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Clostridia: a flexible microbial platform for the production of alcohols

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Solventogenic clostridia are native producers of ethanol and many higher alcohols employing a broad range of cheap renewable substrates, such as lignocellulosic materials and C1 gases (CO and CO_2). These characteristics enable solventogenic clostridia to act as flexible microbial platforms for the production of liquid biofuels. With the rapid development of genetic tools in recent years, the intrinsic intractability of clostridia has been largely overcome, thus, engineering clostridia for production of chemicals and fuels has attracted increasing interests. Here, we provide an overview of recent progress in the production of alcohols based on solventogenic clostridia. Saccharolytic, cellulolytic and gas-fermenting clostridia are discussed, with a special focus on strategies for metabolic engineering to enable and to improve clostridia for the production of higher alcohols.

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Introduction

Alcohols, especially higher alcohols (more than 2 carbons), have attracted increasing interests because of their potentially widespread applications as liquid fuels in the future [1,2]. Compared to petrochemical production, biological processes using industrial microorganisms to produce alcohols possess advantages as well as the potential for deployment within a large market in response to strategic demands [3,4].

Solventogenic clostridia, a class in the phylum *Firmicutes*, are a group of important industrial microorganisms because of their inherent ability to synthesize a variety of bulk chemicals and fuels, especially alcohols. With the rapid progress in genetic tools that can target clostridia in recent years [5•], the original intractability of these anaerobes has been conquered, enabling them to be engineered to produce more non-native alcohols from renewable resources.

Likely due to the diversity of the environments that they inhabit (*e.g.*, soil, aqueous sediments, rumen and intestines), clostridia have evolved and developed exceptional substrate diversity and are capable of using a variety of materials (Table 1), especially lignocellulose and C1 gases. Cellulolytic clostridia are able to directly use lignocelluloses, the most abundant biomass on earth, thus, they have been widely studied for the production of alcohols through consolidated bioprocessing (CBP), a possible ultimate solution for the economical utilization of lignocellulosic biomass [6]. Additionally, a more ideal route is to directly capture carbon before its incorporation into plant biomass, and Clostridia, typified by *Clostridium ljungdahlii*, *Clostridium carboxidivorans* and *Clostridium autoethanogenum*, are important gaseous substrate (*i.e.*, CO and CO_2) biocatalysts. To date, gas-fermenting clostridia have been engineered to produce a wealth of alcohols [7••].

During the past few years, there have been various review articles that summarized the research advances in solventogenic clostridia from different aspects, including physiology [8,9], utilization of feed stocks [10–12], development of genetic tools [5•] and metabolic engineering [13–17]. Here, we intend to specifically highlight recent progresses that are relevant to the clostridia-based production of alcohols (Figure 1). We describe the exceptional substrate diversity of solventogenic clostridia, with a special focus on lignocellulosic and gaseous substrates, two promising cheap feedstocks for further large-scale bioproduction (Table 1). Moreover, we discuss the ability of clostridia to synthesize alcohols, focusing specifically on newly established metabolic engineering strategies to improve alcohol production by clostridia.

Table 1

Summary of typical alcohol-producing clostridia and their substrates. Bold fonts indicate alcohols that were synthesized in non-native hosts

