Minireview

Consolidated bioprocessing for butanol production of cellulolytic Clostridia: development and optimization

Zhiqiang Wen,1 Qi Li,2 Jinle Liu,3 Mingjie Jin1,* and Sheng Yang3,4,**

1 School of Environmental and Biological Engineering, Nanjing University of Science and Technology, Nanjing, 210094, China.
2 College of Life Sciences, Sichuan Normal University, Longquan, Chengdu, 610101, China.
3 Key Laboratory of Synthetic Biology, CAS Center for Excellence in Molecular Plant Sciences, Shanghai Institute of Plant Physiology and Ecology, Chinese Academy of Sciences, Shanghai, 200032, China.
4 Huzhou Center of Industrial Biotechnology, Shanghai Institutes of Biological Sciences, Chinese Academy of Sciences, Shanghai, 200032, China.

Summary

Butanol is an important bulk chemical, as well as a promising renewable gasoline substitute, that is commonly produced by solventogenic Clostridia. The main cost of cellulosic butanol fermentation is caused by cellulases that are required to saccharify lignocellulose, since solventogenic Clostridia cannot efficiently secrete cellulases. However, cellulolytic Clostridia can natively degrade lignocellulose and produce ethanol, acetate, butyrate and even butanol. Therefore, cellulolytic Clostridia offer an alternative to develop consolidated bioprocessing (CBP), which combines cellulase production, lignocellulose hydrolysis and co-fermentation of hexose/pentose into butanol in one step. This review focuses on CBP advances for butanol production of cellulolytic Clostridia and various synthetic biotechnologies that drive these advances. Moreover, the efforts to optimize the CBP-enabling cellulolytic Clostridia chassis are also discussed. These include the development of genetic tools, pentose metabolic engineering and the improvement of butanol tolerance. Designer cellulolytic Clostridia or consortium provide a promising approach and resource to accelerate future CBP for butanol production.

Introduction

Butanol is a bulk chemical and a promising renewable vehicle fuel (Nguyen et al., 2018). Acetone–butanol–ethanol fermentation (with n-butanol as the main product) by solventogenic Clostridia such as Clostridium acetobutylicum or C. beijerinckii has been developed more than a century ago (Moon et al., 2016). During the past 100 years, all aspects of butanol fermentation have progressed significantly, especially with regard to feedstocks. With increasing grain prices and a growing concern about food security by governments, the feedstocks for butanol fermentation are gradually expanding from corn to the less expensive, renewable lignocellulosic biomass and syngas (Gu et al., 2011, 2014; Jiang et al., 2015). Steel mill off-gas is considered to be the most economic source and substrate; however, the performance of current gas-fermenting strains for butanol production and their resistance to stress requires further modification (Durre, 2016; Huang et al., 2016, 2019). At present, butanol production by Clostridium from lignocellulosic biomass hydrolysate is very close to that from grain fermentation (Gu et al., 2011, 2014; Jiang et al., 2015); however, its industrialization bottleneck lies in the addition of exogenous cellulases. It has been estimated that the current cost of cellulases and pretreatment accounts for 35% of the overall cost (Jiang et al., 2015), thus weakening its market competitiveness.

Several steps are involved in microbial butanol production from lignocellulosic biomass including pretreatment, cellulase (cellulosome) secretion, lignocellulose
enzymatic hydrolysis and co-fermentation of hexose and pentose. Compared with other biorefinery processing such as separate hydrolysis and fermentation (SHF), simultaneous saccharification and fermentation (SSF), and simultaneous saccharification and co-fermentation (SSCF), consolidated bioprocessing (CBP) combines all above steps in one reactor, thus decreasing the cost from dedicated cellulase production (Lynd et al., 2005; Olson et al., 2012; den Haan et al., 2015). CBP is considered a promising biorefinery process and has been adapted for cellulosic butanol production, which achieved notable progress (Xin et al., 2019).

The recent progress of synthetic biology technology in Clostridium provides an abundance of tools and approaches towards to realization of CBP (Joseph et al., 2018; McAllister and Sorg, 2019), which effectively drive butanol production by CBP. For example, genome editing and metabolic engineering have been used to modify and optimize CBP-enabling strains or consortia (Wen et al., 2017, 2019). Omics analysis (Patakova et al., 2018; Liu et al., 2019), metabolic and dynamic modelling (Salimi et al., 2010; Liao et al., 2015) and other techniques have been used to analyse butanol tolerance or to optimize the community structure of producers.

This review intends to highlight the CBP progress based on cellulosytic Clostridia driven by synthetic biology technique. Three different synthetic biological approaches to realize CBP are described and compared, with particular focus on the progresses of butanol metabolic engineering in native cellulosytic Clostridia, designer cellulolytic secretion and optimization of clostridial consortia. Progress towards overcoming a number of bottlenecks for butanol production by CBP including the development of genetic tools, xylose utilization and butanol tolerance is also discussed.

**CBP development based on cellulosytic Clostridia**

Consolidated bioprocessing requires microorganisms or microbial systems capable of degrading lignocellulose, while efficiently and simultaneously yielding chemicals. However, few native microorganisms or microbial systems can directly degrade lignocellulose and produce butanol (Wen et al., 2017, 2019).

Currently, three strategies are used to achieve CBP (Higashide et al., 2011; Zuroff and Curtis, 2012; den Haan et al., 2015). These include butanol pathway engineering of cellulosolytic Clostridia, engineering solvento- genic Clostridia to secrete/cell display cellulases or cellulosomes and mixed-culture of cellulosolytic and butanol-producing Clostridia (Fig. 1). Each strategy has its unique advantages and together, and they promote the study of butanol production by CBP.

**Engineering the butanol pathway in cellulosolytic Clostridia**

Several Clostridia natively secrete cellulases and directly grow on lignocellulose with alcohols and organic acids as main products. Examples are *C. thermocellum* (ethanol, acetate and lactate) (Ng et al., 1977), *C. cellulolyticum* (ethanol, acetate and lactate) (Petitdermange et al., 1984) and *C. cellulovorans* (butyrate, ethanol and lactate) (Sléat et al., 1984). Whole genome sequencing and annotation have deepened the current understanding of lignocellulose-degrading enzymes and butanol metabolic networks of the above cellulosolytic Clostridia. Clostridial genetic manipulation techniques provide an opportunity to engineer cellulosolytic Clostridia for n-butanol/isobutanol production by CBP (Wen et al., 2017; Joseph et al., 2018).

*Clostridium cellulolyticum* has been modified to extend the CoA-dependent metabolic pathway for n-butanol production; however, the titre is < 0.12 g l \(^{-1}\) (Gaida et al., 2016). Higashide et al. (2011) introduced a hybrid valine biosynthesis pathway in *C. cellulolyticum* and then diverted its 2-keto acid intermediates towards isobutanol synthesis. This was the first successful report on isobutanol production via metabolic engineering of cellulosolytic clostridia; however, only 0.66 g l \(^{-1}\) of isobutanol was produced within 192 h. Recently, *C. thermocellum* was engineered by the same research group to extend the 2-keto acid metabolic pathway for isobutanol synthesis (Lin et al., 2015), and finally, 5.4 g l \(^{-1}\) isobutanol was produced from cellulose within 75 h. This notably improved titre and productivity, implying promising potential of *C. thermocellum* for CBP.

