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REVIEW

Recent advances in *n*-butanol and butyrate production using engineered *Clostridium tyrobutyricum*

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Abstract

Acidogenic clostridia naturally producing acetic and butyric acids has attracted high interest as a novel host for butyrate and *n*-butanol production. Among them, *Clostridium tyrobutyricum* is a hyper butyrate-producing bacterium, which re-assimilates acetate for butyrate biosynthesis by butyryl-CoA/acetate CoA transferase (CoAT), rather than the phosphotransbutyrylase-butyrate kinase (PTB-BK) pathway widely found in clostridia and other microbial species. To date, *C. tyrobutyricum* has been engineered to overexpress a heterologous alcohol/aldehyde dehydrogenase, which converts butyryl-CoA to *n*-butanol. Compared to conventional solventogenic clostridia, which produce acetone, ethanol, and butanol in a biphasic fermentation process, the engineered *C. tyrobutyricum* with a high metabolic flux toward butyryl-CoA produced *n*-butanol at a high yield of > 0.30 g/g and titer of > 20 g/L in glucose fermentation. With no acetone production and a high C4/C2 ratio, butanol was the only major fermentation product by the recombinant *C. tyrobutyricum*, allowing simplified downstream processing for product purification. In this review, novel metabolic engineering strategies to improve *n*-butanol and butyrate production by *C. tyrobutyricum* from various substrates, including glucose, xylose, galactose, sucrose, and cellulosic hydrolysates containing the mixture of glucose and xylose, are discussed. Compared to other recombinant hosts such as *Clostridium acetobutylicum* and *Escherichia coli*, the engineered *C. tyrobutyricum* strains with higher butyrate and butanol titers, yields and productivities are the most promising hosts for potential industrial applications.

Keywords Acidogenic clostridia · Butyrate · Butanol · *Clostridium tyrobutyricum* · Lignocellulosic biomass · Metabolic engineering

Introduction

Butyric acid, a short-chain volatile fatty acid with broad applications in the chemical industry, is currently produced primarily by chemical synthesis from petroleum-based feedstocks (Wang et al. 2016; Zigova and Sturdik 2000). However, there is a high demand for biobased butyric acid as a natural ingredient for use in animal feeds, cosmetics, foods, and pharmaceuticals (Dwidar et al. 2012; Jha et al. 2014). Therefore, there is an urgent need in developing bacterial strains for butyric acid production from sugars and renewable feedstocks in fermentation (Jiang et al. 2018). *Clostridium tyrobutyricum*, a Gram-positive, strictly anaerobic acidogen, produces acetic and butyric acids as the main products from glucose (see Fig. 1). In the dairy industry, *C. tyrobutyricum* is recognized as the main microbial contaminant affecting cheese quality due to the off flavor from butyrate (D'Incecco et al. 2015; Morandi et al. 2015). On the other hand, *C. tyrobutyricum* has been considered as the most promising microbial cell factory for butyric acid production because of its high metabolic flux toward butyryl-CoA and high butyric acid tolerance (Jiang et al. 2018; Yang et al. 2013a, b). Compared to other butyric acid producing bacteria including native *Clostridium butyricum* (Cummins and Johnson 1971; Sushkova et al. 2013; Zigova et al. 1999) and engineered *Clostridium acetobutylicum* (Jang et al. 2014; Siller et al. 2008) and *Escherichia coli* (Jawed et al. 2016; Kataoka et al. 2017; Saini et al. 2014), *C. tyrobutyricum* can produce more butyrate at a higher titer with a higher

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Fig. 1 Metabolic pathways in engineered *C. tyrobutyricum* for butyrate and *n*-butanol production from glucose, xylose, galactose, and sucrose. (Gene name and abbreviation: *ack*: acetate kinase; *adh*E2: aldehyde/alcohol dehydrogenase; *adh* alcohol dehydrogenase; *bcd*: butyryl-CoA dehydrogenase; *buk*: butyrate kinase; *cat*1: butyryl-CoA/acetate CoA transferase; *ctf*AB: CoA transferase; *crt*: crotonase; *etf*: electron transferring flavoprotein; *fba*: fructose-1,6-diphosphatase; *gal*K: galactokinase; *gal*E: UDP-galactose 4-epimerase; *gal*T: galactose-1-phosphate uridylyltransferase; *gpi*: phosphoglucose isomerase; *hbd*: β-hydroxybutyryl-CoA dehydrogenase; *hyd*A: hydrogenase; *pta*: phosphotransacetylase; *ptb*: phosphotransbutylase; *pfk*A: phosphofructokinase; *pyk*A: pyruvate kinase; *pgm*: phosphoglyceromutase; *scr*B: sucrose-6-phosphate hydrolase; *scr*K: fructokinase; *scr*A: sucrose-specific PTS; *thl*: thiolase; *xyl*A: xylose isomerase; *xyl*B: xylulokinase; *xylT*: D-xylose-proton symporter; HMP pathway: hexose monophosphate pathway)

product yield and purity (Jiang et al. 2018). Figure 1 shows the metabolic pathways involved in butyric acid biosynthesis from glucose and other carbon sources.

In addition to butyric acid, engineered *C. tyrobutyricum* has been affirmed as a superior host for *n*-butanol production, leveraging its high metabolic flux toward butyryl-CoA and tolerance to butanol at high concentrations (> 15 g/L) (Yu et al. 2011). As illustrated in Fig. 1, *n*-butanol can be produced from butyryl-CoA via a bifunctional aldehyde/alcohol dehydrogenase (*adh*E2) from *C. acetobutylicum*. Unlike traditional acetone-butanol-ethanol (ABE) fermentation with solventogenic clostridia such as *Clostridium beijerinckii* and *C. acetobutylicum*, butanol fermentation with engineered *C. tyrobutyricum* overexpressing *adh*E2 does not produce acetone. In addition, the heterologous *n*-butanol biosynthesis pathway in *C. tyrobutyricum* is easier to control as the fermentation does not involve a phase transition from acidogenesis to solventogenesis in the ABE fermentation, which is also suppressed by sporulation under butanol stress (Xu et al. 2017). In addition, industrial ABE fermentation is susceptible to bacteriophage infection (Jones et al. 2000), which has rarely been observed or reported for *C. tyrobutyricum* (Mayer et al. 2010). Therefore, the engineered *C. tyrobutyricum-adh*E2 with a simple

and un-regulated butanol biosynthesis pathway has outperformed any known microbes in *n*-butanol production and achieved the highest titer (> 25 g/L) and yield (> 0.3 g/g from glucose) reported so far (Yu et al. 2011; Zhang et al. 2018).