Species	Substrate	Products	Reference
C. thermocellum	Cellulose	Ethanol, acetate, lactate, formate, **isobutanol**	[18,19•]
C. cellulovorans	Cellulose	Ethanol, acetate, **butanol**	[20•]
C. cellulolyticum	Crystalline cellulose	Acetate, lactate, ethanol, **butanol, isobutanol**	[21,22]
C. clariflavum	Cellulose, hemicellulose	Acetate, ethanol, formate,	[23]
C. stercorarium	Cellulose, hemicellulose	Ethanol, acetate, lactate	[24]
C. phytofermentans	Cellulose, glucan	Ethanol, acetate	[25]
C. acetobutylicum	Starch, sugars (sucrose, glucose, xylose, arabinose, *etc.*)	Butanol, ethanol, acetone, acetate, butyrate, **2,3-butanediol, 1,3-propanediol**	[26–28]
C. beijerinckii	Sugars (sucrose, glucose, xylose, arabinose, *etc.*)	Butanol, ethanol, acetone, acetate, butyrate, 2,3-butanediol	[29•]
C. saccharoperbutylacetonicum	Starch, glucose, xylose, arabinose	Butanol, ethanol, acetone, acetate, butyrate	[30]
C. butyricum	Glycerol, glucose, xylose, arabinose	Butyrate, 2,3-butanediol, lactate, 1,3-propanediol	[31,32]
C. diolis	Glycerol and lignocellulosic hydrolysates	1,3-Propanediol, butyrate	[33••]
C. tyrobutyricum	Glucose, xylose, lactate	Butyrate, acetate, **Butanol**	[34]
C. pasteurianum	Glycerol, glucose, xylose	Butanol, **1,3-propanediol**,butyrate, acetate	[35]
C. ljungdahlii	H_2/CO_2, CO	Acetate, ethanol, butyrate, lactate, 2,3-butanediol, **butanol**	[36–38]
C. carboxidivorans	H_2/CO_2, CO	Acetate, ethanol, butyrate, butanol, hexanol, hexanoate	[39,40]
C. autoethanogenum	H_2/CO_2, CO	Acetate, ethanol, lactate, 2,3-butanediol, **butanol**	[37,41,42]
C. drakei	H_2/CO_2, CO	Acetate, ethanol, butyrate	[43]
C. ragsdalei	H_2/CO_2, CO	2,3-Butanediol, acetate, ethanol, lactate	[37,44]
C. scatologenes	H_2/CO_2, CO	Acetate, ethanol, butyrate	[45]

Recent advances in lignocellulosic and gaseous substrate utilization by clostridia

Clostridia-based ABE (*A*cetone-*B*utanol-*E*thanol) fermentation using starchy substrates (*e.g.*, corn and cassava) or molasses has undergone a long period of industrial-scale production. However, due to the twin shocks of rapidly increased raw material costs and collapsed oil prices, such traditional fermentative routes are currently economically unavailable. Researchers have turned their attention to other cheaper renewable feedstocks, particularly lignocellulosic and gaseous substrates.

Fermentation of lignocellulosic hydrolysates by clostridia

The conventional route for biological utilization of lignocellulosic materials follows the pretreatment-hydrolysis-fermentation (PHF) process. Clostridia is able to utilize a variety of carbohydrates, including hexose and pentose sugars, which make them ideal platforms for fermenting lignocellulosic hydrolysates. However, similar to many other industrial microorganisms, certain typical *Clostridium* species (*e.g.*, *C. acetobutylicum*) possess the carbon catabolite repression (CCR) mechanism [46], in which the presence of preferred substrates will repress the utilization of non-preferable sugars *via* catabolite control protein A (CcpA) and (or) other regulators. There are also exceptions, such as *C. beijerinckii*, another most widely studied *Clostridium* species. Although *C. beijerinckii* also contains CcpA, it did not show severe catabolite repression as that in *C. acetobutylicum* [47].