Similar to *C. thermocellum*, *C. cellulovorans* is a further promising cellulosolytic clostridial chassis for butanol production, since it can grow on lignocellulose and accumulate a high amount of butyrate, the precursor of butanol (Sléat et al., 1984). Yang et al. (2015) overexpressed the aldehyde/alcohol dehydrogenase gene (*adhE2*) in *C. cellulovorans*, which caused the recombinant strain to produce 1.42 g l \(^{-1}\) n-butanol within 252 h. This exemplified the first metabolic engineering in *C. cellulovorans*, but the complicated genetic modification has remained unexplored due to a lack of genetic tools. Wen et al. (2017) verified that genetic tools such as TargeTron and the CRISPR/Cas system were applicable in *C. cellulovorans*. Based on efficient genetic techniques, a CoA-dependent acetone–butanol–ethanol (ABE) pathway was introduced into *C. cellulovorans* to direct carbon flux from butyrate to n-butanol. To further improve n-butanol production, strains with high butanol tolerance were adapted as hosts for metabolic engineering. The generated strains produced 3.47 g l \(^{-1}\) of n-butanol, which is 139-fold of that produced by wild-type *C. cellulovorans* (Wen et al., 2019). Recently, n-butanol
production by \textit{C. cellulovorans} achieved 4.0 g l$^{-1}$ by Bao et al. (2019).

These studies demonstrated that cellulolytic Clostridia are ideal strain platforms for biorefinery by CBP. However, it still remains challenging to simultaneously balance the capacity of efficient cellulose hydrolysis and butanol synthesis in a single strain (Song et al., 2014; Wen et al., 2017). The reason is that the modification of complicated multiple genes is sometimes delayed by the lack of genetic tools (Wen et al., 2019). Moreover, low titre, productivity and yield may also be related to insufficient carbon flux supply (due to inefficient lignocellulolytic hydrolysis) and competing metabolic pathways (Higashide et al., 2011; Yang et al., 2015). Further enhancement of cellulosome and/or cellulases expression and butanol metabolic flux is expected to significantly increase n-butyraldehyde/isobutanol production.

\textbf{Overexpression of cellulosome or cellulases in butanol-producing Clostridia}

Although several butanol-producing Clostridia can secrete xylanase and degrade xylan, they lack major lignocellulose-degrading enzymes (such as endoglucanase, exoglucanase and cellobiohydrolase) (Thomas et al., 2014; Jiang et al., 2018a,b). Basically, Clostridia cannot directly grow on lignocellulose. Fortunately, butanol-producing Clostridia are taxonomically closely related to many cellulolytic Clostridia and have similar codon preference, which laid the foundation for the heterologous production.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig1.png}
\caption{CBP approaches based on cellulolytic clostridia or consortia for butanol production. Purple dotted line of represents ACP dependent pathway (Pasztor et al., 2015), while green and blue dotted lines represent 2-keto acid pathway extended from pyruvate and phosphoenolpyruvate (Chen and Liao, 2016) respectively; \textit{C. cellulovorans} cellulosome or cellulases overexpression of \textit{butanol-producing clostridia}. EGI, endoglucanase; CBBI, cellobiohydrolase I; BGL, \(\beta\)-glucosidase; CBHII, cellobiohydrolase II; CBD, cellulose binding domain; \(\delta\), engineering of consortia composing of cellulolytic and butanol-producing clostridia, taken a twin-clostridia consortium as example(Wen et al., 2017). In the consortium, \textit{C. cellulovorans} secretes cellulosome to degrade AECC (alkali extracted corn cobs) to provide glucose and xylose for \textit{C. beijerinckii} to grow and produce butanol; besides, butyrate produced by \textit{C. cellulovorans} can be re-assimilated by \textit{C. beijerinckii} to produce butanol.}
\end{figure}
expression of cellulase genes in butanol-producing Clostridia.

The expression of soluble cellulases with small molecular weight (such as Cel5A, Cel8C and Cel9M) in *C. acetobutylicum* has been successfully realized (Lopez-Contreras et al., 2003, 2004; Mingardon et al., 2011). However, single type cellulase and very low expression levels (0.5-5 mg l⁻¹, only detectable cellulases activity) cannot support the host to directly utilize lignocellulose for growth and butanol production.

Compared with free and single component cellulase, cellulosome (a multi-enzyme complex) typically achieved much better results. The reason is that cellulosome can adhere to the lignocellulose surface, which contributed to multi-enzyme synergy and locally maintains a relatively high concentration of cellulases (Bayer et al., 2007). Interestingly, although complete gene clusters of cellulosome have been identified in a number of Clostridia (e.g. *C. acetobutylicum*), very small quantities of functional cellulosome were produced (Sabethe et al., 2002).

Currently, studies mainly focus on the development of hybrid cellulosomes and the optimization of heterologous expression. Heterologous production, assembly and secretion of a mini-cellulosome by *C. acetobutylicum* ATCC 824 was first reported by Mingardon et al. in 2005. Subsequently, a number of key issues have been addressed towards the secretion of heterologous cellulosome and cellulases, including the investigation and optimization of leader peptide, promoter strength and molecular chaperones (Mingardon et al., 2011; Fierobe et al., 2012; Hyeon et al., 2013). Recently, allele-coupled exchange (ACE) technology was adapted to stably integrate a hybrid cellulosome operon composed of cellulosomal enzymes and miniscollagen genes into chromosomes (Kovács et al., 2013; Willson et al., 2016). Furthermore, the secreted mini-cellulosome was successfully anchored to the *C. acetobutylicum* cell wall via the native sortase system (Willson et al., 2016). The thus generated recombinant strains displayed an improved growth phenotype on lignocellulosic biomass (Willson et al., 2016). This represents a milestone towards enabling solventogenic Clostridia to be cellulolytic.

However, the number of recombinant solventogenic Clostridia that harboured the overexpression of cellulases or hybrid (chimeric) cellulosomes and that can grow on lignocellulose as the sole carbon source is limited (Wen et al., 2017). Many technical challenges still need to be overcome towards cellulases expression. For example, regulation of selective RNA processing and stabilization on the cellulosome operon indicates the importance of post-transcriptional modification studies (Xu et al., 2015a,b,c). In addition, confirmation of the correct translation, folding and efficient transmembrane transport of cellulases and designer cellulosomes with high molecular weight and complex structure still remain challenging. This aggravates the simultaneous efficient lignocellulose degradation and butanol fermentation (Mingardon et al., 2011).

**Engineering consortia composed of cellulosic and solventogenic Clostridia**

Mixed-culture of cellulosic and butanol-producing Clostridia towards the construction of a cross-species cellulosic butanol pathway can share the metabolic requirements and adjust the abundance of each strain to adapt to the external environment (Song et al., 2014). Moreover, it is advantageous to explore and improve beneficial microbial interactions (Wen et al., 2017) and thus promote butanol production. Consortia engineering has become an emerging strategy for butanol production by CBP (Zuroff and Curtis, 2012; Xin et al., 2019).

Many commensal consortia have been isolated from nature for butanol production from lignocellulose (Jiang et al., 2018a,b); however, it remains difficult to regulate these systems due to their complex community structure and the limitation of available genetic tools. Compared with natural consortia, artificial consortia are relatively simple to construct and regulate.

The sequential co-culture of thermophilic *C. thermocellum* and mesophilic *C. acetobutylicum*, *C. saccharoperbutylacetonicum* N1-4 or *C. beijerinckii* was designed to directly produce n-butanol from cellulose (Yu et al., 1985; Nakayama et al., 2011; Wen et al., 2014a,b). However, the un-matched incubation temperature resulted in a non-isothermal and long fermentation process and, thus, low productivity. Therefore, it is critical to select appropriate strains for the formation of a consortium.

