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Gas Fermentation for Commercial Biofuels Production

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1. Introduction

With diminishing global reserves of crude oil and increasing demand, especially from developing countries, the pressure on oil supply will grow. Although the 2007-2010 financial crisis brought down the price of crude oil (per barrel) from a record peak of US \$145 in July 2008, factors such as recovering global economies and political instability in the Middle East have restored the price of crude oil to the US\$100 mark. At current rate of consumption, the global reserves of petroleum are predicted to be exhausted within 50 years [1, 2]. This, coupled with the deleterious environmental impacts that result from accumulating atmospheric CO₂ from the burning of fossil fuels, the development of affordable, and environmentally sustainable fuels is urgently required. Many countries have responded to this challenge by legislating mandates and introducing policies to stimulate research and development (R&D) and commercialization of technologies that allow the production of low cost, low fossil carbon emitting fuels. For instance, the European Union (EU) has mandated member countries to a target of deriving 10% of all transportation fuel from renewable sources by 2020 [3]. Between 2005 and 2010, renewable energies such as solar, wind, and biofuels have been increasing at an average annual rate of 15-50% [4]. Renewable energy accounted for an estimated 16% of global final energy consumption in 2009 [4].

Biofuels have been defined as solid (bio-char), liquid (bioethanol, biobutanol, and biodiesel) and gaseous (biogas, biosyngas, and biohydrogen) fuels that are mainly derived from biomass [5]. Liquid biofuels provided a small but growing contribution towards worldwide fuel usage, accounting for 2.7% of global road transport fuels in 2010 [4]. The world's largest producer of biofuels is the United States (US), followed by Brazil and the EU [4]. In 2009, US and Brazil accounted for approximately 85% of global bioethanol

production while Europe generated about 85% of the world's biodiesel [6]. The global market for liquid biofuels (bioethanol and biodiesel) increased dramatically in recent years, reaching US\$83 billion in 2011 and is projected to US\$139 billion by 2021 [7].

The use and production of biofuels has a long history, starting with the inventors Nikolaus August Otto and Rudolph Diesel, who already envisioned the use of biofuels such as ethanol and natural oils when developing the first Otto cycle combustion and diesel engines [6]. While fermentative production of ethanol has been used for thousands of years, mainly for brewing beer starting in Mesopotamia 5000 B.C., fermentative production of another potential biofuel butanol, has only been discovered over the last century, but had significant impact. During the World War 1, Chaim Weizmann successfully applied a process called ABE (acetone-butanol-ethanol) fermentation using *Clostridium acetobutylicum* to generate industrial scale acetone (for cordites, the propellant of cartridges and shells) from starchy materials [6, 8]. His contribution was later recognised in the Balfour declaration in 1917 and he became the first President of the newly founded State of Israel [6, 8]. Intriguingly, the enormous potential of butanol produced at that time was not realized and the substance was simply stored in huge containers [6]. ABE fermentation became the second biggest ever biotechnological process (after the ethanol fermentation process) ever performed, but the low demand of acetone following the conclusion of the war led to closure of all the plants [8]. Although ABE fermentation briefly made a comeback during the Second World War, increasing substrate costs and increasing stable supply of low cost crude oil from the Middle East rendered the technology economically unviable. Recently, a resurgence of the technology is underway as some old plants are reopened and new plants are being built or planned in China, the US, the United Kingdom (UK), Brazil, France and Austria [6, 8].

Traditionally sugar substrates derived from food crops such as sugar cane, corn (maize) and sugar beet have been the preferred feedstocks for the production of biofuels. However, world raw sugar prices have witnessed significant volatility over the last decade or so, ranging from US\$216/ton in year 2000 to a 30 year high of US\$795/ton in February 2011 due to global sugar deficits and crop shortfall [9]. This has created uncertainty and raised sustainability issues about its use as a feedstock for large scale biofuel production. This review aims to shed light on the use of syngas and industrial waste gas as feedstocks, and the emerging field of gas fermentation to generate not only biofuels, but also other high-value added products. The advantages of gas fermentation over conventional sugar-based fermentation and thermochemical conversions, and their flexibility in utilizing a spectrum of feedstocks to generate syngas will be discussed. The biochemistry, genetic and energetic background of the microorganisms that perform this bioconversion process will be critically examined, together with recent advances in systems biology and synthetic biology that offer growing opportunities to improve biocatalysts in terms of both the potential products that can be produced and their process performance. The key processes such as gasification, bioreactor designs, media formulation, and product recovery will be analysed. Finally, the state of commercialization of gas fermentation will be highlighted and an outlook will be provided.

2. Advantages of gas fermentation

The production of first generation biofuels relies on food crops such as sugar beet, sugar cane, corn, wheat and cassava as substrates for bioethanol; and vegetable oils and animal fats for biodiesel. Although years of intense R&D have made methods of bioethanol production (typically using the yeast *Saccharomyces cerevisiae*) technologically mature, there remain some serious questions regarding its sustainability. The use of food crops as a source of carbohydrate feedstocks by these processes requires high-quality agricultural land. The inevitable conflict between the increasing diversion of crops or land for fuel rather than food production has been highlighted as one of the prime causes of rising global food prices. Furthermore, corn ethanol producers in the US, have historically enjoyed a 45-cent-a-gallon federal tax credit for years (which ended in early 2012), costing the government US\$30.5 billion between 2005 to 2011, raising questions about its economic competitiveness with gasoline [10, 11].

These arguments have stimulated the search for so-called second generation biofuels, which utilize non-food lignocellulose biomass such as wood, dedicated energy crops, agricultural residues and municipal solid wastes as feedstocks. Biomass consists of cellulose, hemicellulose and lignin, and the latter of which is extremely resistant to degradation. One approach to unlocking the potential in this abundant feedstock is to separate the lignin from the carbohydrate fraction of the biomass via extensive pre-treatment of the lignocellulose involving, for example, steam-explosion and/or acid hydrolysis. These pre-treatments are designed to allow the carbohydrate portion of the biomass to be broken down into simple sugars, for example by enzymatic hydrolysis using exogenously added cellulases to release fermentable sugars [12]. Such approaches have been found to be expensive and rate limiting [6, 12, 13]. Alternatively, processes using cellulolytic microorganisms (such as *C. cellulolyticum*, *C. thermocellum*, and *C. phytofermentans*) to carry out both the hydrolysis of lignocelluloses and sugar fermentation in a single step, termed 'Consolidated Bioprocessing Process (CBP)' [12] have been proposed, however the development of these is still at an early stage, and again low conversion rates seem to be a major limitation that needs to be overcome.