While *n*-butanol has long been produced from sugars and starch in industrial ABE fermentation, currently butanol is almost exclusively produced via petrochemical routes and used mainly in industrial solvents and in the manufacturing of acrylate esters, amino resins, and butylamines, with a worldwide market of ~ 1.5 billion gallons (4.5 million metric tons) (Zhao et al. 2013). Butanol is also an attractive drop-in biofuel with superior fuel properties (high energy density, lower volatility, etc.). It has lower water miscibility, flammability, and corrosiveness than ethanol and is compatible with existing fuel infrastructures and can directly replace gasoline in engines without modification. Increasing demands for biobutanol as a green solvent in food, pharmaceutical, cosmetic and biofuel industries have prompted the search and development of novel bacterial strains for butanol production from renewable feedstocks (Cheng et al. 2019a; Wang et al. 2014; Xue et al. 2017).

This mini-review provides an overview of recent advances in metabolic engineering of *C. tyrobutyricum* for butyrate and *n*-butanol production from sugars and low-cost biomass feedstocks, highlighting strategies and challenges to enhance fermentation efficiency in order to reduce production cost for industrial applications. We also give a brief introduction about *C. tyrobutyricum* and genetic engineering tools, including replicative plasmids for heterologous gene expression and CRISPR-Cas systems for genome editing, available for rational metabolic engineering of clostridia. We conclude with a brief discussion on perspectives for future research and development.

Genomics and metabolic pathway engineering of *C. tyrobutyricum*

The genomes of three *C. tyrobutyricum* strains have been fully sequenced and annotated. For the most widely studied strain ATCC 25755, it has a chromosome of 3,071,606 bp in size, a plasmid of 62,831 bp, and totally 3,220 genes (Lee et al. 2016). The strain CCTCC W428 has a chromosome of 3,011,209 bp, a similar plasmid of 62,833 bp, and totally 3,038 genes (Wu et al. 2017a). The third strain Cirm BIA 2237 has a slightly larger chromosome of 3,159,003 bp but no plasmid and 3,182 genes (Munier et al. 2019). In addition, several draft genome sequences have also been reported for different strains (Bassi et al. 2013; Soggiu et al. 2015; Storari et al. 2015a, b; Wasels et al. 2016). These genome sequences can provide genome-scale information about genes involved in various metabolic and regulatory pathways, which are not fully elucidated yet as many genes have not been annotated or their functions remained unspecified. Nevertheless, the available genomic information has given us a better understanding of the metabolism of *C. tyrobutyricum* and facilitated rational metabolic engineering for creating mutant strains with superior fermentation ability to produce butyric acid and other chemicals such as *n*-butanol. For example, a recent genomic analysis has uncovered that *C. tyrobutyricum* re-assimilates acetic acid for butyric acid biosynthesis through the CoA transferase (CoAT encoded by *cat*1) pathway, rather than the phosphotransbutyrylase-butyrate kinase (PTB-BK) pathway as in other clostridia including *C. butyricum* and solventogenic *Clostridium* species (Lee et al. 2016).

Among the butyric acid producing clostridia, *C. tyrobutyricum* has the smallest genome and a narrower substrate spectrum, but the highest butyric acid production potential based on the final titer and yield attained in fermentation (see Table 1). Since acetate, a major byproduct in butyrate fermentation, can be re-assimilated to generate butyrate via the CoAT pathway in *C. tyrobutyricum*, acetate production can be reduced to minimum with butyrate as the main or only fermentation product (Fu et al. 2017b; 2020), which not only increase butyrate yield but also ease downstream processing for product purification. Recent genomic and proteomic studies of *C. tyrobutyricum* have also suggested that the carbon distribution and energy conservation in *C. tyrobutyricum* favored the biosynthesis of C4 (butyrate) over C2 (acetate) metabolites (Lee et al. 2016; Ma et al. 2015). Although *C. tyrobutyricum* is the leading candidate for fermentation production of biobased butyric acid and *n*-butanol, its prospects for industrial application can be improved by further increasing product yield, productivity, and titer.

Several replicative plasmids with G(+) replicons have been developed for clostridia, including pSOS94 (with pIM13 replicon from *Bacillus subtilis*), pJIR (with pIP404 replicon from *Clostridium perfringens*), pMTL007 (with pCB102 replicon from *C. butyricum*), and pMTL80000 series with replicons pIM13, pCB102, pCD6 (from *Clostridium difficile*), and pBP1 (from *Clostridium botulinum*), respectively (Heap et al. 2007, 2009). These plasmids have been successfully used for gene expression in various *Clostridium* species including *C. tyrobutyricum* (Yu et al. 2011; 2012). Among them, pMTL82151 with pBP1 replicon gave the highest gene expression, plasmid stability, and transformation efficiency (Yu et al. 2012), which are critical to the development of a stable and robust recombinant strain for industrial fermentation. The PMTL plasmids can also be used with the retargeted Group II intron (ClosTron) for gene knockout on the chromosome (Heap et al. 2010). More recently, an endogenous type I-B CRISPR-Cas system with significantly decreased toxicity was developed for genome editing in *C. tyrobutyricum* (Zhang et al. 2018).