Mesophilic cellulosic Clostridia, such as *C. cellulo-lyticum* and *C. cellulovorans*, have been adapted to replace *C. thermocellum* (Petitdemange et al., 1983; Wen et al., 2014a,b). In *C. cellulovorans* and *C. beijerinckii* co-culture, *C. cellulovorans* degrades lignocellulose and provides fermentable sugars to support *C. beijerinckii*’s growth and solvent fermentation; in turn, *C. beijerinckii* re-assimilates and detoxicates butyrate for *C. cellulovorans*. A novel feeding-detoxification relationship between *C. cellulovorans* and *C. beijerinckii* was developed in the multicellular system. Their symbiosis degraded 68.6 g l⁻¹ of alkali extracted deshelled corn cobs (AECC) and produced 8.30 g l⁻¹ n-butanol within 80 h, which demonstrated the promising potential of synthetic consortia in the biorefinery (Wen et al., 2014a,b).

However, the lack of genetic techniques that enable *C. cellulovorans* to be used forms a main obstacle of the further improvement of the twin-Clostridia consortia (Wen et al., 2014a,b). Recently, Wen et al. (2017) developed an electroporation protocol and demonstrated that
several genetic tools, such as TargeTron and the CRISPR system, are functional in *C. cellulosolvens*. Based on available genetic techniques, the overall cross-species n-butanol synthesis pathway in the consortia can be split into four modules. The toxic intermediates (butyrate) metabolism module and detoxification module are installed into *C. cellulosolvens* and *C. beijerinckii*, respectively, which strengthens their feeding-detoxification relationship. Multivariate modular metabolic engineering was adapted to promote butyrate delivery towards the enhancement of n-butanol production. The engineered twin-Clostridia consortia decomposed 83.2 g l\(^{-1}\) AECC and produced 11.5 g l\(^{-1}\) n-butanol, the titre of which approximated ABE output from starchy feedstocks (Wen et al., 2017).

The performance of synthetic consortia significantly depends on the benefit of the interaction between strain species (Song et al., 2014), which can be further improved via genetic engineering of strains and optimization of culture conditions. However, the empirical strategy needs to be combined with model-driven analysis (Salimi et al., 2010; Yoo et al., 2015; Zomorrodi and Segre, 2016), to rationally design and regulate the synthetic consortia towards increased efficiency and robustness (Zomorrodi and Segre, 2016).

Butanol production with varied lignocellulosic feedstocks by CBP is depicted in Table 1. In general, artificial consortia provide a more convenient and feasible approach for butanol production via synergistic utilization of the metabolic pathways of cellulolytic and solventogenic Clostridia. Furthermore, although differences in feedstock exist, the engineered synthetic consortia offered much higher butanol titres and productivity overall, compared with co-culture with wild-type strains and pure culture of recombinant bacteria, including engineered cellulolytic and solventogenic Clostridia. However, it should be pointed out that pure culture is comparatively simple for genetic modification and optimization of culture conditions. These three approaches have the potential to realize large-scale butanol production by CBP. Challenges and bottlenecks of different approaches will likely be resolved by advances in synthetic biology techniques.

**CBP optimization by cellulolytic Clostridia chassis engineering**

Although CBP development has achieved great progress, a number of putative challenges remain that should be addressed. Current CBP-enabling strains or consortia typically exhibit low butanol production, productivity and yield (Wen et al., 2014a,b, 2019). Their further optimization is often delayed by a lack of efficient genetic tools for complex genetic modification (Wen et al., 2017). In addition, the low butanol tolerance of *Clostridium* determines the upper limit of butanol production (Patakova et al., 2018), while inefficient xylose utilization also negatively influences hemicellulose degradation and conversion (Gu et al., 2011, 2014). Driven by advancements of clostridial synthetic biotechnology, progress has been made in the improvement of cellulolytic clostridia chassis to optimize CBP.

**Development of genetic tools**

In the post-genome era, synthetic biology techniques provide resources and approaches for CBP construction and optimization. However, inefficient DNA repair and low plasmid transformation efficiency lead to lack of synthetic biology tools for *Clostridium* (Li et al., 2016a,b), which severely delays CBP development. To accelerate the bottleneck breakthrough, roadmaps or technical guides for the genetic advancement in *Clostridium* have been extensively suggested and reviewed (Pyne et al., 2014; Minton et al., 2016; Joseph et al., 2018).

Milestone studies of the development of genetic manipulation tools are shown in Fig. 2. Before the successful application of the CRISPR/Cas system to *Clostridium*, the TargeTron technology was preferred, because it is dependent on the site-specific insertion of mobile group II introns rather than on homologous recombination (Cui and Davis, 2007; Rodriguez et al., 2009). This system is very suitable for genetic modification of bacteria and was considered intractable to conventional genetic tools. Mesophilic-TargeTron and thermo-TargeTron technology applicable in *Clostridium* have been developed based on Group II A intron LtrB, as well as group II B intron Tel3c and Tel4c respectively (Heap et al., 2007; Shao et al., 2007; Mohr et al., 2013). These have been applied in many *Clostridium* for metabolic engineering and function gene identification due to the manipulation convenience and high reliability they offer (Enyeart et al., 2014; Pyne et al., 2014; Liu et al., 2015). However, a number of native drawbacks exist in the Targetron technology such as incomplete inactivation, off-target and polar effect with low frequency (Pyne et al., 2014; Liu et al., 2015a; Joseph et al., 2018).

Allelic-exchange via single/double-crossover is a classic genetic tool for precision manipulation based on homologous recombination (Argyros et al., 2011; Heap et al., 2012). This tool can realize in-frame deletion or insertion of target genes. The only obstacle is the very low frequency to obtain positive single-crossover or double-crossover mutants, due to inefficient homologous recombination and low plasmid transformation efficiency of *Clostridium* (Argyros et al., 2011; Cartman et al., 2012). Although counter-selection markers such as mazF, tdc (Al-Hinai et al., 2012; Cartman et al., 2012)
| Strain/consortium | CBP approaches | Genotype | Substrate | Titre (g l⁻¹) | Productivity (g l⁻¹ h⁻¹) | Mode | References |
|-------------------|----------------|----------|-----------|--------------|--------------------------|------|------------|
| C. cellulolyticum ATCC 35319 | +kivd yqhD alsS ilvCD | Crystalline cellulose | 0.66⁺ | 0.0031 | Batch | Higashide et al. (2011) |
| C. cellulolyticum ATCC 35319 | +ato hbd crt bcd-adhE2 | Crystalline cellulose | 0.12⁺ | 0.0025 | Batch | Gaida et al. (2016) |
| C. thermocellum DSM 1313 | +thpt+lvBNCD kivD | Crystalline cellulose | 5.4⁺ | 0.072 | Batch | Lin et al. (2015) |
| C. cellulovorans DSM 743B | +adhE2 | Crystalline cellulose | 1.42 | 0.0056 | Batch | Yang et al. (2015) |
| C. cellulovorans DSM 743B | +adhE2 | Pretreated corn cob | 3.36 | 0.028 | Batch | Ou et al. (2017) |
| C. cellulovorans DSM 743B | +adhE2 | Crystalline cellulose | 4.0 | 0.0128 | Batch | Bao et al. (2019) |
| C. cellulovorans DSM 743B | Clocele: +adhE1 ctfAB-ads | Alkali extracted corn cobs | 3.47 | 0.0413 | Batch | Wen et al. (2019) |
| C. acetobutylicum ATCC 824 | +Cel5A, +Cel8C, +Cel9M | Crystalline cellulose | ND | ND | Batch | Mingardon et al. (2011) |
| C. beijerinckii NCIMB 8052 | +celA, +celD | Microcrystalline cellulose | ND | ND | Batch | Lopez-Contreras et al. (2001) |
| C. acetobutylicum ATCC 824 | +Cel8A Cel9B CipA variants | Cellohexaose | ND | ND | Batch | Kovács et al. (2013) |
| C. acetobutylicum ATCC 824 | +Cel9G Cel48F Xyn10A CipC | Untreated wheat straw | ND | ND | Batch | Willson et al. (2016) |
| C. thermocellum and C. saccharoperbutylicolicum strain N1-4 | Wild type | Crystalline cellulose | 7.9 | 0.0299 | Batch | Shunichi et al. (2011) |
| C. cellulovorans DSM 743B and C. beijerinckii NCIMB 8052 | Clocele, +bukΔldhΔadhΔhydΔ; Cbei: +xylT ctfABΔxylR | Alkali extracted corn cobs | 11.8 | 0.0983 | Fed-batch | Wen et al. (2017) |