Microorganisms such as acetogens, carboxytrophs and methanogens are able to utilize the CO₂ + H₂, and/or CO available in such syngas as their sole source of carbon and energy for growth as well as the production of biofuels and other valuable products. However, only acetogens are described to synthesize metabolic end products that have potentials as liquid transportation fuels. While biological processes are generally considered slower than chemical reactions, the use of these microbes to carry out syngas fermentation offers several key advantages over alternative thermo-chemical approaches such as the Fischer-Tropsch' process (FTP). First, microbial processes operate at ambient temperatures and low pressures which offer significant energy and cost savings. Second, the ambient conditions and irreversible nature of biological reactions also avoid thermodynamic equilibrium relationships and allow near complete conversion efficiencies [14, 15]. Third, biological conversions are commonly more specific due to high enzymatic specificities, resulting in higher product yield with the formation of fewer by-products. Fourth, unlike traditional chemical catalysts which require a set feed gas composition to yield desired product ratios or suite, microbial processes have freedom to operate for the production of the same suite of products across a wider range of CO:H₂ ratios in the feed gas [16]. Fifth, bio-

catalysts exhibit a much higher tolerance to poisoning by tars, sulphur and chlorine than inorganic catalysts [6, 16]. However, some challenges have been identified for syngas fermentation to be commercialized, including gas mass transfer limitations, long retention times due to slow cell growth, and lower alcohol production rates and broth concentrations. Recent progress and development to remedy these issues will be highlighted in this review.

3. Feedstock and gasification

Due to the flexibility of the microbes to ferment syngas with diverse composition, virtually any carbonaceous materials can be used as feedstock for gasification. Non-food biomass that can be employed as feedstock for gasification includes agricultural wastes, dedicated energy crops, forest residues, and municipal organic wastes, or even glycerol and feathers [16-20]. Biomass is available on a renewable basis, either through natural processes or anthropogenic activities (e.g. organic wastes). It has been estimated that out of a global energy potential from modern biomass of 250 EJ per year in 2005, only 9 EJ (3.6%) was used for energy generation [18]. The use of existing waste streams such as municipal organic waste also differentiate itself from other feedstocks such as dedicated energy crops because these wastes are available today at economically attractive prices, and they are often already aggregated and require less indirect land use. Alternatively, gasification of non-biomass sources such as coal, cokes, oil shale, tar sands, sewage sludge and heavy residues from oil refining, as well as reformed natural gas are commonly applied as feedstocks for the FTP and can also be used for syngas fermentation [15, 21]. Furthermore, some industries such as steel manufacturing, oil refining and chemical production generate large volume of CO and/or CO₂ rich gas streams as wastes. Tapping into these sources using microbial fermentation process essentially convert existing toxic waste gas streams into valuable commodities such as biofuels. The overall process of gas fermentation is outlined in Figure 1.

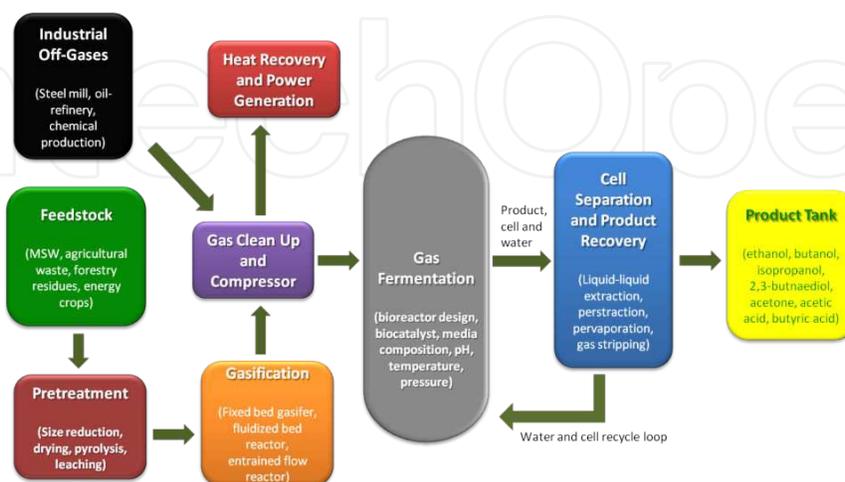


Figure 1. Overview of gas fermentation process

Prior to gasification, biomass generally needs to go through a pre-treatment process encompassing drying, size reduction (e.g. chipping, grinding and chopping), pyrolysis, fractionation and leaching depending on the gasifier configuration [22, 23]. This upstream pre-treatment process can incur significant capital expense and add to the overall biomass feedstock cost, ranging from US\$16-70 per dry ton [22]. Gasification is a thermo-chemical process that converts carbonaceous materials to gaseous intermediates at elevated temperature (600-1000°C), in the presence of an oxidizing agent such as air, steam or oxygen [16, 22]. The resulting syngas contains mainly CO, CO₂, H₂ and N₂, with varying amounts of CH₄, water vapour and trace amount of impurities such as H₂S, COS, NH₃, HCl, HCN, NO_x, phenol, light hydrocarbons and tar [17, 22, 24]. The composition and amount of impurities of syngas depends on the feedstock properties (e.g. moisture, dust and particle size), gasifier type and operational conditions (e.g. temperature, pressure, and oxidant) [17, 22]. Table 1 summarizes typical composition of syngas and other potential gas streams derived from various sources.