Table 1 Clostridial species producing butyric acid as the main fermentation product from various carbon sources

Clostridium species	Substrates	Products [a]	Butyrate Production [b]	Genome (Mbp)	References
tyrobutyricum	Glucose, xylose, fructose, mannitol, lactate, acetate	Butyrate, acetate, CO_2, H_2	43–86.9 g/L, 0.40–0.46 g/g	3.13	Jiang et al. (2011, 2013, 2017), Lee et al. (2016), Liu et al. (2006a), Munier et al. (2019), Storari et al. (2015a, b), Wasels et al. (2016), and Wu et al. (2017a)
butyricum	Starch, glucose, disaccharides, sugars	Butyrate, Acetate, succinate, formate, lactate, CO_2, H_2	7.3–20.0 g/L, 0.24–0.30 g/g	4.54	Li et al. (2016), Mo et al. (2015), Sushkova et al. (2013), and Zigova et al. (1999)
acetobutylicum	Starch, glucose, xylose, sucrose	Butanol, acetone, ethanol, Butyrate, acetate, CO_2, H_2	30.3–32.5 g/L, 0.39–0.41 g/g	4.13	Bao et al. (2011), Jang et al. (2014), Siller et al. (2008), Xu et al. (2017)
beijerinckii	Starch, glucose, xylose, sucrose	Butanol, acetone, ethanol, acetate, butyrate, CO_2, H_2	6.5 g/L, 0.13 g/g	6.00	Alam et al. (1988)
cellulovorans	Cellulose, cellobiose, glucose, xylose, xylan, lactose	Butyrate, Acetate, formate, lactate, CO_2, H_2	~3 g/L, 0.3 g/g	5.26	Tamaru et al. (2010); Yang et al. (2015)
thermobutyricum	Glucose, fructose, xylose, maltose, cellobiose, glucuronic acid, galacturonic acid	Butyrate, acetate, lactate, CO_2, H_2	12.1–44 g/L, 0.35–0.41 g/g [c]	3.40	Wang et al. (2015)

[a] The main fermentation products are indicated with capitalized first letter

[b] Butyrate production in fermentation: Butyrate product titer (g/L) and yield (g/g) from glucose as the carbon source, unless otherwise indicated

[c] Butyrate yield (g/g) based on total sugars present in sorghum juice and bagasse as the carbon source

Furthermore, eliminating native plasmid and type-I restriction endonuclease in *C. tyrobutyricum* increased the transformation efficiency and facilitated genome editing using the CRISPR-Cas9/Cpf1 system which was not applicable in wild-type *C. tyrobutyricum* (Zhang et al. 2020). The ability to perform efficient genome editing with CRISPR-Cas systems in *C. tyrobutyricum* is a major breakthrough that can facilitate multiple gene modifications and create stable strains without requiring a selection pressure (such as antibiotic resistance) suitable for use in industrial fermentation.

Empowered with newly available genomics data and genetic engineering tools, scientists constructed various *C. tyrobutyricum* mutant strains with desirable properties like increased butyrate/butanol titer, yield, productivity, tolerance, and substrate variety and utilization efficiency, which are discussed in the following sections.

Engineering strategies for enhancing butyrate production from glucose

Clostridium tyrobutyricum has been profoundly studied for improving butyrate production from various substrates through rational metabolic engineering strategies (Jiang et al. 2018). Table 2 summarizes metabolic engineering strategies applied to date with notable fermentation performance boosts in final product titer, yield, productivity and purity or selectivity as indicated by the butyric acid to acetic acid ratio (BA/AA). So far most metabolic engineering studies have been focusing on eliminating acetic acid accumulation and overexpressing genes in the butyrate biosynthesis pathway. An earlier attempt to knock out the *pta* and *ack* genes in the acetic acid biosynthesis pathway resulted in mutants (PTA-Em and ACK-Em) with a ~ 14% decrease in acetic acid production and ~ 30% higher butyrate production in fermentation (Liu et al. 2006b; Zhu et al. 2005). For butyrate biosynthesis, *C. tyrobutyricum* uses butyryl-CoA/acetate CoA transferase (*cat1*) to convert butyryl-CoA to butyrate, instead of the phosphotransacetylase and butyrate kinase (PTB-BUK) pathway commonly utilized in other clostridia such as *C. butyricum* and *C. acetobutylicum* (Lee et al. 2016). Overexpressing *cat*1 and crotonase (*crt*) in *C. tyrobutyricum* thus enhanced the flux from acetyl-CoA to butyrate and significantly reduced acetic acid production, which resulted in a 2.24-fold increase in the butyric acid to acetic acid ratio (BA/AA) to 15.76 g/g (Suo et al. 2018a). Meanwhile, overexpressing a [FeFe]-hydrogenase in *C. tyrobutyricum* increased hydrogen and butyrate production (Jo et al. 2010). Since hydrogen production has significant

Table 2 Metabolic engineering of *C. tyrobutyricum* for butyrate production

Engineering strategy	Substrate	Titer (g/L)	Yield (g/g)	Productivity (g/L·h^{-1})	BA/AA (g/g)	References
Wild type	Glucose	20−28.6	0.33−0.34	~0.33	3.0−5.0	Zhu et al. (2005)
	Sugarcane bagasse	20.9	0.51	0.48	~3.48[b]	Wei et al. (2013)
	Corn fiber	29.0	0.47	2.91	10	Zhu et al. (2002)
	Corn husk	21.8	0.39	0.34[b]	5.02	Xiao et al. (2018)
Δ*pta*	Glucose	32.5−51.6	0.38−0.44	0.63	6.55	Liu and Yang, (2006) and Zhu et al. (2005)
Δ*ack*	Glucose	41.7−63.0	0.42	0.23	5.41	Liu et al. (2006a) and Ma et al. (2015)
*cat*1	Glucose	38.2	0.39	0.40	12.65	Suo et al. (2018a)
thl		36.2	0.37	0.38	8.89	
crt		37.9	0.39	0.39	11.11	
*cat*1, *crt*		37.5	0.40	0.39	15.76	
*cat*1, *crt, thl*		36.9	0.40	0.40	16.04	
*pfk*A	Glucose	42.3	0.37	0.44	7.95	Suo et al. (2018b)
*pyk*A		38.6	0.36	0.40	7.45	
*pfk*A, *pyk*A		48.2	0.38	0.50	8.09	
*cat*1, *crt*, *pfk*A, *pyk*A	Glucose	46.8	0.39	0.83	13.2	Suo et al. (2018c)
*tre*S	Glucose	34.6−54.5	0.27−0.43	0.9−1.2	1.9−4.8	Wu et al. (2017b)
*gro*ES	Glucose	44.1−52.2	0.34−0.37	0.39−0.41	7.0−10.4	Suo et al. (2017)
	Corn straw	29.6	0.37[a]	0.31	5.11	Suo et al. (2018c)
	Rice straw	30.1	0.37[a]	0.31	5.16	
*sdr, gro*ES	Corn cob	32.8	0.36[a b]	0.29	3.28[b]	Suo et al. (2019)
*xyl*T, *xyl*A, *xyl*B	Glucose & Xylose	37.5	0.34[a]	0.72	4.57[b]	Fu et al. (2017b)
	Soybean hull	29.7	0.35[a]	0.67	7.43[b]	
	Sugarcane bagasse	42.6	0.36[a]	0.56	7.89[b]	
*scr*A, *scr*B, *scr*K	Cane molasses	45.7	0.39[a]	0.54[b]	9.14[b]	Guo et al. (2020)
*gal*K, *gal*E, *gal*T, *gal*P	Spent coffee grounds	34.3	0.37[a]	0.36	5.56[b]	He et al. (2020)