Clocele, C. cellulovorans; Cbei, C. beijerinckii; ND, not detected; +, overexpression; Δ, deficient or inactivation.

a. Metabolic engineering of cellulolytic Clostridia.
b. Titre of isobutanol.
c. Cellulase expression of butanol-producing Clostridia.
d. Clostridia consortia engineering.
e. Evolved strain.
and endonuclease I-SceI (Zhang et al., 2015) were applied to facilitate the screening of mutant yield during the second exchange, this still suffers from the long operation cycle and low first single-crossover efficiency.

Since 2015, the CRISPR-Cas system has been adapted to increase the frequency of mutants with single- or double-crossover (Wang et al., 2015). However, the low homologous recombination efficiency indicates that DNA double-strand breaks (DSB) cannot be repaired (Koh et al., 2014; Sun et al., 2017); moreover, the low plasmid transformation efficiency further negatively affects the screening of positive transformants. To overcome this particular problem, Xu et al. (2015a,b,c) and Li et al. (2016a,b) introduced Cas9 nickase to C. cellulolyticum and solventogenic Clostridia, respectively, which resulted in a single nick, which triggered homologous recombination and enhanced genome editing efficiency.

Furthermore, Li et al. (2019) developed a base editing system based on cytidine deaminase (rat Apobec1), uracil DNA glycosylase inhibitor (UGI) and CRISPR-Cas9D10A system. Apobec1 and UGI can efficiently convert specific C-G nucleotide base pairs within the CRISPR-Cas9 targeting window sequence to T-A, which can create either missense mutations or null mutations in a gene. It is as precise as Cas9-mediated genome editing, but it does not require DNA cleavage. Therefore, it requires no DNA repair templates. It is very simple to design and theoretically programmable to target all genes in Clostridium.

Except for genome editing, the CRISPR-Cas system can also be used to regulate gene expression, which is similar to classical antisense RNA technology (Desai and Papoutsakis, 1999; Tummal et al., 2003), as well as the recent emergence of sRNA-based gene downregulation techniques (Little et al., 2018). The CRISPR-Cas system can also be combined with traditional transposon technology, which introduces random insertion inactivation into the genome by transposase and constructs a mutant library for screening for specific phenotypes (Lanckriet et al., 2009; Vidal et al., 2009; Cartman and Minton, 2010). More recently, Strecker and Ladha (2019) developed a RNA-guided DNA insertion method that uses CRISPR-associated transposases in Escherichia coli. These can be introduced into Clostridium and facilitate the development of programmable genetic manipulation tools based on non-homologous recombination.

In general, the CRISPR-Cas system is a disruptive technology that almost completely rewrites the patterns of genetic manipulation technology development in Clostridium. However, the homologous recombination capacity of Clostridium itself still requires further improvement by introducing genetic parts such as RecT (Dong et al., 2014).
**Pentose metabolic engineering**

Most Clostridia cannot efficiently utilize pentose (mainly xylose) due to carbon catabolite repression (CCR) (Bruder et al., 2015; Mitchell, 2016), which severely reduces the butanol yield from lignocellulosic biomass, because xylose is the second-most abundant sugar of the lignocellulosic hydrolysates (Jin et al., 2015). The first step to improve the efficiency of xylose uptake is to understand the CCR system, xylose metabolic pathways and transporters in Clostridium. The next step is their modification, as shown in Fig. 1.

Carbon catabolite repression is mediated by the catabolite control protein A (CcpA), which can form a complex with a serine-phosphorylated histone-containing protein (HPr) (Warner and Lolkema, 2003). The resulting complex binds at catabolite responsive element (CRE) sites within the promoter region or the coding sequence of transcriptional units to inhibit transcription (Lorca et al., 2005). Inactivation of ccpA or glcG (encoding EII of the phosphotransferase system (PTS)) can overcome xylose metabolism inhibition (Ren et al., 2010; Xiao et al., 2011). Refined modification such as generation of a ccpA mutant, deficient in co-effecter HPr-Ser46-P binding (Wu et al., 2015), repression of hprK gene (encoding HPr phosphatase/kinase, HPrK) by CRISPR/dCas9 (Bruder et al., 2016) or elimination of CcpA binding sites in xylose and pentose phosphate pathway operon (Bruder et al., 2015) have also been demonstrated. These worked well in the simultaneous utilization of xylose and glucose.

The xylose metabolism is also regulated by other mechanisms. Hu et al. (2011) and Xiao et al. (2011) identified putative transcriptional regulators such as CEA_G2622 and Cbei_2385 (xylR), which are involved in the xylose metabolism in C. acetobutylicum EA 2018 and C. beijerinckii NCIMB 8052 respectively. The inactivation of xylR or araR can upregulate the transcription of the xylose isomerase gene (xylA-II, CAC2610 and xylos isomerase) and the xylose kinase gene (xylB and CAC2612), thus promoting the utilization of the xylose metabolism.

Gu et al. (2010) identified several key genes involved in xylose metabolism in C. acetobutylicum using Targetron combined with other genetic and biochemical methods. The xylose metabolic pathway was reconstructed in C. acetobutylicum according to previous studies and comparative genomic predictions. Subsequently, Liu et al. (2012) reported how C. acetobutylicum metabolizes xylose using both the pentose phosphate pathway and the phosphoketolase pathway via isotope tracing techniques. Based on this, xylose utilization efficiency could be significantly improved by overexpressing genes involved in the xylose metabolic pathway (Gu et al., 2009; Xiao et al., 2011, 2012).

Furthermore, transporter engineering has also achieved great progress. Xiao et al. (2012) identified a D-xylose proton-symporter (encoded by gene cbei-0109) and overexpressed it to enhance the xylose uptake in C. beijerinckii. Recently, Sun et al. (2015) demonstrated that the hexa-protein module XylFII-LytS/YesN-XylFGH is associated with xylose utilization in C. beijerinckii. Li et al. (2017) further confirmed that it is a new ‘three-component’ xylose response and regulatory system whose molecular mechanisms were deeply analysed.

The above-mentioned xylose metabolism-related functional genes basically mapped the framework of pentose transport, metabolism and regulation in solventogenic Clostridia (Fig. 1). Studies of xylose metabolic engineering in solventogenic Clostridia provide a reference for cellulolytic clostridia used in CBP (Xiong et al., 2018).

**Improvement of butanol tolerance**

Butanol toxicity is considered a major barrier for butanol accumulation in fermentation media to high titre since this might cause microbial growth inhibition and cell death (Papoutsakis, 2008; Patakova et al., 2018). To date, n-butanol output did not exceed 21 g l−1, which was achieved by C. beijerinckii BA101in 1999 (Chen and Blaschek, 1999). A low butanol titre increases the cost of product separation, which impedes the economics of the CBP process.

Traditional random mutations and optimization of culture methods have often been adapted to obtain high butanol-producing and tolerant strains, such as BA101, GS4-3, SA-1 and BKM19 (Chen and Blaschek, 1999; Jang et al., 2013; Sandoval-Espinola et al., 2013; Li et al., 2016a,b). These provide an opportunity to observe, describe or hypothesize the butanol tolerance mechanism.