4. Fixed bed gasifier

Depending on the direction of the flows of carbonaceous fuel and oxidant (air or steam), fixed bed gasifier can be further categorized into updraft or downdraft reactor. In the updraft (counter-current) version of the fixed bed gasifier, biomass enters from the top while gasifying agent from the bottom. The biomass moves down the reactor through zones of drying (100°C), pyrolysis (300°C), gasification (900°C) and finally oxidation zone (1400°C) [18]. Although this mode of gasifier is often associated with high tar content in the exit gas, recent advances in tar cracking demonstrated that very low tar level is achievable [31]. The direct heat exchange of the oxidizing agent with the entering fuel feed results in low gas exit temperature and hence high thermal efficiency [18, 23]. The downdraft (co-current) gasifier has very similar design as the updraft reactor, except the carbonaceous fuel and oxidizing agent flow in the same direction. In comparison to the updraft gasifier, the downdraft reactor has lower tar content in the exit gas but exhibit lower thermal efficiency [23]. Due to the size limitation in the constriction (where most of the gasification occurs) of the reactor, this mode of gasifier is considered unsuitable for large scale operation [18].

5. Fluidized bed reactor

In fluidized bed reactor, the carbonaceous fuel is mixed together with inert bed material (e.g. silica sand) by forcing fluidization medium (e.g. air and/or steam) through the reactor. The inert bed facilitates better heat exchange between the fuel materials, resulting in nearly isothermal operation conditions and high feedstock conversion efficiencies [18, 22]. The maximum operating temperature of the gasifier is typically around 800 - 900°C, which is limited by the melting point of the bed material [18]. Furthermore, the geometry of the reactor and excellent mixing properties also means that fluidized bed reactors are suitable for

up-scaling [18, 22]. Due to these properties, fluidized bed reactor is currently the most commonly used gasifier for biomass feedstock [32]. However, this mode of gasifier is not suitable for feedstocks with high levels of ash and alkali metals because the melting of these components causes stickiness and formation of bigger lumps, which ultimately negatively affect the hydrodynamics of the reactor [18].

	Composition vol%, dry basis						Ref.
	CO	CO ₂	H ₂	N ₂	CH ₄	Other	
Non-biomass source							
Coal gasification	59.4	10	29.4	0.6	0	0.6	[25]
Coke oven gas	5.6	1.4	55.4	4.3	28.4	4.9	[25]
Partial oxidation of heavy fuel oil	47	4.3	46	1.4	0.3	1	[26]
Hardwood chips + 20 wt%liquid crude glycerol	19.73	11.67	19.38	NR*	3.82	NR*	[19]
Steam reforming of natural gas	15.5	8.1	75.7	0.2	0.5	0	[25]
Steam reforming of Naphtha	6.7	15.8	65.9	2.6	6.3	2.7	[25]
Water gas	30	3.4	31.7	13.1	12.2	9.6	[25]
Steel Mill	44	22	2	32	0	0	[27]
Biomass and organic waste source							
Demolition wood + sewage sludge	10.53	15.02	8.02	60.46	3.19	2.78	[28]
Cacao shell	8	16.02	9.02	61.45	2.34	3.17	[28]
Dairy biomass	8.7	15.7	18.6	56	0.6	0.4	[29]
Switchgrass	14.7	16.5	4.4	56.8	4.2	3.4	[13]
Kentucky bluegrass straw	12.9	17.4	2.6	64.2	2.1	0.8	[30]
Willow	9.4	17.1	7.2	60.42	3.3	2.58	[28]

Note: NR, not reported

The factors that determine which type of gasifier to employ are scale of operation, feedstock size and composition, tar yield and sensitivity towards ash [18]. Currently, three main types of gasifier are commercially employed: fixed bed, fluidized bed and entrained flow reactors [18].

Table 1. Typical composition of syngas and other potential gas streams from various sources

6. Entrained flow reactor

Entrained flow reactor is the preferred route for large scale gasification of coal, petcoke and refinery residues because of high carbon conversion efficiencies and low tar production [22]. This mode of gasifier does not require inert bed material but relies on feeding the feedstocks co-currently with oxidizing agent at high velocity to achieve a pneumatic transport regime

[18]. At operating temperature of 1200-1500°C, this method is able to convert tars and methane, resulting in better syngas quality [18]. Importantly this technology requires the feedstocks to be pulverised into fine particles of ~50 µm before feeding, which is not a major issue for coal but very difficult and costly for biomass sources [18, 22].

7. Microbes and biochemistry of gas fermentation

Acetogens are defined as obligate anaerobes that utilize the reductive acetyl-CoA pathway for the reduction of CO₂ to the acetyl moiety of acetyl-coenzyme A (CoA), for the conservation of energy, and for the assimilation of CO₂ into cell carbon [33]. In addition to the reductive acetyl-CoA pathway, four other biological pathways are known for complete autotrophic CO₂ fixation: the Calvin cycle, the reductive tricarboxylic acid (TCA) cycle, the 3-hydroxypropionate/methyl-CoA cycle and the 3-hydroxypropionate/4-hydroxybutyrate cycle [34]. Since the earlier atmosphere of earth was anoxic and the acetyl-CoA pathway is biochemically the simplest among the autotrophic pathways (the only linear pathway, whereas the other four pathways are cyclic), it has been postulated to be the first autotrophic process on earth [35, 36]. The reductive acetyl-CoA pathway is also known as the 'Wood-Ljungdahl' pathway, in recognition of the two pioneers, Lars G. Ljungdahl and Harland G. Wood, who elucidated the chemical and enzymology of the pathway using *Moorella thermoacetica* (formerly: *Clostridium thermoaceticum*) [35] or CODH/ACS pathway after the key enzyme of the pathway Carbon Monoxide dehydrogenase/Acetyl-CoA synthase. This ancient pathway is diversely distributed among at least 23 different bacterial genera: *Acetivomaculum*, *Acetonaerobium*, *Acetobacterium*, *Acetohalobium*, *Acetonema*, *Alkalibaculum*, "*Bryantella*", "*Butyrivacterium*", *Caloramator*, *Clostridium*, *Eubacterium*, *Holophaga*, *Moorella*, *Natroniella*, *Natronincola*, *Oxobacter*, *Ruminococcus*, *Sporomusa*, *Syntrophococcus*, *Tindallia*, *Thermoacetogenium*, *Thermoanaerobacter*, and *Treponema* [33]. A selection of mesophilic and thermophilic acetogens are presented in Table 2. Acetogens are able to utilize gases CO₂ + H₂, and/or CO to produce acetic acid and ethanol according to the following stoichiometries:



