by glucose connected by β-1,4-glycosidic bonds,[66] and it can be selectively hydrolysed to glucose by cellulolytic enzymes (*e.g. exo*-1,4-β-glucanases, *endo*-1,4-β-glucanase and β-glucosidases). The enzymatic hydrolysis efficiency of cellulose is affected by many parameters. For example, when the binding ability of cellulase is weak at high solid-phase load,[67] the surface coverage of cellulose is low, which lowers the cellulose hydrolysis efficiency. Improving the enzymatic hydrolysis efficiency of cellulose in fermentation substrates to obtain high levels of fermentable sugars to produce cellulose butanol is a major goal.[68]

Hemicellulose, another cheap and abundant substrate, accounts for ~25 to 35% of lignocellulose. It is a polyphase polymer composed of several different types of monosaccharides, the main structural units are xylose (>50%), mannose, glucose, arabinose and galactose, and fermenting hemicellulose results in more butanol per unit mass of lignocellulose.[41] Compared with the β 1→4 linkages of cellulose, glycoside bonds of hemicellulose are more reactive and can be broken by chemical hydrolysis at milder conditions.[69] However, completely hydrolysing different types of glycosidic bonds requires a specific family of glycoside hydrolases, such as *endo*-β-1,4-xylanase, *endo*-β-1,4-man-nanase, α-galactosidase, and *endo*-galactanase. Therefore, although several clostridia can secrete some hemicellulases, the extracellular activity of enzymes is not adequate for direct utilisation of hemicellulose,[70] making butanol production from hemicellulose relatively inefficient due to expensive pretreatment processes.[71] At present, dilute-acid hydrolysis,[72] steam explosion,[73] and autohydrolysis/liquid hot water extraction,[74] have been evaluated for producing fermentable hemicellulosic sugars. For example, after treatment of hemicellulosic pre-hydrolysates by flocculation, followed by simultaneous detoxification with *Ureibacillus thermosphaericus* and *Cupriavidus taiwanensis* co-culture and hydrolysis with *Paenibacillus campinasensis*, a reduction of phenolic compounds up to 56% was achieved after flocculation, and ABE fermentation could produce 6.8 g L^{-1} of butanol after 116 h.[75]

3. Limitations and alleviation strategies for lignocellulosic butanol fermentation

Essential pretreatment and hydrolysis steps limit the fermentation of lignocellulosic biomass, accounting for a large portion of the process cost. Therefore, it is crucial to develop more cost-efficient pretreatment methods that minimise the generation of inhibitors, lower energy consumption, diminish operating costs, and simultaneously maximise fermentable sugar yields with a careful consideration of feedstock properties.[76] Due to

the low transport and utilization efficiency of fermentable sugars, low butanol yield is another major issue for lignocellulosic fermentation. Additionally, the accumulation of toxic intermediates, or overproduction of foreign proteins during butanol efficient fermentation often caused unwanted cellular stresses, resulting in a decrease in overall cell fitness. Therefore, increase of tolerance and robustness of strain is also important and necessary.[77,78]

3.1 Lignocellulose fermentation substrate treatment

Catabolite repression, release of inhibitors, and low sugar concentrations have proved to be the limiting factors for lignocellulosic butanol fermentation, and more cost-effective processes are needed to convert hemicellulose and cellulose into fermentable hydrolysates with appropriate concentrations of sugars, inhibitors, and stimulators.