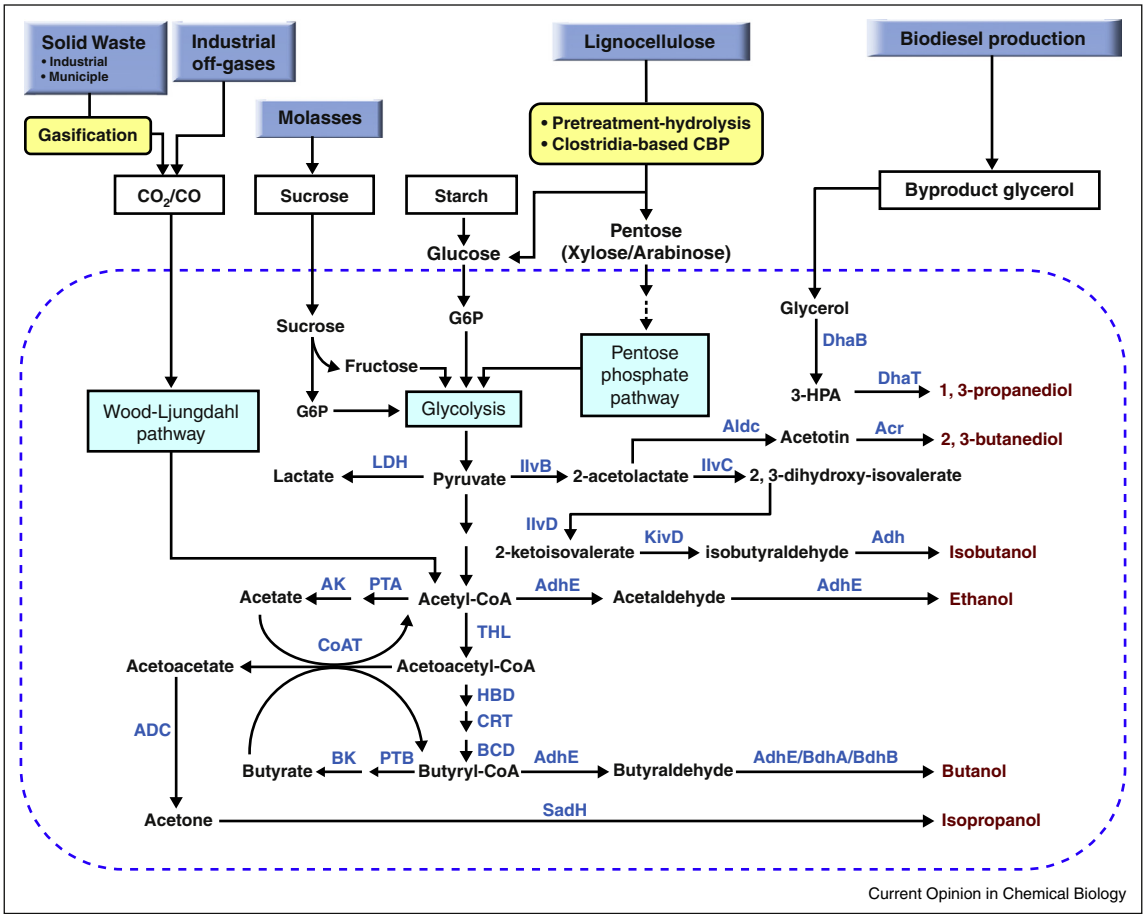
To address these challenges, many efforts have been made, such as inactivating key factors responsible for CCR [48,49], mutating catabolite responsive element (CRE) [50], disrupting specific repressors of the xylose degradation pathway [47], and strengthening the xylose degradation pathway [51,52]. All of these strategies have enabled *C. acetobutylicum* and *C. beijerinckii* to efficiently co-ferment a mixture of sugars.

Additionally, the tolerance of clostridia to the toxicity of lignocellulosic hydrolysates is also a key issue. A variety of toxic inhibitors, such as phenolic compounds and furan derivatives [53•], will form during the pretreatment process of lignocellulosic biomass. Commonly, detoxification depends on physical and chemical approaches [54], which will inevitably increase the preparation cost of hydrolysates; thus, this is unlikely to be the optimal choice for industrialization. Similar problems have also occurred with biotechnological routes, *i.e.*, microbial and enzymatic detoxification [55]. Therefore, a promising strategy is to seek or construct alcohol producing strains with high tolerance to inhibitors; however, to date, this is still a challenging task for clostridia, and little progress has been made [56,57].