The Acetyl-CoA pathway is essentially a terminal electron-accepting process that assimilates CO₂ into biomass [35]. It constitutes an *Eastern* (or Carbonyl) branch and a *Western* (or Meth-

Species	Substrate	Product(s)	T _{opt} (°C)	pH _{opt}	Genome Status	Ref.
Mesophilic Microorganisms						
<i>Acetobacterium woodii</i>	H ₂ /CO ₂ , CO	Acetate	30	6.8	Available	[41, 42]
<i>Acetoneama longum</i>	H ₂ /CO ₂	Acetate, butyrate	30-33	7.8		[43]
<i>Alkalibaculum bacchi</i>	H ₂ /CO ₂ , CO	Acetate, ethanol	37	8.0-8.5		[44, 45]
<i>Blautia producta</i>	H ₂ /CO ₂ , CO	Acetate	37	7		[46]
<i>Butyribacterium methylotrophicum</i>	H ₂ /CO ₂ , CO	Acetate, ethanol, butyrate, butanol	37	6		[47-49]
<i>Clostridium aceticum</i>	H ₂ /CO ₂ , CO	Acetate	30	8.3	Under construction	[50-52]
<i>Clostridium autoethanogenum</i>	H ₂ /CO ₂ , CO	Acetate, ethanol, 2,3-butanediol, lactate	37	5.8-6.0		[27, 53]
<i>Clostridium carboxidivorans</i> or "P7"	H ₂ /CO ₂ , CO	Acetate, ethanol, butyrate, butanol, lactate	38	6.2	Draft	[54, 55]
<i>Clostridium drakei</i>	H ₂ /CO ₂ , CO	Acetate, ethanol, butyrate	25-30	5.8- 6.9		[55-57]
<i>Clostridium formicoaceticum</i>	CO	Acetate, formate	37	NR		[50, 58, 59]
<i>Clostridium glycolicum</i>	H ₂ /CO ₂	Acetate	37-40	7.0-7.5		[60, 61]
<i>Clostridium ljungdahlii</i>	H ₂ /CO ₂ , CO	Acetate, ethanol, 2,3-butanediol, lactate	37	6	Available	[27, 62, 63]
<i>Clostridium magnum</i>	H ₂ /CO ₂	Acetate	30-32	7.0		[64, 65]
<i>Clostridium mayombei</i>	H ₂ /CO ₂	Acetate	33	7.3		[66]
<i>Clostridium methoxybenzovorans</i>	H ₂ /CO ₂	Acetate, formate	37	7.4		[67]
" <i>Clostridium ragsdalei</i> " or "P11"	H ₂ /CO ₂ , CO	Acetate, ethanol, 2,3-butanediol, lactate	37	6.3		[68]
<i>Clostridium scatologenes</i>	H ₂ /CO ₂ , CO	Acetate, ethanol, butyrate	37-40	5.4-7.5		[55, 56]
<i>Eubacterium limosum</i>	H ₂ /CO ₂ , CO	Acetate	38-39	7.0-7.2	Available	[41, 69]
<i>Oxobacter pfennigii</i>	H ₂ /CO ₂ , CO	Acetate, butyrate	36-38	7.3		[70]
Thermophilic Microorganisms						
<i>Moorella thermoacetica</i>	H ₂ /CO ₂ , CO	Acetate	55	6.5-6.8	Available	[71-73]
<i>Moorella thermoautotrophica</i>	H ₂ /CO ₂ , CO	Acetate	58	6.1		[74]
<i>Thermoanaerobacter kiuvi</i>	H ₂ /CO ₂	Acetate	66	6.4		[72]
Notes: NR, not reported						

Table 2. Acetogens

yl) branch (Figure 2.). The *Western* branch employs a series of enzymes to carry out a six-electron reduction of CO₂ to the methyl group of acetyl-CoA, starting from the conversion of CO₂ to formate by formate dehydrogenase. Formyl-H₄folate synthase then condenses formate with H₄folate to form 10-formyl-H₄folate, which is then converted to 5,10-methenyl-H₄folate by a cyclohydrolase. This is followed by a dehydrogenase that reduces methenyl- to 5,10-methylene-H₄hydrofolate, before (6S)-5-CH₃-H₄folate is formed by methylene-H₄folate reductase [37]. A B12-dependent methyltransferase (MeTr) then transfer the methyl group of (6S)-5-CH₃-H₄folate to corrinoid iron-sulphur protein (CoFeSP) of the bi-functional carbon monoxide dehydrogenase/acetyl-CoA synthase (CODH/ACS) complex [37]. The bi-functional CODH/ACS enzyme complex is formed by two autonomous proteins, an α₂β₂ tetramer (CODH/ACS) and a γδ heterodimer (CoFeSP), and the genes are often arranged in an operon, together with MeTr [37, 38]. In the *Eastern* branch, the CODH component catalyzes the reduction of CO₂ to CO. The central molecule, acetyl-CoA, is finally generated when CO, methyl group (bound to CoFeSP) and CoASH are condensed by ACS. Given the pivotal role of CODH/ACS, it is unsurprising that this complex was found to be the most highly expressed transcripts under autotrophic conditions in *C. autoethanogenum* [27], and can represent up to 2% of the soluble cell protein of an acetogen [39]. CODH/ACS is not unique to acetogenic bacteria, as it is also present in sulphate-reducing bacteria, desulfitobacteria, and Archaea (methanogens and Archaeoglobus) [38, 40].