3.1.1 Low-concentration fermentable sugars. During ABE fermentation, the shift from the acidogenesis phase toward the solventogenesis phase is strongly dependent on the concentration of the carbon source after growth phase;[79] increasing the concentration of the carbon source can promote fermentation toward the solventogenic phase, and thereby increase the butanol : acetone ratio.[80] Therefore, mismatching hydrolysis and fermentation processes, in which relatively low levels of sugars can be achieved by typical enzymatic hydrolysis of cellulose, is a major obstacle for lignocellulosic butanol production.[68] Achieving high concentrations of fermentable sugars is crucial for lignocellulosic butanol, and several strategies have been developed to increase the total sugar concentration from treatment process:[81] (1) using a fractionation pretreatment (*e.g.*, organosolv pretreatment) to generate a cellulose-rich pretreated solid; (2) preparation of hydrolysates by simultaneous enzymatic hydrolysis of cellulose and hemicellulose,[82] or supplementing with starch for ABE fermentation,[83] but carbon catabolite repression (CCR) may be an issue;[84] (3) concentrating hydrolysates by filtration or evaporation, increasing the total sugar concentration to 30–80 g L^{-1}.[85]

3.1.2 Inhibitors and detoxification processes

3.1.2.1 Lignocellulose-derived inhibitors. During pretreatment of lignocellulose, several lignocellulose-derived microbial inhibitory compounds (LDMICs)[86] are often generated, in which the amounts and species of inhibitors are dependent on raw materials and/or pretreatment processes.[54] These inhibitors can be classified into weak organic acids, furan derivatives, and phenolics, with toxicity at the same dose against ABE production ranked formic acid > phenols > furfurals.[87] More interestingly, clostridia have different tolerances of and stress responses to these inhibitors.[88] Compared with *C. beijerinckii*,[89] *C. acetobutylicum* is more sensitive to formic acid, and 0.1 M formic acid induces an 'acid crash' that completely inhibits butanol production and cell growth.[90] However, low concentrations (0.5–2 g L^{-1}) of furfural and 5-hydroxymethyl furfural can stimulate *Cupriavidus basilensis*, *C. beijerinckii* BA101,[91] *C. beijerinckii* P260 (ref. 92) and *C. acetobutylicum* ATCC 824.[93]

3.1.2.2 Lignin-derived compounds. Compared with weak organic acids and furan derivatives, partial decomposition of (poly)phenolic aromatic compounds in lignin, such as syringealdehyde, vanillic acid and ferulic acid,[94] is more toxic to clostridia.[95–97] More importantly, combinations of acids and furan aldehydes, as well as different phenolics, can have synergistic effects on ABE fermentation,[98] but the sophisticated physiological regulation mechanism remains unclear.[99] For example, during ABE fermentation, production of butanol and total solvent was decreased to 6.07 g L^{-1} from 8.08 g L^{-1}, and to 11.71 g L^{-1} from 13.95 g L^{-1}, respectively, under 0.5 g L^{-1} of ferulic acid stress,[100] and the biosynthesis of organic acids was severely inhibited at lower concentrations of phenolics (0.2 g L^{-1} vanillin or/and vanillic acid).[98] Furthermore, lignosulfonates, lignin-based organic polymers, can also significantly downregulate glycolysis and the butanol biosynthetic pathway in clostridia due to the unique properties of these anionic, water-soluble, highly acidic compounds.[101] For example, augmenting the lignosulfonate concentration >0.5 g L^{-1} led to a significant decrease in solvent titre (ABE ~1.50 g L^{-1}), while >1 g L^{-1} of low-molecular-weight lignosulfonate seriously inhibited solvent synthesis, and even completely blocked the process of ABE fermentation.[102]

3.1.2.3 Hemicellulose-derived inhibitors. Hexoses and pentoses could be obtained *via* the chemical hydrolysis of hemicellulose, however, often accompanying with the formation of 5-hydroxymethyl furfural (HMF) and furfural due to dehydration reactions.[103,104] More importantly, furan compounds can be also subjected to rehydration reactions to form levulinic acid and formic acid under acidic conditions, providing inhibitory effect on *C. acetobutylicum* due to the acid crash.[67,90] In addition, acetic acid is also produced through hydrolysis of the acetyl group of hemicellulose, and 10 g L^{-1} of acetic acid slightly increases the ABE fermentation performance, but the higher levels significantly inhibit the fermentation performance of *C. acetobutylicum*.[89] Furthermore, the presence of monosaccharides, oligomers, and some ingredients of hemicellulosic hydrolysates can also inhibit cellulase activity, and then negatively influence the hydrolysis efficiency of cellulose.[105]