ack: acetate kinase; *cat*1: butyryl-CoA/acetate CoA transferase; *crt*: crotonase; *gal*K: galactokinase; *gal*E: UDP-galactose 4-epimerase; *gal*T: galactose-1-phosphate uridylyltransferase; *gal*P: non-phosphorylating transporter of galactose; *pfk*A: phosphofructokinase; ppcc: *gro*ES: heat shock protein; *pta*: phosphotransacetylase; *pyk*A: pyruvate kinase; *scr*A: sucrose-specific PTS; *scr*B: sucrose-6-phosphate hydrolase; *scr*K: fructokinase; *sdr*: short-chain dehydrogenase/reductase; *thl*: thiolase; *tre*S: trehalose synthase; *xyl*A: xylose isomerase; *xyl*B: xylulokinase; *xyl*T: D-xylose-proton symporter;

[a]Yield on total sugar

[b]Value of calculation depending on experimental data

effects on electron balance and product distribution, adding artificial electron carriers, such as benzyl viologen (BV) and methyl viologen (MV), in the fermentation medium was found to inhibit hydrogen production and shift the metabolism from acetic acid production to reassimilation for butyrate production, which resulted in a high BA/AA of 58 g/g or a product purity of 98.3% in batch fermentation (Choi et al. 2012; Fu et al. 2017b).

To increase butyrate productivity from glucose, 6-phosphofructokinase (*pfk*A) and pyruvate kinase (*pyk*A*)* in the EMP pathway were overexpressed individually or simultaneously in *C. tyrobutyricum* to enhance glucose catabolism (Suo et al. 2018b). Then, genes involved in butyric acid biosynthetic pathway, including thiolase (*thl*), crotonase (*crt*), and butyryl-CoA/acetate CoA transferase (*cat*1), were further investigated for enhancing the butyrate titer and yield (Suo et al. 2018c). Finally, *C. tyrobutyricum* mutant strain co-expressing *crt, cat*1, *pfk*A, and *pyk*A was shown to produce the highest level of butyric acid of 46.8 g/L with a productivity of 0.83 g/L·h and butyrate/acetate ratio of 13.22 g/g in batch fermentation, which were 33.7%, 69.4% and 83.1% increase, respectively, as compared to the wild-type *C. tyrobutyricum* (Suo et al. 2018c).

Butyrate production in fermentation is strongly inhibited by butyric acid, which at > 10 g/L would reduce cell growth and metabolic activities by more than 80% (Wu and Yang 2003; Zhu and Yang 2003). Several Class I heat shock proteins (including *dna*J, *dna*K, *grp*E, *gro*ES, *gro*EL, and *htp*G)

known to play important roles in resisting environmental stress were investigated for their effects on butyrate tolerance in *C. tyrobutyricum*. Among them, the overexpression of *gro*ESL significantly improved the butyrate tolerance and the mutant gave a high level of butyric acid production of up to 52.2 g/L, which was a 15.2% increase compared to the wild type strain (Suo et al. 2017). Wu et al. reported that overexpressing trehalose synthase (TreS), which converted maltose to trehalose, in *C. tyrobutyricum* increased the host's oxidative resistance and robustness under hypoxic and aerobic conditions (Wu et al. 2017b). Interestingly, compared to the wild type strain the mutant also produced significantly more butyrate in batch fermentations under acidic conditions (pH 4.0 and 5.0). In addition, many earlier studies have focused on enhancing cell butyrate tolerance through adaptation in immobilized-cell bioreactor such as the fibrous bed bioreactor (FFB) (Jiang et al. 2011; Zhu and Yang 2003). While the original strain was unable to grow in the presence of 40 g/L butyric acid, cells immobilized in a FBB were able to produce up to 86.9 g/L butyric acid from glucose in a repeated fed-batch fermentation process (Jiang et al. 2011). The adapted cells in the FBB had an elongated rod morphology and significantly elevated intracellular pH, which might have contributed to the higher butyric acid tolerance.

Engineering strategies for enhancing *n*-butanol production from glucose

Yu et al. first introduced *adh*E2 into various strains of *C. tyrobutyricum* for *n*-butanol production from glucose, achieving a high butanol yield of 0.27 g/g (Yu et al. 2011). After optimizing the conjugative plasmid expression system, *C. tyrobutyricum* Δ*ack-adh*E2 produced 20.5 g/L of *n*-butanol with a high yield of 0.33 g/g with mannitol as the substrate (Yu et al. 2012). However, large amounts of acids (acetate and butyrate) were also produced. To overcome this problem, CoA transferase (encoded by *ctf*AB) from *C. acetobutylicum* was co-overexpressed with *adh*E2 in *C. tyrobutyricum* to facilitate the reassimilation of butyrate for *n*-butanol production, leading to over twofold increase in butanol productivity and yield (Yu et al. 2015a). However, acetone was also produced in the fermentation. More recently, using the native CRISPR-Cas system, Zhang et al. successfully knocked out *cat*1 with *adh*E2 insertion on the genome of C. *tyrobutyricum* and the mutant strain Δ*cat1*::*adhE2* produced 26.2 g/L *n*-butanol with a yield of 0.23 g/g and very little butyrate production (Zhang et al. 2018). However, large amounts of acetate and ethanol were also produced by this mutant in the fermentation. Additional metabolic and process engineering efforts are thus required to direct more carbon flux toward C4 compounds in order to further enhance *n*-butanol production, which might also be limited by NADH availability. These metabolic engineering strategies along with additional studies described in the following sections are summarized in Table 3.