The reducing equivalents required for fixation of CO₂ carbon into acetyl-CoA come from the oxidation of molecular hydrogen under chemolithoautotrophic growth, or NADH and reduced ferredoxin under heterotrophic growth [75]. An extensive review by Calusinska *et al.* (2010) highlighted the diversity of ubiquitous hydrogenases that Clostridia possess although only one acetogen *C. carboxidivorans* was included in this study [76], which catalyze the reversible oxidation of hydrogen:



The direction of the hydrogenase reaction is directed by the redox potential of the components able to interact with the enzyme. Hydrogen evolution occurs when electron donor is available, whereas the presence of electron acceptor results in hydrogen oxidation [77]. Hydrogenases can be classified into three phylogenetically distinct classes of metalloenzymes: [NiFe]-, [FeFe]-, and [Fe]-hydrogenases [76]. In *Methanosarcina barkeri*, the Ech hydrogenase, a [NiFe]-hydrogenase, was demonstrated to oxidize H₂ to reduce ferredoxin [78]. During acetoclastic methanogenesis, Ech hydrogenase oxidize ferredoxin to generate H₂ [78]. Although genome analysis revealed the presence of Ech-like hydrogenase in *C. thermocellum*, *C. phytofermentans*, *C. papyrosolvans*, and *C. cellulolyticum*, their physiological roles remained unknown [76]. Clostridia harbour multiple distinct [FeFe]-hydrogenases, perhaps reflecting their ability to respond swiftly to changing environmental conditions [76]. The monomeric, soluble [FeFe]-hydrogenase of *C. pasteurianum* is one of the best studied. It transfer electrons from reduced ferredoxins or flavodoxins to protons, forming H₂ [79]. A trimeric [FeFe]-hydrogenase found in *C. difficile*, *C. beijerinckii*, and *C. carboxidivorans* were hypothesized to

couple formate oxidation to reduce protons into H_2 [76]. In *Thermotoga maritima*, an electron bifurcating, trimeric [FeFe]-hydrogenase was identified, that was shown to simultaneously oxidize reduced ferredoxin and NADH to evolve hydrogen under low H_2 partial pressure [80]. Under high H_2 partial pressure, the authors hypothesized that the NADH is oxidized to produce ethanol. *In silico* analysis revealed homologs of this bifurcating hydrogenase in a few Clostridia including *C. beijerinckii* and *C. thermocellum* [80]. In addition to classical hydrogenases, CODH/ACS and pyruvate:ferredoxin oxidoreductase (PFOR) from *M. thermoacetica* were shown to have hydrogen evolving capability, possibly as a mean of disposing excess reducing equivalents when electron carriers are limited and/or CO concentration is sufficient to inhibit conventional hydrogenases [81].

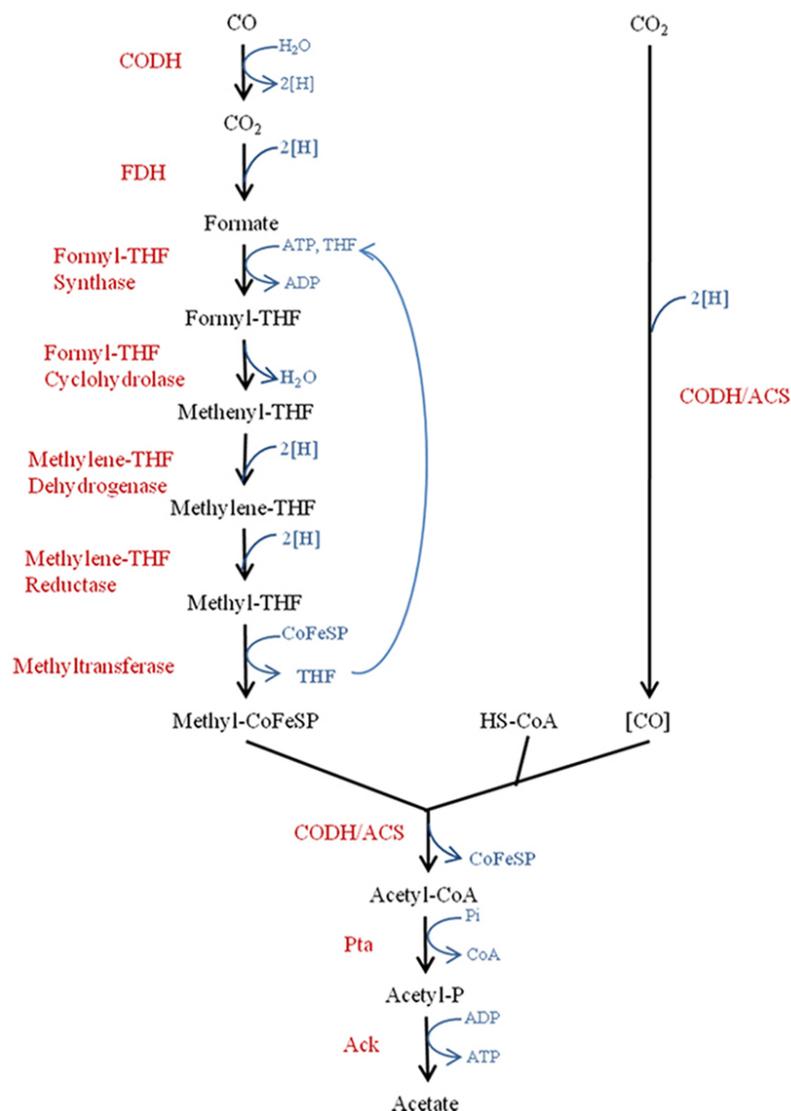


Figure 2. Wood-Ljungdahl Pathway. Ack, acetate kinase; ACS, acetyl-CoA synthase; CODH, carbon monoxide dehydrogenase; CoFeSP, corrinoid iron sulfur protein; FDH, formate dehydrogenase; Pta, phosphotransacetylase; THF, tetrahydrofolate.