3.1.2.4 Strategies to alleviate inhibition. In general, levels of inhibitors and fermentable sugars in hydrolysates are dependent on the feedstock and the conditions of pretreatment and hydrolysis processes.[106] Thus, several strategies have been developed to counteract inhibition problems, including (1) selecting less recalcitrant feedstocks; (2) utilising mild pretreatment conditions; (3) biological hydrolysis of hemicelluloses with specific enzymes, or co-culture of microorganisms producing hydrolysing enzymes[107] to effectively decrease the formation of inhibitors (but this can increase several incumbent costs);[108] (4) combining pretreatment, hydrolysis, fermentation and product recovery into a single consolidated process;[109] (5) integrating several detoxification methods, such as electrodialysis,[110] liming/overlimiting,[111] activated carbon/charcoal,[112] *in situ* extraction with oleyl alcohol,[113] and filtration or centrifugation, and resin treatments[114] to remove solids and undissolved lignin in lignocellulosic hydrolysates. However, although these inhibitors can be effectively removed during the detoxification process,[115] partial fermentable sugars are also

removed from lignocellulosic hydrolysates,[116] making the detoxification process impractical for commercial application.[117] Thus, it is necessary to develop a cost- and energy-efficient pretreatment stage to improve the conversion of lignocellulosic biomass with higher solid loading and low enzyme dosage under mild treat conditions, thereby maximising hemicellulose and cellulose recovery with minimal degradation of lignin, or minimise inhibitor formation to allow fermentation without detoxification.[65]

3.2 Transport and utilization efficiency of fermented sugars

The transportation and metabolic pathways of substrates during butanol synthesis have been dissected alongside stoichiometric glucose reactions.[30,118] Clostridia use PEP (phosphoenolpyruvate)-dependent PTSs (phosphotransferase systems) and/or non-PTS mechanisms to assimilate glucose during ABE fermentation, and PTSs are predominant in the solventogenic phase. However, some clostridia are dominated by non-PTSs (ATP-dependent glucokinases) during the solventogenic stage, such as the butanol-hyperproducing mutant strain *C. beijerinckii* BA101, derived from NCIMB 8052.[119] PTSs transport and phosphorylate sugar substrates, play a central role in metabolic regulation. For example, there are 13 PTSs in Clostridium related to acetone and butanol,[120] among which 12 are encoded by chromosomal genes and can be used to transport glucose, mannose, fructose, lactose, cellulose-disaccharide, sucrose, galactose and maltose. The other PTS is encoded by genes on the large PSOL1 plasmid, and is used for transporting mannose and fructose. Some PTSs can transport multiple sugars simultaneously.[121] The uptake of sugars such as pentose proceeds through non-PTS pathway; xylose and arabinose enter the cell through their specific transporters, then converge into xylose-5-phosphate through isomerisation and phosphorylation, respectively, and enter central metabolism through PPP (pentose phosphate pathway) or PK (pyruvate kinase) pathways.[122]