Compared to butyric acid, each mole of butanol produced from butyryl-CoA requires additional two moles of NADH (see Fig. 1), which may cause redox imbalance. To increase NADH availability for butanol biosynthesis from glucose, Nguyen et al. (2018) knocked out the redox-sensing transcriptional repressor gene (*rex*A) and replaced NAD^+-dependent 3-hydroxybutyryl-CoA dehydrogenase (*hbd*) with a heterologous $NADP^+$-dependent 3-hydroxybutyryl-CoA dehydrogenase (*hbd*1) in *C. acetobutylicum*. They also replaced the native thiolase (*thl*A) with a heterologous acetoacetyl-CoA thiolase/synthase (*ato*B) to increase the flux from C2 (acetyl-CoA) to C4 (butyryl-CoA) and knocked out CoA transferase (*ctf*AB), butyrate kinase (*buk*) and phosphotransbutyrylase (*ptb*). The resulting mutant produced *n*-butanol as the main metabolic product at a high yield of 0.34 g/g glucose. Replacing NAD^+-dependent 3-hydroxybutyryl-CoA dehydrogenase with $NADP^+$-dependent one thus should have a positive effect on NADH availability for *n*-butanol biosynthesis in *C. tyrobutyricum*, which remains to be verified.

Some process engineering strategies have also been applied to improve *n*-butanol production. For example, adding MV as an artificial electron carrier in the fermentation by *C. tyrobutyricum* Δ*ack-adh*E2 reduced acetate and butyrate production by more than 80–90% and increased *n*-butanol production to 14.5 g/L with a high yield of > 0.3 g/g (Du et al. 2015). The MV effect on increased butanol production can be attributed to its effects on inhibiting hydrogen production and thus increasing available NADH for butanol biosynthesis. In addition, FBB was applied to immobilize *C. tyrobutyricum adh*E2, which not only dramatically increased cell density, but also improved butanol titer, yield, and productivity with reduced acid production (Huang et al. 2019).

Engineering *C. tyrobutyricum* for butyrate/butanol production from low-cost feedstocks

Although high-titer *n*-butanol and butyrate can be produced from glucose with a high yield because few byproducts are coproduced in the fermentation, especially with the addition of MV (Du et al. 2015), *C. tyrobutyricum* has a narrow substrate spectrum and can use only a few monosaccharides (glucose, xylose, and fructose), mannitol, and lactate for growth (Dwidar et al. 2012). This can be attributed to the fact that *C. tyrobutyricum*'s relatively small genome, compared to *C. butyricum* and *C. acetobutylicum*, is lacking genes for starch and disaccharides, such as maltose and sucrose, transport and catabolism (Jiang et al. 2018). In order to expand the substrate spectrum of *C. tyrobutyricum*, heterologous sucrose, maltose, and galactose catabolism pathways have been successfully introduced into *C. tyrobutyricum*.

Table 3 Metabolic engineering of *C. tyrobutyricum* for *n*-butanol production

Engineering strategy	Substrate	Titer (g/L)	Yield (g/g)	Productivity (g/L·h^{-1})	References
Δ*ack-adh*E2	Glucose	10.0	0.27	0.03[b]	Yu et al. (2011)
	Mannitol	16.0	0.31	0.06[b]	
	Mannitol	20.5	0.33	0.32	Yu et al. (2012)
	Glucose	14.5	0.28	0.13	Du et al. (2015)
	Cassava bagasse	13.0	0.34	0.26	Huang et al. (2019)
	Corn fiber, cotton stalk, soybean hull, sugarcane bagasse	15.0	0.30	0.30	Li et al. (2019)
Δ*ack-adh*E2, *ctf*AB	Glucose	12.0	0.26	0.35	Yu et al. (2015a)
Δ*ack-adh*E2, *scr*A, *scr*B, *scr*K	Sucrose	14.8	0.21	0.15	Zhang et al. (2017b)
	Sugarcane juice	12.8	0.21	0.53	
Δ*ack-adh*E2, *aglu*I	Maltose	17.2	0.20	0.29	Yu et al. (2015b)
	Soluble starch	16.2	0.17	0.19	
Δ*ack-adh*E2, *xyl*T, *xyl*A, *xyl*B	Glucose/Xylose	12.0	0.12[a]	0.17	Yu et al. (2015c)
	Soybean hull	15.7	0.24	0.29	
Δ*cat*1:*adh*E2	Glucose	26.2	0.23	0.16[b]	Zhang et al. (2018)
	Paper mill sludge	16.5	0.26	0.17[b]	Cao et al. (2020)

ack: acetate kinase; *aglu*I: α-glucosidase; *cat*1: butyryl-CoA/acetate CoA transferase; *ctf*AB: butyryl-CoA/acetate CoA transferase; *scr*A: sucrose-specific PTS; *scr*B: sucrose-6-phosphate hydrolase; *scr*K: fructokinase; *xyl*A: xylose isomerase; *xyl*B: xylulokinase; *xyl*T: D-xylose-proton symporter;

[a]Yield on total sugar

[b]Value of calculation depending on experimental data

For sucrose catabolism, sucrose-specific PTS (*scr*A), sucrose-6-phosphate hydrolase (*scr*B), and fructokinase (*scr*K) from *C. acetobutylicum* were co-expressed in *C. tyrobutyricum* (Guo et al. 2020). The mutant strain was able to utilize cane molasses as both nitrogen and carbon sources and produced 45.7 g/L butyric acid with a yield of 0.39 g/g in fed-batch fermentation. Similarly, *C. tyrobutyricum* was engineered to co-express *adh*E2 with *scr*K*, scr*B, and *scr*A for *n*-butanol production from sucrose (Zhang et al. 2017a). The mutant produced 16 g/L *n*-butanol with a yield of 0.24 g/g sugars from sugarcane juice supplemented with corn steep liquor (CSL) (Zhang et al. 2017b). Compared to glucose as the substrate, the feedstock cost was reduced by ~ 50% when cane molasses or sugarcane juice was used in the fermentation.