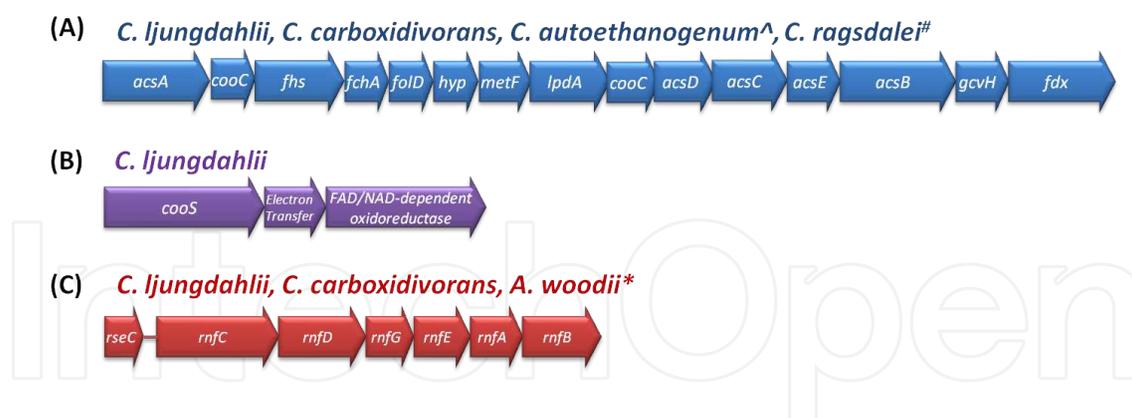


Figure 3. The organization of genes involved in acetogenesis and energy conservation from sequenced key acetogens. (A) Wood-Ljungdahl cluster; (B) carbon monoxide dehydrogenase (CODH) cluster; (C) Rnf complex cluster. *acsA*, CODH subunit; *acsB*, ACS subunit; *acsC*, corrinoid iron-sulfur protein large subunit; *acsD*, corrinoid iron-sulfur protein small subunit; *acsE*, methyltransferase subunit; *cooC*, gene for CODH accessory protein; *cooS*, CODH; *fchA*, formimido-tetrahydrofolate cyclodeaminase; *fdx*, ferredoxin; *fhs*, formyl-tetrahydrofolate synthase; *folD*, bifunctional methylene-tetrahydrofolate dehydrogenase/formyl-tetrahydrofolate cyclohydrolase; *gcvH*, gene for glycine cleavage system H protein; *hyp*, hypothetical protein; *lpdA*, dihydrolipoamide dehydrogenase; *metF*, methylene-tetrahydrofolate reductase; *rnfA*, *rnfB*, *rnfC*, *rnfD*, *rnfE*, *rnfG*, electron transport complex protein subunits; *rseC*, sigma E positive regulator. \wedge , truncated *acsA*. #, truncated *fdx*. *, lack *rseC*.

Most acetogens are also able to utilize another gas carbon monoxide (CO). In contrast to CO₂, CO can serve as both a source of carbon but also as source of electrons such that hydrogen is not necessarily required. With a CO₂/CO reduction potential of -524 to -558mV, CO is approximately 1000-fold more capable of generating extremely low potential electrons than NADH, capable of reducing cellular electron carriers such as ferredoxin and flavodoxin [38, 82]. The reducing equivalents generated from CO oxidation can be coupled to reduction of CO₂ into acetate, butyrate and/or methane, evolution of molecular hydrogen from protons, reduction of nitrate/nitrite, reduction of sulfur species and reduction of aldehydes into alcohols [35, 83]. However, relatively few microorganisms are able to utilize CO as sole carbon and energy source, probably due to growth inhibition from sensitivity of their metallo-proteins and hydrogenases towards CO [38, 83]. During exponential growth of *Pseudomonas carboxydovorans* (an aerobic carboxydotroph), it was demonstrated via immunological localization studies that 87% of the key enzyme CODH is associated with the inner cytoplasmic membrane, but this association was lost at the end of the exponential growth phase and a reduction in CO-dependent respiration rate was observed [84, 85]. It should be mentioned that aerobic and anaerobic CODH enzymes are structurally very different. CODH has been reported to be a very rapid and efficient CO oxidizer at rates between 4,000 and 40,000 s⁻¹, and reduces CO₂ at 11s⁻¹ [86, 87]. Other electron donors commonly used by acetogens include formate, CH₃Cl, lactate, pyruvate, alcohols, betaine, carbohydrate, acetoin, oxalate and citrate [88]. CODH is able to split water in a biological water-gas shift reaction into hydrogen and electron according to the stoichiometry:



The operation of this water gas shift reaction is the biochemical basis for the tremendous flexibility that acetogens have in terms of input gas composition. Via this reaction these organisms can flexibly use CO or H₂ as a source of electrons. Recently, some acetogens such as *C. ljungdahlii*, *C. acetivum*, *M. thermoacetica*, *Sporomusa ovata*, and *S. sphaeroides* have additionally been shown to utilize electrons derived from electrodes to reduce CO₂ into organic compounds such as acetate, formate, fumarate, caffeine, and 2-oxo-butyrate [89]. Termed microbial electrosynthesis, this nascent concept offers another route for acetogens to harvest the electrons generated from sustainable sources (e.g. solar and wind) to reduce CO₂ into useful multi-carbon products such as biofuels [90].