Apart from dissimilarities in uptake systems utilised for sugars, carbon catabolic repression can also lead to the consumption of sugars at different rates.[123] After hydrolysis of lignocellulosic biomass, various ratios of fermentable sugars containing a mixture of pentoses and hexoses are released into the culture broth. Unfortunately, wild-type clostridia are poor at consuming mixed fermentable sugars due to CCR, and the utilisation of pentose sugars can be decreased or inhibited markedly in the presence of glucose.[84] Therefore, efficient utilisation of mixtures of sugars has become a prerequisite for effective lignocellulosic butanol fermentation. To alleviate CCR in mixed sugar fermentation systems, several valuable strategies have been employed, including engineering metabolic pathways (such as knocking out ccpA to facilitate xylose and glucose utilisation[124]), mixed culture consolidated bioprocess,[125] exogenous supplementation with trace elements[126] and semi-hydrolysis strategies.[127] For example, the synergistic effect of calcium and zinc was investigated in ABE fermentation by *C. acetobutylicum* using glucose, xylose and glucose/xylose mixtures as carbon sources. As a result, glucose/xylose utilisation, cell growth, acid re-assimilation and butanol biosynthesis were significantly improved, with butanol and ABE production increased from 11.7 and 19.4 g L^{-1} to 16.1 and 25.9 g L^{-1}, respectively, from 69.3 g L^{-1} glucose.[128] Similarly, semi-hydrolysates with low enzyme loading using H_2SO_4-pretreated rice straw was also employed to further improve butanol fermentation efficiency through preferential production of cellobiose and xylose (instead of glucose). As a result, butanol productivity was correspondingly increased from 0.0628 g L^{-1} h^{-1} to 0.265 g L^{-1} h^{-1} during fermentation of undetoxified semi-hydrolysates with a high cell density.[127]

Therefore, efficient transport and utilization of the available sugars in lignocellulosic hydrolysate without causing or alleviating the glucose carbon repression effect could be a potential exploring direction to improve the efficiency of lignocellulosic butanol production in the future.

3.3 Fermentation strain with good physiological characteristics

There are various stress responses under varying environment stresses in the process of butanol production using industrialized fermentation of lignocellulose, such as various inhibitors in the fermentation matrix after cellulose pretreatment and hydrolysis,[129] intermediate metabolites (organic acids such as butyric acid and acetic acid accumulate) and fermentation end-products (accumulation of solvents such as butanol, ethanol, and acetone) during ABE fermentation,[130] strains exposed to physical pressures such as inappropriate pH, temperature, dissolved oxygen and osmotic pressure due to the inhomogeneity of large-scale fermentation system, and overproduction of exogenous proteins caused by various modifications or accumulation of toxic intermediates for improving the fermentation yield or output.[78] In light of it, ideal strains for butanol production by lignocellulose possess should physiological functions such as favorable adaptability and robustness for maintaining highly-active metabolic flux with good substrate and environmental tolerance of butanol during industrial fermentation.

4. Strategy for improving lignocellulosic butanol production

4.1 Improving lignocellulosic fermentation using microbial consortia or utilizing microorganisms cooperate

In lignocellulosic butanol fermentation, the complexity of the lignocellulose degradation process and the butanol metabolic pathway can significantly burden the metabolism of single strains, resulting in low butanol production using monocultures. In nature, lignocellulosic biomass can be entirely degraded and assimilated by microbial consortia containing fungi and cellulose-degrading bacteria.[131] In this regard, natural microbial communities were mimicked to construct a synthetic 'Y-shaped' consortium consisting of two strains with the same butanol biosynthetic pathway and orthogonal capacity for glucose and xylose metabolism. The resulting consortium could not only adapt to environmental perturbations, but also

simultaneously utilise C5/C6 sugars in different ratios, with the most efficient butanol production from mixed sugars through equally efficient orthogonal consumption of C5 and C6 sugars.[132] Similarly, cellulosic butanol could be produced using anaerobic co-cultures of *C. saccharoperbutylacetonicum* and white-rot fungus *Phlebia* sp. MG-60-P2. Following knockout of pyruvate decarboxylase, co-cultures of KO77 and *C. saccharoperbutylacetonicum* could displayed synergistically enhanced saccharification and butanol production was further increased from 2.5 g L^{-1} to 3.2 g L^{-1} compared with co-cultures of MG-60-P2 and *C. saccharoperbutylacetonicum*.[133] Therefore, constructing microbial consortia with the capacity for butanol synthesis and degrading lignocellulosic biomass has potential for improving lignocellulosic butanol production.