Metabolic engineering of *C. tyrobutyricum* for *n*-butanol production from maltose and soluble starch was also studied (Yu et al. 2015b). Two α-glucosidase genes, *aglu*I and *aglu*II, from *C. acetobutylicum* were cloned and co-expressed with *adh*E2 in *C. tyrobutyricum* Δ*ack*. The mutant expressing *aglu*I demonstrated robust activity in breaking down the α-1,4-glycosidic bonds in maltose and starch and produced 17.2 g/L butanol from maltose with a yield of 0.20 g/g and productivity of 0.29 g/L·h in batch fermentation. With soluble starch, 16.2 g/L butanol was produced with a yield of 0.17 g/g and productivity of 0.20 g/L·h. Because of the inherent higher butanol tolerance, the mutant was able to produce more butanol at a remarkably higher productivity as compared to *C. acetobutylicum* ATCC 824 (11.2 g/L at 0.10 g/L·h from maltose and 8.8 g/L at 0.10 g/L·h from soluble starch).

For galactose catabolism, the recombinant *C. tyrobutyricum kept* was constructed by co-expressing UDP-galactose 4-epimerase (*gal*E), galactokinase (*gal*K), phosphoglucomutase (*gal*P), and galactose-1-phosphate uridylyltransferase (*gal*T) genes from *C. acetobutylicum*, which utilized glucose and galactose simultaneously without glucose-mediated carbon catabolite repression (CCR) (He et al. 2020). When using hydrolyzed coffee ground (rich in galactose) as the substrate, the mutant strain produced 34.3 g/L butyric acid with a yield of 0.37 g/g, which were 78.6% and 56.5%, respectively, higher than those from the wild-type strain.

C. tyrobutyricum can also use xylose as the sole carbon source in fermentation (Liu and Yang 2006). The product (butyrate and butanol) yields from xylose were comparable to those from glucose although xylose utilization involves the hexose monophosphate pathway that would give slightly less ATP but more NADH and thus somewhat different product profiles under different pH conditions (Zhu and Yang 2004). However, in the presence of glucose, xylose utilization was greatly inhibited by glucose-mediated CCR, which could be alleviated by overexpressing three xylose catabolism genes (*xyl*B: xylulokinase, *xyl*T: D-xylose-proton

symporter, and *xyl*A: xylose isomerase) from *C. acetobutylicum* (Fu et al. 2017a; Yu et al. 2015c).

Compared to *C. butyricum* and *C. acetobutylicum*, engineered *C. tyrobutyricum* strains with heterologous galactose, maltose, and sucrose catabolism genes gave better fermentation performance due to their higher tolerance to butyrate and butanol. The engineered *C. tyrobutyricum* strains thus can provide more robust and cost-effective processes for industrial butyrate and butanol production from food processing wastes such as sugarcane molasses and spent coffee ground.

Engineering strategies for using lignocellulosic biomass hydrolysates

Lignocellulosic biomass (LCB) is the most abundant renewable resource on the planet (Kumar et al. 2013). The feasibility of using LCB hydrolysates as low-cost feedstock has thus also been explored for butyric acid and butanol production by *C. tyrobutyricum* (Baroi et al. 2015; Cao et al. 2020; Chen et al. 2017; Huang et al. 2011; 2016a; 2016b; 2019; Liu et al. 2013; Oh et al. 2019; Sjoblom et al. 2016; Song et al. 2011; Wei et al. 2013; Xiao et al. 2018; Zheng et al. 2018; Zhu et al. 2002). The application of LCB in fermentation requires relatively harsh chemical, physical, and/or thermal pretreatments before enzymatic hydrolysis of cellulose. The pretreatment process usually generates chemical inhibitors derived from the degradation of lignin and sugars (Amiri and Karimi 2018; Sharma et al. 2019). In general, immobilized cells had better resistance to the hydrolysate inhibitors, especially after adaptation in bioreactors. Compared to free-cell fermentation, significantly higher butyrate and butanol titers and productivities were obtained from LCB hydrolysates when *C. tyrobutyricum* cells were immobilized in fibrous bed bioreactor (FBB) (Fu et al. 2017b; Li et al. 2019; Wei et al. 2013; Xiao et al. 2018). Various detoxification approaches (chemical, physical or biological methods) have been developed to remove inhibitors in the hydrolysates prior to fermentation (Jönsson et al. 2013). For example, an *in-situ* detoxification process using Tween 80 as a surfactant was found to be effective in removing hydrolysate inhibitors in pretreated rice straw hydrolysate, which after detoxification could be directly added in *C. tyrobutyricum* fermentation broth for butyrate production with comparable performance to that from pristine sugars (Lee et al. 2015).

However, detoxification is not always effective and can be costly (Jönsson et al. 2013). Improving cell tolerance to LCB-derived inhibitors via metabolic engineering was thus investigated. One study showed that the overexpression of Class I heat shock protein genes (*gro*ESL) improved the fermentation performance of *C. tyrobutyricum* with a significantly higher butyrate production from glucose (Suo et al. 2017) as well as LCB (corn straw and rice straw) hydrolysates as compared to the wild type (Suo et al. 2018b). More recently, a short-chain reductase (SDR) and aldo/keto reductases (AKR) from *C. beijerinckii* were investigated for enhancing the fermentability of undetoxified corncob acid hydrolysate (Suo et al. 2019). SDR and AKR can catalyze the reduction of furfural and 5-hyroxymethyl furfural (HMF) to corresponding alcohols, which are less toxic than the aldehydes (Suo et al. 2019). Compared to the parental strain, butyrate fermentation productivity was improved to 0.29 g/L·h with the butyric acid titer increased by 28.1% when *sdr* and *gro*ESL genes were co-overexpressed in *C. tyrobutyricum* (Suo et al. 2019).