Recently, many synthetic biological techniques, such as comparative genomics, transcriptomics, metabolomics and proteomics, have attempted to analyse the mechanisms by which Clostridia respond to butanol stress (and to identify putative gene targets). The complex responses are reflected in the aspects of overall cell, cell envelope and cytoplasm (Liu et al., 2017; Patakova et al., 2018), including cell wall and cell membrane modifications (Cfa, a cyclopropane fatty acid synthase gene (Zhao et al., 2003) and Lyt-1, a cell wall lytic enzymes gene (Van Der Westhuizen et al., 1982)), stress protein production (GroESL and DnaK (Jones et al., 2016; Liao et al., 2017)), butanol excretion by efflux pumps (formate transporter FocA (Reyes et al., 2011) and multidrug efflux pump AcrB (Fisher et al., 2014)), and other mechanisms involved in n-butanol tolerance (AdhE mutation (Tian et al., 2019) and Spo0A overexpression (Alsaker et al., 2004)).
Based on the above known or not yet fully understood mechanisms (and gene targets), synthetic biology strategies have been introduced to enhance butanol tolerance and thus further understand the underlying mechanisms. Xu et al. (2015a,b,c) obtained the C. acetobutylicum mutant JB200 with high butanol production of 21 g l\(^{-1}\) through evolution in a fibrous bed bioreactor (FBB). Comparative genomic analysis and reverse metabolic engineering confirmed that the cac_3319 gene encodes histidine kinase (HK), which is responsible for the enhanced n-butanol tolerance. In a further adaptation study in a chemostat, Tian et al. (2019) showed that a D494G mutation in the adhE gene or adhE deletion can enhance the butanol tolerance of C. thermocellum from 5 to 15 g l\(^{-1}\).

More recently, Wen et al. (2019) developed a novel adaptive laboratory evolution (ALE) approach based on spo0A-deficient strains to enhance the positive ratio of evolved mutants. The generated strain can tolerate 12 g l\(^{-1}\) n-butanol. Moreover, Wen et al. adapted the evolved strain as host for further metabolic engineering, which achieved much higher butanol production than using the wild-type strain as chassis for metabolic engineering. This indicates that the butanol tolerance enhancement of Clostridium can promote butanol production (Nicolaou et al., 2010). However, it must be pointed out that butanol tolerance and butanol production are not always tightly associated (Liu et al., 2013). Butanol tolerance only determines the upper limit of butanol production and is not the decisive factor for high butanol production.

**Conclusions and outlook**

Consolidated bioprocessing is the logic end of the evolution of biorefinery routes towards the production of cellulosic butanol. Here, cellulolytic Clostridia are more important than saccharolytic and gas-fermenting Clostridium species. CBP development based on cellulolytic Clostridia has achieved great progress in the aspects of butanol pathway engineering of native cellulolytic clostridia. This enables solventogenic Clostridia to be cellulolytic, as well as the engineering of consortia composed of cellulolytic and butanol-producing Clostridia. However, improvement and optimization of CBP-enabling Clostridia (consortia) have been delayed due to a lack of efficient genetic tools. Moreover, low butanol tolerance and inefficient xylose utilization in Clostridia negatively affect butanol titre and yield respectively. In addition to these bottlenecks, many other problems need to be addressed, such as hydrolysis inhibitors resistance, balance of species population competition and cooperation.

Recently, advances have been achieved in the study of synthetic biotechnology accelerated CBP based on cellulolytic Clostridia. For instance, programmable genome editing tool-assisted metabolic engineering provided a variety of reliable approaches and resources for the construction and optimization of designer cellulosomes, microbes or consortia. Moreover, omics data (genome, transcriptome, proteome and metabolome) and various models (genome-scale metabolic network models (GSSM), kinetic models and population interaction models) will promote the design and re-construction of CBP-enabling Clostridia. CBP driven by clostralid synthetic biotechnology is a promising technique to achieve large-scale production of cellulosic butanol in the near future.

**Acknowledgements**

This work is supported by the grant from the National Natural Science Foundation of China (21706133, 21825804, 31670094), the Fundamental Research Funds for the Central Universities (3091801130), Natural Science Foundation of Shanghai (Shanghai Natural Science Foundation, 18ZR1446500), Key Laboratory of Biomass Chemical Engineering of Ministry of Education, Zhejiang University (2018BCE003).

**Conflict of interest**

None declared.

**References**

Al-Hinai, M.A., Fast, A.G., and Papoutsakis, E.T. (2012) Novel system for efficient isolation of clostridium double-crossover allelic exchange mutants enabling markerless chromosomal gene deletions and DNA integration. *Appl Environ Microbiol* 78: 8112–8121.

Alsaker, K.V., Spitzer, T.R., and Papoutsakis, E.T. (2004) Transcriptional analysis of spo0A overexpression in *Clostridium acetobutylicum* and its effect on the cell’s response to butanol stress. *J Bacteriol* 186: 1959–1971.

Argyros, D.A., Tripathi, S.A., Barrett, T.F., Rogers, S.R., Feinberg, L.F., Olson, D.G., et al. (2011) High ethanol titers from cellulose by using metabolically engineered thermophilic, anaerobic microbes. *Appl Environ Microbiol* 77: 8288–8294.

Bao, T., Zhao, J., Li, J., Liu, X., and Yang, S.-T. (2019) n-Butanol and ethanol production from cellulose by *Clostridium cellulovorans* overexpressing heterologous aldehyde/ alcohol dehydrogenases. *Biorec Technol* 285: 121316.

Bayer, E.A., Lamed, R., and Himmel, M.E. (2007) The potential of cellulosases and cellulosomes for cellulosic waste management. *Curr Opin Biotechnol* 18: 237–245.

Bruder, M., Moo-Young, M., Chung, D.A., and Chou, C.P. (2015) Elimination of carbon catabolite repression in *Clostridium acetobutylicum*-a journey toward simultaneous use of xylose and glucose. *Appl Microbiol Biotechnol* 99: 7579–7588.
Bruder, M.R., Pyne, M.E., Moo-Young, M., Chung, D.A., and Chou, C.P. (2016) Extending CRISPR-Cas9 technology from genome editing to transcriptional engineering in the genus clostridium. *Appl Environ Microbiol* 82: 6109–6119.

Cartman, S.T., and Minton, N.P. (2010) A mariner-based transposon system for in vivo random mutagenesis of *Clostridium difficile*. *Appl Environ Microbiol* 76: 1103–1109.

Cartman, S.T., Kelly, M.L., Heeg, D., Heap, J.T., and Minton, N.P. (2012) Precise manipulation of the *Clostridium difficile* chromosome reveals a lack of association between the tcdC genotype and toxin production. *Appl Environ Microbiol* 78: 4683–4690.

Chen, C.K., and Blaschek, H.P. (1999) Acetate enhances solvent production and prevents degeneration in *Clostridium beijerinckii* BA101. *Appl Microbiol Biotechnol* 52: 170–173.

Chen, C.T., and Liao, J.C. (2016) Frontiers in microbial 1-butanol and isobutanol production. *Fems Microbiology Letters* 363: 1–13.

Cui, X., and Davis, G. (2007) Mobile group II intron targeting: applications in prokaryotes and perspectives in eukaryotes. *Fronti Biosci* 12: 4972–4985.

Desai, R.P., and Papoutsakis, E.T. (1999) Antisense RNA strategies for metabolic engineering of *Clostridium acetobutylicum*. *Appl Environ Microbiol* 65: 936–945.