4.2 Application of advanced fermentation strategies

Following physical, chemical or biological pretreatment processes, four fermentation processes, hydrolysis and fermentation (SHF), simultaneous saccharification and fermentation (SSF), simultaneous saccharification and co-fermentation (SSCF), and the processes consolidated bioprocessing (CBP), have been applied to overcome the complex characteristics of feedstocks and feedback inhibition for lignocellulosic butanol production (Table 2).[134,135]

4.2.1 Simultaneous saccharification and fermentation (SSF). Compared with SHF, simultaneous saccharification and fermentation (SSF)_has fewer processing steps, lower operating costs, less contamination risk, and lower feedback inhibition of sugars on enzymes, which improves the efficiency of hydrolysis and fermentation, resulting in a higher yield of butanol.[68] However, the SSF mode of operation is challenging in a number of ways: (1) during the SSF process, matching the optimum temperatures between enzymolysis (45–50 °C) and ABE fermentation (35–37 °C) is a major hurdle to achieving high cellulase activity, sugar yield, and butanol production.[136,137] To this end, *C. acetobutylicum* with improved thermotolerance at 39–45 °C was engineered and used in high-temperature SSF of pretreated corn stover, and the optimised SSF process at 42 °C with a 12 h pre-hydrolysis yielded 10.8 g L^{-1} of butanol and 18.2 g L^{-1} of ABE from overlimited acid-treated liquid;[138] (2) an imbalance in the rates of sugar formation by enzymatic hydrolysis and sugar consumption by clostridia is another challenge.[139] In this regard, water-soluble cellulose oligomers have been used to modify the SSF process, in which chemically prepared water-soluble oligomers and pretreated cellulose were simultaneously subjected to enzymatic hydrolysis and ABE fermentation in a process named simultaneous co-saccharification and fermentation (SCSF).[140] The SCSF process achieved a higher yield of butanol/lignocellulosic biomass (g/g), and is suited for pretreatment and enzymatic hydrolysis using lignocellulosic biomass as the substrate.[141] Recently, a novel fed-batch SSFR (simultaneous saccharification, fermentation and recovery) strategy was developed and applied for ABE fermentation from alkaline-pretreated rice straw. After optimisation of initial solids and enzyme loadings by batch SSF assays, the maximum butanol concentration and butanol productivity were increased to 24.80 g L^{-1} and 0.344 g L^{-1} h^{-1}, respectively, through three biomass feedings, showing that SSFR can significantly enhance the performance of lignocellulose fermentation when accompanied by efficient enzyme use.[142]

4.2.2 Consolidated bioprocesses. Recently, CBP that integrates cellulase production, lignocellulose hydrolysis, and fermentation of mixtures of sugars into a single consecutive process with a single microbe or a microbial consortium has been developed for lignocellulosic fermentation.[143,144] For lignocellulosic butanol fermentation, several CBP processes have been carried out either by genetically engineering strains[145–147] or co-culturing biofuel-producing strains with saccharolytic strains such as *C. cellulolyticum*[148] and *C. thermocellum*.[149] For example, a microbial consortium combining *Thermoanaerobacterium thermosaccharolyticum* M5 and *C. acetobutylicum* NJ4 was constructed for efficient ABE fermentation using xylan *via* CBP. In this study, strain M5 could efficiently degrade xylan, accumulating 19.73 g L^{-1} of xylose within 50 h, and strain NJ4 could efficiently utilise xylose to effectively decrease substrate inhibition on xylanase and xylosidase. As a result, the synergy of the two strains could produce 13.28 g L^{-1} of butanol from 70 g L^{-1} of xylan under optimal conditions.[150]