Cellulolytic clostridia-based consolidated bioprocessing (CBP) for lignocellulosic biomass utilization

Compared to PHF route, an alternative strategy for the bioconversion of lignocellulosic materials to chemicals and biofuels is CBP, which depends on cellulolytic

Figure 1



Schematic of native and non-native alcohol production in clostridia. CBP, consolidated bioprocessing; G6P, glucose 6-phosphate; LDH, lactate dehydrogenase; PTA, phosphotransacetylase; AK, acetate kinase; PTB, phosphotransbutyrylase; BK, butyrate kinase; THL, thiolase; HBD, 3-hydroxybutyryl-CoA dehydrogenase; CRT, 3-hydroxybutyryl-CoA dehydratase; BCD, crotonase; AdhE, bifunctional alcohol and aldehyde dehydrogenase; BdhA, butanol dehydrogenase A; BdhB, butanol dehydrogenase B; CoAT, acetoacetyl-CoA:acetate/butyrate:coenzyme A transferase; ADC, acetoacetate decarboxylase; SadH, primary/secondary alcohol dehydrogenase; DhaB, glycerol dehydratase; 3-HPA, 3-hydroxypropionaldehyde; DhaT, 1,3-propanediol oxidoreductase; IlvB, acetolactate synthase; IlvC, acetohydroxy-acid isomeroreductase; IlvD, dihydroxyacid dehydratase; KivD, α-ketoisovalerate decarboxylase; Adh, alcohol dehydrogenase; Aldc, acetolactate decarboxylase; Acr, acetoin reductase.

microorganisms that are capable of both hydrolysing lignocellulosic materials and fermenting sugars. CBP combines three steps (production of saccharolytic enzymes, hydrolysis of polysaccharides and fermentation of sugars) within one reactor, which can significantly decrease biomass processing costs [6]. Many *Clostridium* species, such as *C. thermocellum*, *C. cellulolyticum* and *C. cellulovorans*, have been found to be able to secrete cellulase and can directly grow on lignocellulose and hemicellulose (Table 1). Moreover, some cellulolytic clostridia have been engineered for CBP-based biorefinery to produce ethanol and other higher alcohols [18,20•] (Table 2). However, industrial-scale application of CBP still remains challenging due to the low saccharification rate of lignocellulosic biomass, which severely drags down the conversion efficiency of the whole system.

Utilization of gaseous substrates by clostridia

To bypass the recalcitrance of lignocellulosic materials to degradation, which makes the development of an economical process extremely challenging, one alternative is to directly fix inorganic carbon before incorporation into plants. Autotrophic *Clostridium* species can use the Wood-Ljungdahl (WL) pathway (also known as the reductive acetyl-CoA pathway) to capture carbon (CO or CO_2), allowing the focus to be on the biological utilization of gaseous substrates, *e.g.*, 'synthesis gas' (mixtures of CO, CO_2 and H_2) and waste gases from industry [58••,59].

Briefly, the WL pathway consists of two branches, the methyl and the carbonyl branches, which are responsible for the reduction of CO_2 to methyl and CO, respectively [60]. Among all known six natural pathways for autotrophic

Table 2

Recent advances in metabolic engineering of Clostridia to produce alcohols

Organism	Target	Substrate	Genes ectopically expressed	Host genotype	Titer (g/L)	Reference
C. acetobutylicum	Butanol	Glucose	*adhE1*^{D485G}	*Δbuk Δpta*	18.9	[28]
C. acetobutylicum	Butanol	Glucose	*pfkA, prkA*	WT	13.07	[68]
C. acetobutylicum	Butanol	Glucose	*thl* ^{9G4}	WT	12.4	[70]
C. acetobutylicum	IBE (isopropanol-butanol-ethanol)	Glucose	*sadh,hydG*	WT	27.9	[83]
C. beijerinckii	Butanol	Glucose	–	*ΔnuoG*	9.5	[69]
C. tyrobutyricum	Butanol	Glucose, xylose	*adhE2, xylT, xylA, xylB*	*Δack*	15.7	[79]
C. cellulovorans	Butanol	Cellulose	*adhE2*	WT	1.42	[20•]
C. thermocellum	Ethanol	Avicel	–	*Δhpt ΔhydG Δldh Δpfl Δpta-ack*	3.4	[18]
C. thermocellum	Isobutanol	Cellulose	*kivD, ilvB, ilvN, ilvC, ilvD*	WT	5.4	[19•]
C. autoethanogenum	Butanol	CO	*thlA, bcd, ctfA, ctfB, buk, ptb, hbd*	WT	1.9	[84]

carbon fixation, the WL pathway is the shortest CO_2-fixing pathway, only using eight enzymatic reaction steps to convert CO_2 to acetyl-CoA [60]; moreover, the WL pathway is also the most energetically efficient pathway for acetyl-CoA production, consuming approximately one ATP for the synthesis of one acetyl-CoA [60,61].