Dong, H., Tao, W., Gong, F., Li, Y., and Zhang, Y. (2014) A functional recT gene for recombineering of *Clostridium*. *J Biotecnol* 173: 65–67.

Durre, P. (2016) Butanol formation from gaseous substrates. *FEMS Microbiol Lett* 363: 1–7.

Enyeart, P.J., Mohr, G., Ellington, A.D. and Lambowitz, A.M. (2014) Biotechnological applications of mobile group II introns and their reverse transcriptases: gene targeting, RNA-seq, and non-coding RNA analysis. *Mobile DNA* 5: 1–19.

Fierobe, H.P., Mingardon, F., and Chanal, A. (2012) Engineering cellulase activity into *Clostridium acetobutylicum*. In *Cellulases*. Gilbert, H.J. (ed). San Diego: Elsevier Academic Press Inc, pp. 301–316.

Fisher, M.A., Boyarsky, S., Yamada, M.R., Kong, N., Bauer, S., and Tullman-Ercek, D. (2014) Enhancing tolerance to short-chain alcohols by engineering the *Escherichia coli* AcrB efflux pump to secrete the non-native substrate n-butanol. *ACS Synth Biol* 3: 30–40.

Gaida, S.M., Liedtke, A., Jengetes, A.H.W., Engels, B., and Jennnewein, S. (2016) Metabolic engineering of *Clostridium cellulolyticum* for the production of n-butanol from crystalline cellulose. *Microb Cell Fact* 15: 1.

Gu, Y., Li, J., Zhang, L., Chen, J., Niu, L., Yang, Y., et al. (2009) Improvement of xylose utilization in *Clostridium acetobutylicum* via expression of the talA gene encoding transaldolase from *Escherichia coli*. *J Biotecnol* 143: 284–287.

Gu, Y., Ding, Y., Ren, C., Sun, Z., Rodionov, D.A., Zhang, W.W., et al. (2010) Reconstruction of xylose utilization pathway and regulators in *Firmicutes*. *BMC Genom* 11: 255.

Gu, Y., Jiang, Y., Wu, H., Liu, X., Li, Z., Li, J., et al. (2011) Economical challenges to microbial producers of butanol: feedstock, butanol ratio and titer. *Biotecnol J* 6: 1349–1357.

Gu, Y., Jiang, Y., Yang, S., and Jiang, W.H. (2014) Utilization of economical substrate-derived carbohydrates by solventogenic clostridia: pathway dissection, regulation and engineering. *Curr Opin Biotechnol* 29: 124–131.

den Haan, R., van Rensburg, E., Rose, S.H., Gorgens, J.F., and van Zyl, W.H. (2015) Progress and challenges in the engineering of non-cellulolytic microorganisms for consolidated bioprocessing. *Curr Opin Biotechnol* 33: 32–38.

Heap, J.T., Pennington, O.J., Cartman, S.T., Carter, G.P., and Minton, N.P. (2007) The Clostron: a universal gene knock-out system for the genus *Clostridium*. *J Microbial Methods* 70: 452–464.

Heap, J.T., Ehsaan, M., Cooksley, C.M., Ng, Y.K., Cartman, S.T., Winzer, K., and Minton, N.P. (2012) Integration of DNA into bacterial chromosomes from plasmids without a counter-selection marker. *Nucleic Acids Res* 40: e59.

Higashide, W., Li, Y., Yang, Y., and Liao, J.C. (2011) Metabolic engineering of *Clostridium cellulolyticum* for production of isobutanol from cellulose. *Appl Environ Microbiol* 77: 2727–2733.

Hu, S., Zheng, H., Gu, Y., Zhao, J., Zhang, W., Yang, Y., et al. (2011) Comparative genomic and transcriptomic analysis revealed genetic characteristics related to solvent formation and xylose utilization in *Clostridium acetobutylicum* EA 2016. *BMC Genom* 12: 93.

Huang, H., Chai, C., Li, N., Rowe, P., Minton, N.P., Yang, S., et al. (2016) CRISPR/Cas9-based efficient genome editing in *Clostridium ljungdahlii*, an autotrophic gas-fermenting bacterium. *Acs Synthetic Biology* 5: 1355–1361.

Huang, H., Chai, C., Yang, S., Jiang, W., and Gu, Y. (2019) Phage serine integrase-mediated genome engineering for efficient expression of chemical biosynthetic pathway in gas-fermenting *Clostridium ljungdahlii*. *Metab Eng* 52: 293–302.

Hyeon, J.E., Jeon, S.D., and Han, S.O. (2013) Cellulosome-based, Clostridium-derived multi-functional enzyme complexes for advanced biotechnology tool development: advances and applications. *Biotechnol Adv* 31: 936–944.

Jang, Y.S., Malaviya, A., and Lee, S.Y. (2013) Acetone-butanol-ethanol production with high productivity using *Clostridium acetobutylicum* BKM19. *Biotechnol Bioeng* 110: 1646–1653.

Jiang, Y., Liu, J.L., Jiang, W.H., Yang, Y.L., and Yang, S. (2015) Current status and prospects of industrial bio-production of n-butanol in China. *Biotechnol Adv* 33: 1493–1501.

Jiang, Y., Guo, D., Lu, J., Duerre, P., Dong, W., Yan, W., et al. (2018a) Consolidated bioprocessing of butanol production from xylan by a thermophilic and butanologenic *Thermoanaerobacterium* sp M5. *Biotechnol Biofuels* 11: 1–14.

Jiang, Y., Zhang, T., Lu, J., Duerre, P., Zhang, W., Dong, W., et al. (2018b) Microbial co-culturing systems: butanol production from organic wastes through consolidated bioprocessing. *Appl Microbiol Biotechnol* 102: 5419–5425.

Jin, M., Slingier, P.J., Dien, B.S., Waghmode, S., Moser, B.R., Orjuela, A., et al. (2015) Microbial lipid-based lignocellulosic biofinery: feasibility and challenges. *Trends Biotechnol* 33: 43–54.

Jones, A.J., Venkataramanan, K.P. and Papoutsakis, T. (2016) Overexpression of two stress-responsive, small,
non-coding RNAs, 6S and tmRNA, imparts butanol tolerance in *Clostridium acetobutylicum*. FEMS Microbiol Lett **363**: 1–6.

Joseph, R.C., Kim, N.M., and Sandoval, N.R. (2018) recent developments of the synthetic biology toolkit for Clostridium. Front Microbiol **9**: 1–13.

Koh, C.M.J., Liu, Y.B., Moehnins Du, M.G. and Ji, L.H. (2014) Molecular characterization of KU70 and KU80 homologues and exploitation of a KU70-deficient mutant for improving gene deletion frequency in *Rhodosporidium toruloides*. BMC Microbiol **14**: 1–10.

Kovács, K., Wilsson, B.J., Schwarz, K., Heap, J.T., Jackson, A., Bolam, D.N., et al. (2013) Secretion and assembly of functional mini-cellulosomes from synthetic chromosomal operons in *Clostridium acetobutylicum* ATCC 824. Biotechnol Biofuels **6**: 117.

Lancrriet, A., Timbermont, L., Happenon, L.J., Pajunen, M.I., Pasmans, F., Haesebrouck, F., et al. (2009) Generation of single-copy transposon insertions in *Clostridium perfringens* by electroporation of phage mu DNA transposition complexes. Appl Environ Microbiol **75**: 2638–2642.

Li, Q., Chen, J., Minton, N.P., Zhang, Y., Wen, Z.Q., Liu, J.L., et al. (2016a) CRISPR-based genome editing and expression control systems in *Clostridium acetobutylicum* and *Clostridium beijerinckii*. Biotechnol J **11**: 961–972.