4.2.3 Two-stage and multi-stage fermentation. Compared with batch fermentation, single-stage continuous fermentation can achieve high solvent productivity, but lower solvent concentrations, indicating that single-stage continuous fermentation is not suitable for industrial-scale applications.[151] However, a continuous bioreactor with immobilised cells and multistage fermentation has been applied for continuous ABE fermentation.[152] Two-stage and multi-stage fermentation systems can completely utilise substrates and effectively assimilate acids to solvents, achieving increased productivity and solvent concentrations over long-term fermentation.[152] For example, a four-stage continuous fermentation with cane molasses was employed to enhance butanol titre and productivity, achieving 13.75 g L^{-1} of solvents with a productivity of 0.439 g L^{-1} h^{-1} with a gradient dilution mode of 0.15–0.15–0.125–0.1 h^{-1}.[153] In addition, a novel two-stage fermentation process, comprising an acidogenic fermentation process followed by an ABE fermentation process, was also developed and introduced to maximise sugar utilisation and butanol yield with alkaline-pretreated rice straw.[154] A sugar-rich hydrolysate (90.4 g L^{-1} reducing sugar) and a high acid level in the fermentation broth (33.9 g L^{-1} butyric acid) were obtained. Also, final butanol production was significantly increased to 15.9 g L^{-1} with 3-fold lower cellulase loading, yielding 149 g butanol and 36 L hydrogen gas from 1 kg rice straw, respectively.[154]

4.2.4 Improving fermentation processes for lignocellulosic butanol. In general, pretreatment processes have three main drawbacks:[155] (1) requiring specific instruments and facilities to pretreat lignocellulosic materials under strict conditions; (2) the formation and release of inhibitors that negatively impact enzymatic hydrolysis and fermentation, resulting in the need for an additional detoxification step, which increases total costs;[156] (3) using 'non-green' chemicals as catalysts in several pretreatment methods, with consequent negative environmental impacts.[157] To overcome these drawbacks, a 'semi-

Table 2 Summary of pretreatment and detoxification techniques applied to lignocellulosic materials for ABE fermentation[a]

	Pretreatment and/hydrolysis	Detoxification	Organism	Fermentation conditions	ABE, g L^{-1}	Butanol, g L^{-1}	Ref
Rice straw	Acid hydrolysis + shear stress	Filtering with a sterile cotton cloth	*C. acetobutylicum* NCIM233	Batch fermentation in 2 L bioreactor	20.56	13.5	162
Corn cob	NaOH + enzymatic hydrolysis	NA	*C. saccharobutylicum* DSM 13864	Batch fermentation	19.44	12.27	163
Corn straw	Acid pre-impregnated steam explosion (APSE) pretreatment	Concentrating with Rotavapor; and then lime-treated hydrolysate with $Ca(OH)_2$	*C. acetobutylicum* ATCC 824	Batch fermentation	0.31 g g^{-1} sugar	0.18 g g^{-1} sugar	164
Switchgrass	Alkali + enzyme	NA	*C. saccharobutylicum* DSM13864	Batch fermentation	22.7	13	165
Switchgrass	Thermal hydrolysis pretreatment	Without detoxification process	*C. beijerinckii* ATCC 51743	Batch fermentations	18.5	NA	166
Wheat straw	Biological treatment	NA	Coculture of *C. beijerinckii* 10132 with *C. cellulovorans* 35296	Batch fermentation	23.30	14.20	167
Wheat straw	Ammonium sulfite pretreatment	NA	*C. acetobutylicum* ATCC 824	Simultaneous saccharification and fermentation	19.83	12.64	168
Barley straw hydrolysate	Mechanical + acidic pretreatment	Overliming method	*C. beijerinckii* P260	Fermentation with product removal by gas stripping	47.20	30.86	169
Mango peel waste	Mechanical + enzyme hydrolysis	NA	*C. acetobutylicum* NCIM 2878	Batch fermentation	15.13	10.50	170
Apple peel waste	Mechanical	NA	*C. acetobutylicum* DSM 792	Batch fermentation	20.00	14.00	171
Apple pomace	Acid pretreatment + enzymatic hydrolysis	NA	*C. beijerinckii* P260	Batch fermentation	10.8	5.6	19
Sago waste	Acid hydrolysis + enzyme hydrolysis	NA	*Clostridium bifermentans* and *Bacillus coagulans*	Batch fermentation	9.01	3.36	172
Wheat red dog	Enzymatic digests	NA	*C. beijerinckii* NCIMB 8052	Batch fermentation	20.0	15.0	173
Spent sulfite liquor (SSL)	NA	Coupling nanofiltration, ultrafiltration and ion exchange	*C. acetobutylicum* ATCC 824	Submerged fermentation	21.09	12.96	174
Cellulosic material	NA	NA	White-rot fungus *Phlebia* sp. MG-60-P2 and *C. saccharoperbutylacetonicum*	Anaerobic co-culture in consolidated bioprocessing	NA	3.2	133
Sugarcane straw	Microwave-assisted alkali pretreatment + acid hydrolysis	NA	*C. beijerinkii* NBRC 109359	Batch fermentation	18.7	NA	63
Industrial vinegar residue	Steam explosion pretreatment + enzymatic hydrolysis	NA	*C. acetobutylicum*	Batch fermentation	12.59	7.98	175
Food waste	Liquefied and saccharified	NA	*C. saccharoperbutylacetonicum*	Continuous immobilized-cell fermentation system at dilution rate of 0.1 h^{-1}	19.65	NA	176
Yellow top (*Physaria fendleri*)	Dilute acid + enzymatic hydrolysis	NA	*C. beijerinckii* P260	Batch fermentation	28.8	NA	177