Under chemolithoautotrophic conditions, acetogenesis must not only fix carbon but also conserve energy. Approximately 0.1 mol of ATP is required for generation of 1g of dry biomass in anaerobes [82]. Acetyl-CoA is an energy rich molecule that through the combined actions of Pta (phosphotransacetylase) and Ack (acetate kinase), one ATP can be generated via substrate level phosphorylation (SLP). However, the activation of formate to 10-formyl-H₄folate in the methyl-branch of Acetyl-CoA pathway consumes one ATP so no net gain in ATP is achieved via this mechanism [35, 75]. Furthermore, the reduction of CO₂ to the carbonyl group also requires energy, estimated at one third of ATP equivalent [35]. Recent advances indicated that other modes of energy conservation such as electron transport phosphorylation (ETP) or chemiosmotic processes that are coupled to the translocation of protons or sodium ions are implicated in acetogens. Acetogens such as *M. thermoacetica* harbour membrane-associated electron transport system containing cytochrome, menaquinones, and oxidoreductases that translocate H⁺ out of the cell [33]. For acetogens that lack such membranous electron transport system, such as *Acetobacterium woodii* and *C. ljungdahlii*, a membrane-bound corrinoid protein is hypothesized to facilitate extrusion of Na⁺ or protons during the transfer of methyl group from methyl-H₄F to CODH/ACS [75]. However, all enzymes involved are predicted to be soluble rather than membrane bound. Recent evidence suggested coupling to an Rnf complex in *A. woodii*, and *C. ljungdahlii* (Figure 3) which acts as ferredoxin:NAD⁺-oxidoreductase [62, 91-93]. The Rnf complex is also found in other Clostridia (but not in ABE model organism *C. acetobutylicum*) and bacteria, and was originally discovered in *Rhodobacter capsulatus* where it is involved in nitrogen fixation [93]. Using reduced ferredoxin (Fd²⁻) generated from CO oxidation, carbohydrate utilization and/or hydrogenase reactions, this membrane-bound electron transfer complex is predicted to reduce NAD⁺ with concomitant translocation of Na⁺/ H⁺. The ion gradient generated from the above processes is harvested by H⁺- or Na⁺- ATP synthase to generate ATP [33, 93]. The recent genome sequencing of *A. woodii* revealed that Rnf complex is likely to be the only ion-pumping enzyme active during autotrophic growth and the organism's entire catabolic metabolism is optimized to maximize the Fd²⁻/NAD⁺ ratio [42]. Recently, a third mechanism of energy conservation which involves bifurcation of electrons by hydrogenases was proposed for anaerobes [94] and demonstrated for enzymes hydrogenase (see above; [80]), butyryl-CoA dehydrogenase [94, 95], or an iron-sulfur flavoprotein Nfn [96]. A similar mechanism has also been proposed for the methylene-THF reductase of the reductive acetyl-CoA pathway, which would enable this highly exergonic reduction step ($\Delta G^{\circ} = -22$ kJ/mol)

to be coupled with the Rnf complex for additional energy conservation [62]. However, no experimental proof to support this hypothesis has been published to date.

In an attempt to generate an autotrophic *E. coli*, the genes encoding MeTr, the two subunits of CODH/ACS, and the two subunits of CoFeSP from *M. thermoacetica* were cloned and heterologously expressed in *E. coli* [97]. Although the MeTr was found to be active, the other subunits misassembled hence no active enzymes were found [97]. Autotrophic capability is clearly a very complex process that involves many genes other than the CODH/ACS complex and tetrahydrofolate pathway, including compatible cofactors, electron carriers, specific chaperones and energy conservation mechanisms. For instance, more than 200 genes are predicted to be involved in methanogenesis and energy conservation from CO₂ and H₂ in methanogens [98]. A recent patent application described the introduction of three Wood-Ljungdahl pathway genes encoding MeTr, CoFeSP subunit α and β from *C. difficile* into *C. acetobutylicum* [99]. The recombinant strain was shown to incorporate more CO₂ into extracellular products than wild-type [99].

8. Products of gas fermentation

Acetyl-CoA generated via the Wood-Ljungdahl pathway serves as key intermediate for synthesis of cell mass as well as products. All acetogens are described to produce acetate, in order to gain energy via SLP to compensate for the energy invested in activating formate in the *Western* branch of the reductive acetyl-CoA pathway. Acetate and ATP are formed via acetyl-phosphate through the successive actions of Pta and Ack. *pta* and *ack* are arranged in the same operon and they were reported to be constitutively expressed [100]. With CO₂ and H₂ as substrate, only acetate has been observed as major product [44], with minor amounts of ethanol produced in rare cases with *C. ljungdahlii* [101], *C. autoethanogenum* [53], or "*Moor-ella sp.*" [102, 103]. Using the more reduced substrate CO, production of a range of other products have been reported, such as ethanol, butanol, butyrate, 2,3-butanediol [104], and lactate (Figure 4.) [105]. From a biofuel perspective, ethanol and butanol are of particular interest. Ethanol and butanol have even been described as the main fermentation products over acetate in some acetogens under specific conditions. Ethanol producers include *C. ljungdahlii* [62, 63], *C. autoethanogenum* [53], "*C. ragsdalei*" ("*Clostridium* strain P11") [106, 107], "*Moorella sp.*" [102, 103], *Alkalibaculum bacchii* [44], *C. carboxidivorans* ("*Clostridium* strain P7") [54, 55], and *B. methylotrophicum* [49, 108]. The latter two have also been described to produce butanol.

Due to historical roles in ABE fermentation, organisms like *C. acetobutylicum*, *C. beijerinckii*, *C. saccharobutylicum*, and *C. saccharoperbutylacetonicum* have been much more extensively characterized than acetogenic Clostridia [95]. Since *C. acetobutylicum* was the first *Clostridium* to be fully sequenced [109] and it remains the most commonly used species for industrial production of solvents to date [110], it provides a model for study of solventogenesis. Although sugar- and starch-utilizing ABE Clostridia and acetogens exhibit clear distinctions in substrate utilization and thus metabolism, they share some similarities in the biochemical

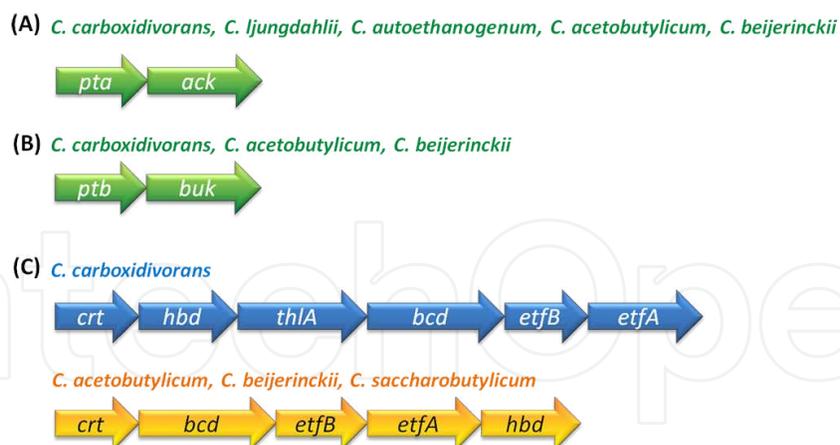


Figure 5. Similarity of acidogenesis and butanol formation gene clusters of acetogens and sugar-utilizing Clostridia. (A) Acetate-forming operon; (B) butyrate-forming operon; (C) butanol-forming operon. *ack*, acetate kinase; *buk*, butyrate kinase; *bcd*, butyryl-CoA dehydrogenase; *crt*, crotonase; *etfA*, electron-transferring flavoprotein subunit A; *etfB*, electron-transferring flavoprotein subunit B; *hbd*, 3-hydroxybutyryl-CoA dehydrogenase; *ptb*, phosphotransbutyrylase; *thlA*, thiolase.