During gas fermentation, CO can act as both the substrate and the electron donor, while CO_2 is the only carbon source, requiring an extra supply of electrons. Additionally, reducing equivalents can be obtained directly or indirectly from electricity [62] and the catabolism of other compounds, such as sugars [63]. For the latter, one effective approach is 'acetogenic mixotrophy', which makes full use of the complementarity of WLP and glycolysis, whereby the two molecules of CO_2 and eight electrons yielded from glycolysis are captured and reused again by WLP to form an extra acetyl-CoA [64].

It is known that low gas–liquid mass transfer rate is a distinct constraint for the gas bioconversion rate [65]. To overcome this hurdle, a variety of bioreactor systems have been tested, including agitated and non-agitated reactors [66], among which hollow fiber membrane bioreactors (HFMBR) have been suggested for their advantages in terms of mass transfer capability over conventional reactors [66]. For example, such a reactor configuration has enabled the production of a considerable ethanol concentration (over 23 g/L) in the fermentation of syngas using *C. carboxidivorans* P7 [67]. However, considering manufacturing and running costs, it is uncertain whether HFMBR is feasible in industrial-scale gas fermentation.

Production of alcohols in native clostridial hosts

The typical clostridia-based ABE fermentation using saccharolytic *Clostridium* species (*C. acetobutylicum*, *C. beijerinckii*, *C. saccharobutylicum* and *C. saccharoperbutylacetonicum*) primarily produces two types of important alcohols, 1-butanol and ethanol, normally comprising 60–70% and 10% of total fermentation products, respectively. Because 1-butanol has an obvious advantage over ethanol as a biofuel (*e.g.*, lower oxygen content, similar octane values compared with gasoline and lower vapour pressure), many metabolic engineering strategies have attempted to improve the titer and yield of 1-butanol produced during clostridial ABE fermentation, such as inactivating by-product synthetic pathways [28], increasing intracellular NAD(P)H levels [68,69], and strengthening the metabolic flux of some related pathways [70,71••] (Table 2). Of note, Jang *et al.* reinforced a 'hot channel' for direct 1-butanol production in *C. acetobutylicum*, realizing a significant increase in both the titer (18.9 g/L) and yield (0.71 mol/mol glucose) of 1-butanol produced by batch fermentation [28].

For cellulolytic clostridia, ethanol is the only known alcohol that is natively produced, and many efforts aiming to improve ethanol production by these anaerobes have been reported [72]. However, to date, the undesired ethanol-producing ability of cellulolytic clostridia is still a major obstacle for commercial scale production. A more promising strategy is to construct a cross-species reaction system by co-cultivating cellulolytic clostridia and other specific microbial producers [73]. For example, the co-cultivation of engineered *C. thermocellum* and *Thermoanaerobacterium saccharolyticum* realized 38 g/L ethanol within 145 h using avicel [74]. This strategy is also used to produce higher alcohols. For instance, co-cultivation of *C. thermocellum* and saccharolytic *C. beijerinckii* has enabled the production of 1-butanol directly from a variety of lignocellulosic materials [73]. Although all of these observations indicate the potential of synthetic consortia in the biorefinery of lignocellulosic materials, it cannot meet the industrial requirements currently due to the low conversion efficiency (over 100 h of bioconversion process).

Alcohols natively produced by autotrophic gas-fermenting clostridia include not only ethanol but also some

higher alcohols, *e.g.*, 1-butanol, 2,3-butanediol and hexanol [37,39]; however, the concentrations of these alcohols are very low in batch fermentation. In response, new methods and systems (strain evolution, medium optimization, unconventional bioreactors and process control) have been tested, whereby high productivity of ethanol has been achieved, which was well summarized by Liew *et al.* [58••]. It is worth noting that the real potential of autotrophic clostridia resides in their ability to produce high-value higher alcohols other than ethanol (Table 1); however, the ability to produce higher alcohols by these acetogens has increased very little.