Table 2 (*Contd.*)

	Pretreatment and/hydrolysis	Detoxification	Organism	Fermentation conditions	ABE, g L^{-1}	Butanol, g L^{-1}	Ref
Banana crop residue	Dilute alkali and acid + enzymatic saccharification	NA	*C. beijerinckii*	Batch fermentation	20.5	14.0	178
Sugarcane bagasse	Hemicellulosic hydrolysate	Without detoxification process	*C. saccharoperbutylacetonicum*	Batch fermentation	7.11	5.85	179
Hardwood hemicelluloses	Hydrolysate + enzyme	Coagulation–flocculation + inhibitors removal with bacteria	*C. acetobutylicum* ATCC 824	Batch fermentation	11.8	6.8	75
Paper pulp	Enzymatic hydrolysis	NA	*C. saccharoperbutylacetonicum* ATCC 13564	Simultaneous saccharification and fermentation with *in situ* butanol recovery	NA	19.2–22.0	158

[a] NA, data not available.

hydrolysis' strategy was proposed to utilise lignocellulosic material containing oligosaccharides and monosaccharides using less hydrolytic enzyme, then further improving the butanol yield, enzyme loading, and overall butanol productivity.[127] For example, a consolidated fermentation system was established in which enzymatic semi-hydrolysis of paper pulp (93.2% glucan) without pretreatment and with low enzyme loading produced high levels of cellobiose (13.9 g L^{-1}) and glucose (21.3 g L^{-1}) *via* ABE fermentation, which was as efficient as fermentation using commercial sugars without inhibitors.[158]

Furthermore, physical, chemical or biological detoxification methods have been developed for eliminating toxic inhibitors, but the resulting wastewater, increased energy costs, and sugar loss remain barriers to economic viability and environmental sustainability at industrial scale.[17,159] Therefore, mutagenesis, genetic manipulation and metabolic perturbations have been applied to construct inhibitor-tolerating strains that can achieve lignocellulosic butanol fermentation without detoxification or wastewater generation. For example, a mutant overexpressing PTS^{GlcG} exhibited improved inhibitor tolerance, and produced 10.1 g L^{-1} of butanol using corn stover hydrolysate (CSH) culture without detoxification, an increase by 300% and 400% compared with 2.5 and 2.0 g L^{-1} achieved control and PTS^{GlcG}-deficient strains, respectively.[160] Similarly, introduction of a 'push–pull' strategy in *C. cellulovorans* DSM 743B diverted carbon flux from acetyl-CoA to butyryl-CoA by overexpressing a *trans*-enoyl-coenzyme A reductase gene (*ter*), and an acid reassimilation pathway uncoupled from acetone synthesis was also constructed to redirect carbon flux from butyrate and acetate toward butyryl-CoA. Xylose metabolism was engineered by overexpressing xylT (CA_C1345) and inactivating xylR (Clocel_0594) and araR (Clocel_1253), and final production of butanol was increased to 4.96 g L^{-1} directly from alkali-extracted corncobs.[161]