LCB hydrolysates contain glucose and xylose as two main types of monosaccharide. Although most of clostridia, including *C. acetobutylicum* and *C. tyrobutyricum*, can use xylose as the sole carbon source, xylose utilization in the presence of glucose was greatly inhibited by CCR, leading to poor xylose consumption and low fermentation productivity (Xiao et al. 2012). To overcome the CCR in glucose/xylose co-fermentation, three xylose catabolism genes *xyl*B, *xyl*T, and *xyl*A from *C. acetobutylicum* were expressed in *C. tyrobutyricum* Δ*ack* and Δ*ack*:*adh*E2 for butyrate and butanol production, respectively (Fu et al. 2017a; Yu et al. 2015c). Glucose and xylose co-utilization with significantly reduced residual xylose was achieved in batch fermentations with these mutants. The mutant Ct-pTBA was evaluated with the hydrolysates of sugarcane bagasse, rice straw, corn fiber, wheat straw, and soybean hull. A high butyric acid titer of 42.6 g/L with a yield of 0.36 g/g and productivity of 0.56 g/L·h was obtained from sugarcane bagasse hydrolysate (Fu et al. 2017b), which were significantly higher than those from the wild type (see Table 2). In batch fermentation with *C. tyrobutyricum* Δ*ack*:*adh*E2-pTBA, 15.7 g/L *n*-butanol with a yield of 0.24 g/g was produced from soybean hull hydrolysate (Yu et al. 2015c). Clearly, expressing *xyl*A, *xyl*B, and *xyl*T alleviated the CCR bottleneck in *C. tyrobutyricum* and was effective in enhancing butyrate and butanol production from LCB hydrolysates containing glucose and xylose. Table 3 summarizes notable metabolic engineering strategies applied to *C. tyrobutyricum* for *n*-butanol production from various substrates.

Comparison to other bacterial hosts for butyrate and *n*-butanol production

Compared to the best recombinant microbes engineered to date for butyrate and *n*-butanol production, engineered *C. tyrobutyricum* strains generally gave higher product titer, yield, and productivity and thus would have greater potential for industrial application (see Table 4). Native solventogenic *C. acetobutylicum* produces acetone, butanol, and ethanol as the main products at a mass ratio of 6:3:1 with a relatively low butanol titer (10 – 14 g/L) and yield (~ 0.2 g/g). After

Table 4 Comparison of *C. tyrobutyricum* with engineered *C. acetobutylicum* and *E. coli* for butyrate and *n*-butanol production

	C. tyrobutyricum		*C. acetobutylicum*	*Escherichia coli*
Butyrate (reference)	Liu et al. (2006a)	Suo et al. (2018c)	Jang et al. (2014)	Saini et al. (2014)
Strain with engineered genes	Δ*ack*	*cat*1, *crt, pfk*A, *pyk*A	Δ*pta,* Δ*buk,* Δ*ctf*AB, Δ*adh*E1	Δ*frd*A, Δ*ldh*A, Δ*pta*, Δ*adh*E *ter, hbd, crt, pha*A, *ato*DA
Titer (g/L)	41.7–63.0	46.8	30.3	~11
Yield (g/g)	0.42	0.39	0.41	0.36
Productivity (g/L·h)	0.23	0.83	0.82	~0.23
BA/AA ratio (g/g)	5.41	13.2	27.1	143
Comments	Much higher titer of > 80 g/L and BA/AA ratio of 58 were obtained with MV	Overexpressing *cat*1 increased butyrate productivity and selectivity	Knocking out some solventogenic genes turned the host into an acidogen	Multiple gene knockouts and overexpression of heterologous genes turned the host into a butyrate producer
Butanol (reference)	Yu et al. (2012)	Zhang et al. (2018)	Nguyen et al. (2018)	Shen et al. (2011)
Strain with engineered genes	Δ*ack*-*adh*E2	Δ*cat*1:*adh*E2	Δ*buk*, Δ*ptb*, Δ*ldh*A, Δ*ctfAB*, Δ*rex*A, Δ*thl*A, *ato*B, Δ*hbd*, *hbd*1	Δ*adh*E, Δ*ldh*A, Δ*frd*BC, Δ*pta* *hbd, crt, ter, adh*E2, *fdh*
Titer (g/L)	20.5	26.2	~10	~15
Yield (g/g)	0.33	0.23	0.34	0.28
Productivity (g/L·h)	0.32	0.16	NA	0.07
Comments	Butanol was the main product with only small amounts of butyrate and acetate as byproducts when MV was added in the fermentation	Knocking out *cat*1 eliminated butyrate biosynthesis and resulted in *n*-butanol as the major fermentation product at a high final titer, but a comparable amount of acetate was also co-produced and resulted in a lower butanol yield	Genes for acetone and butyrate biosynthesis were effectively eliminated through pathway engineering so that butanol was the only major product with a high yield in the fermentation	By introducing heterologous butanol biosynthesis pathway genes and knocking out acids forming genes, the recombinant strain was able to produce butanol at an appreciable titer and yield. However, titer and productivity were relatively low

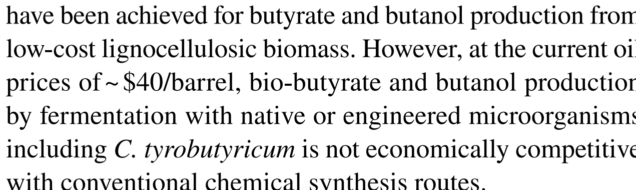
ack acetate kinase; *adh*E1 aldehyde-alcohol dehydrogenase; *ato*AD acetoacetyl-CoA transferase; *ato* Bacetoacetyl-CoA thiolase/synthase; *buk* butyrate kinase; *cat*1 butyryl-CoA/acetate CoA transferase; *crt* 3-hydroxybutyryl-CoA dehydratase; *ctf*AB butyryl-CoA/acetate CoA transferase; *fdh* formate dehydrogenase; *frd*A, subunit of fumarate reductase; *frd*BC fumarate reductase Fe-S subunit and membrane anchor subunit; *hbd* NAD^+-dependent 3-hydroxybutyryl-CoA dehydrogenase; *hbd*1 $NADP^+$-dependent 3-hydroxybutyryl-CoA dehydrogenase; *ldh*A lactate dehydrogenase; *pfk*A phosphofructokinase; *pyk*A pyruvate kinase; *pha*A β-ketothiolase; *pta* phosphotransacetylase; *ptb* phosphotransbutyrylase, *rex*A redox-sensing transcriptional repressor; *ter* trans-enoyl-coenzyme A (CoA) reductase; *thl*A thiolase; NA Not available