Li, S.B., Qian, Y., Liang, Z.W., Guo, Y.,Zhao, M.M. and Pang, Z.W. (2016b) Enhanced butanol production from cassava with *Clostridium acetobutylicum* by genome shuffling. *World J Microbiol Biotechnol* **32**: 1–10.

Li, J.X., Wang, C.Y., Yang, G.H., Sun, Z., Guo, H., Shao, K., et al. (2017) Molecular mechanism of environmental D-xylose perception by a XyFlII-LytS complex in bacteria, pp. 8235–8240.

Li, Q., Seys, F.M., Minton, N.P., Yang, J., Jiang, Y., Jiang, W. and Yang, S. (2019) CRISPR-Cas9(D10A) nickase-assisted base editing in the solvent producer *Clostridium beijerinckii*. Biotechnol Bioeng **116**: 1475–1483.

Liao, C., Seo, S.O., Celik, V., Liu, H., Kang, W., Wang, Y., et al. (2015) Integrated, systems metabolic picture of acetone-butanol-ethanol fermentation by Clostridium acetobutylicum, pp. 8505–8510.

Liao, Z., Zhang, Y., Luo, S., Suo, Y., Zhang, S., and Wang, J. (2017) Improving cellular robustness and butanol titers of *Clostridium acetobutylicum* ATCC824 by introducing heat shock proteins from an extremophilic bacterium. J Biotechnol **252**: 1–10.

Lin, P.P., Mi, L., Morioka, A.H., Yoshino, K.M., Konishi, S., Xu, S.C., et al. (2015) Consolidated bioprocessing of cel lulose to isobutanol using *Clostridium thermocellum*. Metab Eng **31**: 44–52.

Little, G.T., Willson, B.J., Heap, J.T., Winzer, K. and Minton, N.P. (2016) The butanol producing microbe *Clostridium beijerinckii* NCIMB 14988 manipulated using forward and reverse genetic tools. Biotechnol J **13**: 1–9.

Liu, L., Zhang, L., Tang, W., Gu, Y., Hua, Q., Yang, S., et al. (2012) Phosphoketolase pathway for xylose catabolism in *Clostridium acetobutylicum* revealed by C-13 metabolic flux analysis. J Bacteriol **194**: 5413–5422.

Liu, X.B., Gu, Q.Y., and Yu, X.B. (2013) Repetitive domestication to enhance butanol tolerance and production in *Clostridium acetobutylicum* through artificial simulation of bio-evolution. Bioresour Technol **130**: 638–643.

Liu, Y.-J., Zhang, J., Cui, G.-Z., and Cui, Q. (2015) Current progress of targetron technology: development, improvement and application in metabolic engineering. Biotechnol J **10**: 855–865.

Liu, S., Qureshi, N. and Hughes, S.R. (2017) Progress and perspectives on improving butanol tolerance. *World J Microbiol Biotechnol* **33**: 855–865.

Liu, H., Zhang, J., Yuan, J., Jiang, X., Jiang, L., Zhao, G., et al. (2019) Omics-based analyses revealed metabolic responses of *Clostridium acetobutylicum* to lignocellulose-derived inhibitors furfural, formic acid and phenol stress for butanol fermentation. Biotechnol Biofuels **12**: 1–20.

Lopez-Contreras, A.M., Martens, A.A., Sziarto, N., Mooibroek, H., Claassen, P.A.M., van Der Oost, J., and De Vos, W.M. (2003) Production by *Clostridium acetobutylicum* ATCC 824 of CelG, a cellulosomal glycoside hydrolase belonging to family 9. *Appl Environ Microbiol* **69**: 869–877.

Lopez-Contreras, A.M., Gabor, K., Martens, A.A., Renckens, B.A., Claassen, P.A., Van Der Oost, J., and De Vos, W.M. (2004) Substrate-induced production and secretion of cellulases by *Clostridium acetobutylicum*. *Appl Environ Microbiol* **70**: 5238–5243.

Lorca, G.L., Chung, Y.J., Barabote, R.D., Weyler, W., Schilling, C.H., and Saier, M.H. Jr (2005) Catabolite repression and activation in *Bacillus subtilis*: dependency on CcpA. HPr, and HprK, J Bacterial **187**: 7826–7839.

Lynd, L.R., van Zyl, W.H., McBride, J.E., and Laser, M. (2005) Consolidated bioprocessing of cellulose biomass: an update. *Curr Opin Biotechnol* **16**: 577–583.

McAllister, K.N. and Sorg, J.A. (2019) CRISPR genome editing systems in the genus Clostridium: a timely advancement. *J Bacteriol* **201**: e00219–19.

Mingardon, F., Perret, S., Belaich, A., Tardif, C., Belaich, J.P., and Fierobe, H.P. (2005) Heterologous production, assembly, and secretion of a minicellulosome by *Clostridium acetobutylicum* ATCC 824. *Appl Environ Microbiol* **71**: 1215–1222.

Mingardon, F., Chanal, A., Tardif, C., and Fierobe, H.P. (2011) The issue of secretion in heterologous expression of clostridium cellulolyticum cellulase-encoding genes in *Clostridium acetobutylicum* ATCC 824. *Appl Environ Microbiol* **77**: 2831–2838.

Minton, N.P., Ehsaan, M., Humphreys, C.M., Little, G.T., Baker, J., Henstra, A.M., et al. (2016) A roadmap for gene system development in Clostridium. *Anaerobe* **41**: 104–112.

Mitchell, W.J. (2016) Sugar uptake by the solventogenic clostridia. *World J Microbiol Biotechnol* **32**: 1–10.

Mohr, G., Hong, W., Zhang, J., Cui, G.-Z., Yang, Y., Cui, Q., et al. (2013) A targetron system for gene targeting in thermostable *Clostridium thermocellum* cocultured with *Rhodosporidium toruloides*. FEMS Microbiol Lett **322**: 1–10.

Moon, H.G., Jang, Y.S., Cho, C., Lee, J., Binkley, R. and Lee, S.Y. (2016) One hundred years of clostridial butanol fermentation. FEMS Microbiol Lett **363**: 1–15.

Nakayama, S., Kiyoshi, K., Kadokura, T., and Nakazato, A. (2011) Butanol production from crystalline cellulose by cocultured *Clostridium thermocellum* and *Clostridium acetobutylicum* through artificial simulation of bio-evolution. Bioresour Technol **130**: 638–643.
saccharoperbutylicetonicum N1-4. Appl Environ Microbiol 77: 6470–6475.

Ng, T., Weimer, P., and Zeikus, J. (1977) Cellulolytic and physiological properties of Clostridium thermocellum. Arch Microbiol 114: 1–7.

Nguyen, N.P.T., Raynaud, C., Meynial-Salles, I. and Soucaille, P. (2018) Reviving the Weizmann process for commercial n-butanol production. Nat Commun 9: 1–8.

Nicolaou, S.A., Gaida, S.M., and Papoutsakis, E.T. (2010) A comparative view of metabolite and substrate stress and tolerance in microbial bioprocessing: from biofuels and chemicals, to biocatalysis and bioremediation. Metab Eng 12: 307–331.

Olson, D.G., McBride, J.E., Shaw, A.J., and Lynd, L.R. (2012) Recent progress in consolidated bioprocessing. Curr Opin Biotechnol 23: 396–405.

Papoutsakis, E.T. (2008) Engineering solventogenic clostridia. Curr Opin Biotechnol 19: 420–429.

Patakova, P., Kolek, J., Sedlar, K., Koscova, P., Branska, B., Kupkova, K., et al. (2018) Comparative analysis of high butanol tolerance and production in clostridia. Biotechnol Adv 36: 721–738.