Production of alcohols in non-native clostridial hosts

The efficient production of alcohols in non-native clostridial hosts will require the design and implementation of new metabolic pathways as well as pathway optimization to adapt them to a global metabolic network. The tools used to date have enabled us to achieve the objectives in these anaerobes [5•]. Recently, CRISPR/Cas9 genome editing system has been successfully used in several Clostridia species [75–78].

1-Butanol, as mentioned above, is a major higher alcohol produced by clostridia. However, due to the high toxicity of 1-butanol to cells, typical ABE fermentation cannot achieve a breakthrough in 1-butanol production. An alternative effective approach is to reconstruct a 1-butanol synthetic pathway in *Clostridium* species with higher 1-butanol tolerance. A recent study attempted to introduce the 1-butanol synthetic pathway into *C. tyrobutyricum*, and realized as high as 15.7 g/L of 1-butanol [79] (Table 2); meanwhile, because of the native deficiency in acetone synthesis, a high yield of 1-butanol was obtained using recombinant *C. tyrobutyricum* [80]. Additionally, isobutanol, another attractive biofuel and platform chemical, can be synthesized through metabolic engineering of cellulolytic clostridia [19•] (Table 2).

Isopropanol is a bulk chemical and can also be used as a gasoline additive. Although some clostridial species are capable of naturally producing isopropanol, more efforts were made to engineer ABE-producing *C. acetobutylicum*, a widely studied species once used in large-scale application, to produce isopropanol more efficiently [81,82]. As *C. acetobutylicum* can synthesize acetone, isopropanol can be easily obtained *via* only a one-step reduction in acetone by introducing the secondary alcohol dehydrogenase from *Clostridium beijerinckii* NRRL B-593 [82]. This enables the conversion of typical ABE (acetone-butanol-ethanol) fermentation into IBE (isopropanol-butanol-ethanol) fermentation, realizing the production of mixed alcohols by clostridia with high titers (21–28 g/L) [81,83] (Table 2).

Heterologous genes have also been introduced into cellulolytic and autotrophic gas-fermenting clostridia to realize the production of non-native higher alcohols. For the former, introduction of the CoA-dependent pathway and aldehyde/alcohol dehydrogenase enabled *C. cellulolyticum* and *C. cellulovorans*, respectively, to produce 1-butanol [20•,85] (Table 2); introduction of the keto acid pathway into *C. cellulolyticum* and *C. thermocellum* realized the production of isobutanol [19•,22] (Table 2). For the latter, *C. ljungdahlii* and the closely related *C. autoethanogenum* have been engineered recently to produce more alcohols in addition to ethanol, *e.g.*, 1-butanol [36] and 2,3-butanediol [37,86], using CO and CO_2 as the carbon sources (Table 2). This further demonstrated the importance of clostridia-based gas fermentation as a flexible platform in producing short-chain biofuels and chemicals.

Conclusion

To date, among potential biofuels, ethanol is still the closest to commercial scale production. However, higher alcohols are known to demonstrate better properties over ethanol, appearing to play essential roles in the future usage of biofuels, specifically advanced biofuels.

Solventogenic clostridia are of great interest due to their exceptional substrate and metabolite diversity. Compared to other major industrial microbial hosts, *e.g.*, *E. coli* and yeast, solventogenic clostridia is able to directly utilize more types of renewable feedstocks and wastes, such as lignocelluloses and C1 gases, and simultaneously produce a variety of distinctive metabolites. Furthermore, solventogenic clostridia still maintain the highest production ability of certain higher alcohols, such as 1-butanol. Thus, this group of acetogens can be used as an important platform for biomanufacturing.

Currently, the principal factor that limits the competitiveness of the biological production of alcohols relative to the petrochemical route is the high cost of feedstocks. In this regard, cellulolytic and gas-fermenting *Clostridium* species may play more roles in the near future.

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