multiple gene manipulations (overexpression and deletion of multiple genes) Nguyen et al. (2018), were able to engineer *C. acetobutylicum* to produce mainly *n*-butanol without acetone, achieving a high butanol yield of 0.34 g/g. Several mutant strains of *C. acetobutylicum* ATCC 824 with *pta*, *ctf*B, and *adh*E1 knockouts were able to produce up to 31 g/L butyric acid with a high BA/AA ratio of 31.3 g/g and negligible solvent production when *buk* was also inactivated (Jang et al. 2014). In addition, *E. coli*, which has the most well-developed genetic tools and has been extensively studied as a robust host for production of a variety of chemicals, has also been metabolically engineered to produce butyrate and *n*-butanol. For example, Shen et al. (2011) engineered *E. coli* to express a chimeric *n*-butanol biosynthetic pathway with increased NADH availability to achieve a high *n*-butanol titer of ~ 15 g/L with a yield of 0.28 g/g (~ 70% theoretical). Metabolically engineered *E. coli* strains were also constructed to produce butyrate at a high yield (0.31 – 0.43 g/g) with minimal acetate production, achieving a high selectivity with the highest BA/AA ratio of 143 g/g obtained from 20 g/L glucose and 8 g/L acetate in an LB medium (Saini et al. 2014). However, *E. coli* has relatively poor tolerance to butyric acid and butanol, and the highest butyrate and butanol titers produced so far were much lower than those from clostridial fermentations. Although *C. tyrobutyricum* is more difficult to engineer because of limited genetic engineering tools and its relatively low transformation efficiency, overall it is a better host with superior fermentation performance in product titer, yield, and productivity.

Moreover, compared to *C. acetobutylicum* and other solventogenic clostridia used in industrial ABE fermentation, *C. tyrobutyricum* is not as susceptible to sporulation (Xu et al. 2017) and bacteriophage infection (Jones et al. 2000). Although several strains of *C. tyrobutyricum* (NCIMB 9582, NCIMB 701753 and 701756) were found to be susceptible to the phage φCTP1 isolated from a landfill site (Mayer et al. 2010), no bacteriophage infection of *C. tyrobutyricum* ATCC 25755 has ever been observed in a continuous or fed-batch fermentation process operated for an extended period (over a month). It is noted that phage-resistant strains can be obtained through screening/isolation (Liu et al. 2017) or genetic engineering to clone and express a potent restriction/modification system (such as using CRISPR/Cas9 technology for double-strand DNA cleavage) targeting selected phage genes (e.g., endolysin) (Baltz et al. 2018).

Conclusions and prospects for further developments

Clostridium tyrobutyricum has attracted a great deal of interest as a robust host for butyrate and butanol production. To date, impressive progresses in strain and process engineering



have been achieved for butyrate and butanol production from low-cost lignocellulosic biomass. However, at the current oil prices of ~ $40/barrel, bio-butyrate and butanol production by fermentation with native or engineered microorganisms including *C. tyrobutyricum* is not economically competitive with conventional chemical synthesis routes.

There are challenges and opportunities in further engineering *C. tyrobutyricum* for efficient utilization of lignocellulosic biomass hydrolysates to attain desirable product titer, yield and productivity suitable for industrial application. Genome-scale analyses, including comparative genomics, transcriptomics, and metabolomics analyses, are valuable in guiding rational metabolic engineering at a systems level and have been applied to clostridia (Yoo and Soucaille 2020; Ou et al. 2020) but not *C. tyrobutyricum* yet. Further strain engineering may also require more sophisticated strategies and approaches such as multivariate modular metabolic engineering (Biggs et al. 2014), which would require a well-characterized "toolbox" including replicon (ori), ribosomal binding sites (RBS), promoters, and reporters (Joseph et al. 2018). Replicon plays a significant role in plasmid copy number and transformation efficiency (Yu et al. 2012). RBS and promoter are important in regulating gene expression and balancing metabolic flux and redox potential, which are critical to optimizing cell growth and metabolic activities. Efficient reporter systems suitable for anaerobes, such as the one based on a flavin mononucleotide (FMN)-dependent fluorescent protein Bs2 (Cheng et al. 2019b), can facilitate the evaluation and screening of promoters with different strengths and thus would be valuable in promoter engineering. These novel genetic engineering toolkits and CRISPR-Cas9 genome-editing systems have rapidly advanced synthetic biology (Kwon et al. 2020; Joseph et al. 2018) and should facilitate the further development of *C. tyrobutyricum* for butyrate and *n*-butanol production.

In addition to metabolic engineering, adaptation or evolutionary engineering has also been demonstrated as an efficient strategy to enhance cell tolerance to toxic chemicals such as butyric acid and *n*-butanol. Cells highly tolerant to butyric acid or *n*-butanol were obtained after prolonged exposure to the corresponding metabolite produced in fed-batch or repeated batch fermentation in a FBB (Jiang et al. 2011; Yang and Zhao 2013; Zhu and Yang 2003). Comparative genomic analysis revealed that the butanol tolerant mutant strain *C. acetobutylicum* JB200 had a single-base deletion in a histidine kinase (encoded by *cac3319*). This finding led to the development of *cac3319* knockout mutant with 45% higher butanol production (~ 18.2 g/L vs. ~ 12.6 g/L for the parental strain) and a 90% higher productivity (Xu et al. 2015). Histidine kinase is involved in the phosphorylation or activation of Spo0A, a global regulator in clostridia which is known to control not only sporulation but also stress response and solventogenesis

in *C. acetobutylicum* (Steiner et al. 2011). It has also been reported that inactivating the sporulation transcription factor (*spo*0A) enhanced the butanol tolerance and production ability of *Clostridium cellulovorans* after adaptation (Wen et al. 2019). Therefore, we can speculate that knocking out histidine kinase and/or *spo*0A in *C. tyrobutyricum* may also enhance its ability to produce more butyric acid and butanol.

Finally, the engineered *C. tyrobutyricum* with enhanced tolerance can be used in an integrated process with in situ or on-line product separation, such as liquid–liquid extraction for butyric acid (Wu and Yang 2003) and gas stripping for butanol (Du et al. 2015; Lu et al. 2013), to further increase product titer, productivity, and yield, allowing for economical production of these metabolites in fermentation (Yang and Lu 2013).

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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