Petitdemange, E., Caillet, F., Giallo, J., and Gaudin, C. (2010) Identiﬁcation and inactivation of pleiotropic regulators in Clostridium cellulovorans sp. nov. Appl Environ Microbiol 48: 88–93.

Shao, L., Hu, S., Yang, Y., Gu, Y., Chen, J., Yang, Y., et al. (2007) Targeted gene disruption by use of a group II intron (targetron) vector in Clostridium acetobutylicum. Cell Res 17: 963–965.

Slet, R., Mah, R.A., and Robinson, R. (1984) Isolation and characterization of an anaerobic, cellulolytic bacterium, Clostridium cellulovorans sp. nov. Appl Environ Microbiol 48: 88–93.

Song, H., Ding, M.-Z., Jia, X.-Q., Ma, Q., and Yuan, Y.-J. (2014) Synthetic microbial consortia: from systematic analysis to construction and applications. Chem Soc Rev 43: 6954–6981.

Strecker, J. and Ladha, A. (2019) RNA-guided DNA insertion with CRISPR-associated transposases. Science 365: 48–53.

Sun, Z., Chen, Y., Yang, C., Yang, S., Gu, Y., and Jiang, W. (2015) A novel three-component system-based regulatory model for D-xylose sensing and transport in Clostridium beijerinckii. Mol Microbiol 95: 576–589.

Tian, L., Cervenka, N.D., Low, A.M., Olson, D.G. and Lynd, L.R. (2019) A mutation in the AdhE alcohol dehydrogenase of Clostridium thermocellum increases tolerance to several primary alcohols, including isobutanol, n-butanol and ethanol. Sci Rep 9: 1–7.

Thomas, L., Joseph, A., and Gottumukkala, L.D. (2014) Xylanase and cellulase systems of Clostridium sp.: an insight on molecular approaches for strain improvement. Biorese Technol 158: 343–350.

Van Der Westhuizen, A., Jones, D.T., and Woods, D.R. (1982) Autolytic activity and butanol tolerance of Clostridium acetobutylicum. Appl Environ Microbiol 44: 1277–1281.

Vidal, J.E., Chen, J., Li, J., and McClane, B.A. (2009) Use of an EZ-Tn5-based random mutagenesis system to identify a novel toxin regulatory locus in Clostridium perfringens strain 13. PLoS ONE 4: e6232.

Warner, J.B., and Lolkema, J.S. (2015) A synthetic O2-tolerant butanol pathway exploiting native fatty acid biosynthesis in Escherichia coli. Nat Commun 6: 641.

Wen, Z., Wu, M., Lin, Y., Yang, L., Lin, J., and Cen, P. (2014a) Artificial symbiosis for acetone-butanol-ethanol (ABE) fermentation from alkali extracted deshelled corn cobs by co-culture of Clostridium beijerinckii and Clostridium cellulovorans. Microbiol Cell Fact 13: 1–11.

Wen, Z., Wu, M., Lin, Y., Yang, L., Lin, J., and Cen, P. (2014b) A novel strategy for sequential co-culture of Clostridium thermocellum and Clostridium beijerinckii to produce solvents from alkali extracted corn cobs. Process Biochem 49: 1941–1949.
Wen, Z., Minton, N.P., Zhang, Y., Li, Q., Liu, J., Jiang, Y., and Yang, S. (2017) Enhanced solvent production by metabolic engineering of a twin-clostridial consortium. *Metab Eng* **39**: 38–48.

Wen, Z., Ledesma-Amaro, R., Lin, J., Jiang, Y. and Yang, S. (2019) Improved n-butanol production from *Clostridium cellulovorans* by integrated metabolic and evolutionary engineering. *Appl Environ Microbiol* **85**: e02560–18.

Willson, B.J., Kovacs, K., Wilding-Steele, T., Markus, R., Winzer, K., and Minton, N.P. (2016) Production of a functional cell wall-anchored minicellulosome by recombinant *Clostridium acetobutylicum* ATCC 824. *Biotechnol Biofuels* **9**: 109.

Wu, Y., Yang, Y.P., Ren, C., Yang, C., Yang, S., Gu, Y., and Jiang, W.H. (2015) Molecular modulation of pleiotropic regulator CcpA for glucose and xylose couterilization by solvent-producing *Clostridium acetobutylicum*. *Metab Eng* **28**: 169–179.

Xiao, H., Gu, Y., Ning, Y., Yang, Y., Mitchell, W.J., Jiang, W., and Yang, S. (2011) Confirmation and elimination of xylose metabolism bottlenecks in glucose phosphoenolpyruvate-dependent phosphotransferase system-deficient *Clostridium acetobutylicum* for simultaneous utilization of glucose. *Xylose, and Arabinose, Applied and Environmental Microbiology* **77**: 7886–7895.

Xiao, H., Li, Z.L., Jiang, Y., Yang, Y.L., Jiang, W.H., Gu, Y., and Yang, S. (2012) Molecular engineering of D-xylose pathway in *Clostridium beijerinckii* to optimize solvent production from xylose mother liquid. *Metab Eng* **14**: 569–578.

Xin, F., Dong, W., Zhang, W., Ma, J., and Jiang, M. (2019) Biobutanol production from crystalline cellulose through consolidated bioprocessing. *Trends Biotechnol* **37**: 167–180.

Xiong, W., Reyes, L.H., Michener, W.E., Maness, P.-C., and Chou, K.J. (2018) Engineering cellulolytic bacterium *Clostridium thermocellum* to co-ferment cellulose- and hemicellulose-derived sugars simultaneously. *Biotechnol Bioeng* **115**: 1755–1763.

Xu, C., Huang, R., Teng, L., Jing, X., Hu, J., Cui, G., et al. (2015a) Cellulose stoichiometry in *Clostridium cellulolyticum* is regulated by selective RNA processing and stabilization. *Nat Commun* **6**: 6900.

Xu, M.M., Zhao, J.B., Yu, L., Tang, I.C., Xue, C., and Yang, S.T. (2015b) Engineering *Clostridium acetobutylicum* with a histidine kinase knockout for enhanced n-butanol tolerance and production. *Appl Microbiol Biotechnol* **99**: 1011–1022.

Xu, T., Li, Y.C., Shi, Z., Hemme, C.L., Li, Y., Zhu, Y.H., et al. (2015c) Efficient genome editing in *Clostridium cellulolyticum* via CRISPR-Cas9 nickase. *Appl Environ Microbiol* **81**: 4423–4431.

Yang, X., Xu, M., and Yang, S.T. (2015) Metabolic and process engineering of *Clostridium cellulovorans* for biofuel production from cellulose. *Metab Eng* **28**: 39–48.

Yoo, M., Bestel-Corre, G., Croux, C., Riviere, A., Meynial-Salles, I., and Soucaille, P. (2015) A Quantitative System-Scale Characterization of the Metabolism of *Clostridium acetobutylicum*, *mBio* **6**: e01808–e01815.

Yu, E., Chan, M.-H., and Saddler, J. (1985) Butanol production from cellulosic substrates by sequential co-culture of *Clostridium thermocellum* and *Clostridium acetobutylicum*. *Biotech Lett* **7**: 509–514.

Zhang, N., Shao, L., Jiang, Y., Gu, Y., Li, Q., Liu, J., et al. (2015) I-SceI-mediated scarless gene modification via allelic exchange in *Clostridium*. *J Microbiol Methods* **108**: 49–51.

Zuroff, T.R., and Curtis, W.R. (2012) Developing symbiotic consortia for lignocellulosic biofuel production. *Appl Microbiol Biotechnol* **93**: 1423–1435.