4.3 A strategy for engineering physiological functionalities

Good physiological performance of microbes is crucial for successful biological fermentation. Strain improvement through physiological engineering relies on integration of knowledge on physiological functionality and efficient engineering approaches.[130] For example, introduction of pro (precursor region)–mtg (microbial transglutaminase) into *C. acetobutylicum* improves oxidative-stress resistance, growth performance, and solvent production of the host.[180] In the past, many strains obtained by metabolic engineering strategies failed to steady exhibit the expected phenotype because the physiological stress responses of microorganisms were ignored. Therefore, microorganisms should not only have strong metabolic capacity, but also possess strong physiological robustness and adaptability in order to work effectively in actual biological processes.

5. Future prospects

Clostridial species generally perform ABE fermentation using the cellulose and hemicellulose fraction of lignocellulosic

feedstocks.[181] However, essential pretreatment steps limit the fermentation of lignocellulosic biomass, accounting for a large portion of the process cost. Therefore, it is crucial to develop more cost-efficient pretreatment processes that minimise the formation of inhibitors, lower energy consumption, diminish operating costs, and concurrently maximise fermentable sugar production with careful consideration of feedstock properties.[76] Carbon loss can occur in the form of xylose during biomass pretreatment, and a large amount of carbon in the form of CO_2 is emitted during fermentation and co-generation processes.[182,183] An innovative way to valorise off-gas streams is to produce alcohols through gas fermentation, in which acetogens ferment CO_2 plus H_2 to produce VFAs (volatile fatty acids) and alcohols *via* the Wood–Ljungdahl pathway, as demonstrated with *Clostridium carboxidivorans*.[184–186] Furthermore, effective utilisation of xylose in hemicellulose is also a key factor to reduce the production cost of biobutanol. To increase the overall competitiveness of lignocellulosic butanol, cost-effective and industrially feasible strategies for lignocellulosic biomass pretreatment and hydrolysis must be further optimised. Furthermore, an ideal lignocellulosic butanol-producing strain is also needed to maintain high butanol metabolic flux activity, efficient sugar transport, the use of fermentable mixed sugar, and good substrate tolerance in the process of industrial butanol fermentation, which can further improve the fermentation performance and lower fermentation costs.

The performance of lignocellulosic fermentation could be improved as follows:

• Process integration and intensification measures to further improve the performance and economic competitiveness of lignocellulosic butanol fermentation, such as integration of (1) pretreatment with hydrolysis, (2) hydrolysis with fermentation, and even (3) hydrolysis, fermentation, and product recovery.

• Adapting and improving the physiological characteristics of target microorganisms: it can achieve rapid transformation of hexose and pentose; good tolerance to the target product butanol, the intermediate product butyric acid, and inhibitors in raw materials; under low pH stress conditions, and in the presence of organic acids and butanol, the metabolic activity can remain high; good metabolic flux can be maintained throughout the fermentation process.

• Production of butanol by lignocellulose is so complicated that might lead to unnecessary cell stress. In that case, the toxicity problem of the butanol fermentation process and the heterogeneity of industrial production should be considered although it is essential to orient at breaking through a single problem. More precisely, lignocellulose pretreatment, microbial physiological process and characteristics, carbon efficient utilization or butanol recovery technology should be adopted to improve the physiologic performance of microbes, achieving both the enhanced yield and stable output in conjunction with interleaving functions of biological robustness.

Conflicts of interest

The authors declare that they have no competing interests.

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