Similar to sugar- and starch-utilizing ABE Clostridia, acetogens such as *C. carboxidivorans* [111, 112], *C. ljungdahlii* [113], and *C. autoethanogenum* [27] also typically undergo biphasic fermentation under autotrophic conditions. The first phase involves the production of carboxylic acids (acidogenic), H₂ and CO₂ during exponential growth. This is followed by the solventogenic phase in which part of the produced acids are reassimilated or reduced into solvents, which usually occurs during stationary growth phase [114]. This shift from acidogenesis to solventogenesis is of industrial importance and several transcriptional analysis on *C. acetobutylicum* [100, 115], and *C. beijerinckii* [116] have been performed to shed light on this process. In both organisms, the onset of solventogenesis coincides with an increase in expression of master sporulation/solventogenesis regulator gene *spo0A*, solventogenic genes such as *ald*, *ctfA-ctfB*, and *adc*, as well as down-regulation of chemotaxis/motility genes [100, 115, 116]. Physiologically, the signals that induce solventogenesis were hypothesized to involve temperature, low pH, high concentrations of undissociated acetic and butyric acids, limiting concentrations of sulphate or phosphate, ATP/ADP ratio and/or NAD(P)H levels [117].

For Clostridia such as acetogen *C. carboxidivorans* [54], which harbour the genes thiolase (*thlA*), 3-hydroxybutyryl-CoA dehydrogenase (*hbd*), crotonase (*crt*) and butyryl-CoA dehydrogenase (*bcd*), the two carbon acetyl-CoA can be converted to four carbon butyryl-CoA [95]. ThlA compete with the activities of Pta, Ald (aldehyde dehydrogenase), and PFOR to condense two acetyl-CoA into one acetoacetyl-CoA, and plays a key role in regulating the C2:C4 acid ratio [110, 118]. Since the formation of acetate yields twice as much ATP per mole of acetyl-CoA relative to butyrate formation, thiolase activity indirectly affects ATP yield [118]. Under physiological conditions, Crt catalyzes dehydration of β-hydroxybutyryl-CoA to crotonyl-CoA [119]. Bcd was shown to require a pair of electron transfer flavoproteins (EtfA and EtfB) to convert crotonyl-CoA to butyryl-CoA [120]. Furthermore, the Bcd was dem-

onstrated to form a stable complex with EtfA and EtfB, and they were shown to couple the reduction of crotonyl-CoA to butyryl-CoA with concomitant generation of reduced ferredoxins, which can be used for energy conservation via Rnf complex [94, 119]. Subsequent actions of phosphotransbutyrylase (*ptb*) and butyrate kinase (*buk*) then generate ATP and butyrate from butyryl-CoA [118].

Under low extracellular pH of 4-4.5, the secreted undissociated acetic acid (pK_a 4.79) and/or butyric acid (pK_a 4.82) diffuse back into cell cytoplasm and then dissociate into the respective salts and protons because of the more alkaline intracellular conditions. Without further interventions, the result of this is abolishment of the proton gradient and inevitable cell death [95]. The conversion of acetate and butyrate into solvents increase the pH, thus provide some time for the organism to sporulate and secure long term survival. However, the solvents produced are toxic because they increase membrane fluidity and disrupt critical membrane-associated functions such as ATP synthesis, glucose uptake and other transport processes [114, 121]. In *C. acetobutylicum*, it has been demonstrated that the addition of 7-13 g/l of butanol, or up to 40 g/l of acetone and ethanol resulted in 50% growth inhibition [122]. The bacterium is likely to experience a different cytotoxic effect from endogenously produced solvents because the organism has time to adapt to increasing amount of solvents.

The reassimilation of acetate and butyrate into the respective acyl-CoA and acetoacetate is catalyzed by acetoacetyl-CoA:acetate/butyrate CoA transferase (CtfA and CtfB) [110, 117, 118]. Acetoacetate is deconstructed by acetoacetate decarboxylase (Adc) into acetone and CO₂. This enzyme is missing in acetogenic *C. carboxidivorans* compared to the ABE strains [54, 123]. Some ABE strains such as *C. beijerinckii* NRRL B593 also possess a primary/secondary alcohol dehydrogenase that converts acetone to isopropanol [124]. In acetogenic "*C. ragsdalei*", reduction of acetone to isopropanol was also observed although the mechanism of this reduction is as yet unknown [124, 125]. Again, *C. carboxidivorans* lacks this activity [125]. The recycled acetyl-CoA and butyryl-CoA can be converted to ethanol and butanol through the actions of coenzyme A-acylating aldehyde dehydrogenase (Ald) and alcohol dehydrogenase (Adh) [110, 118]. Ald converts acyl-CoA into aldehydes, and the enzyme has been purified from *C. beijerinckii* NRRL B593 and was shown to be NADH-specific, exhibit higher affinity with butyraldehyde than acetaldehyde, but possess no Adh activity [126]. In *C. ljungdahlii*, two variants of aldehyde:ferredoxin oxidoreductases (AOR) are present in the genome, and they are hypothesized to couple reduced ferredoxin from CO oxidation via the CODH (see above) to perform the reversible reduction of acetate into acetaldehyde, which can be further reduced into ethanol [62].

The final step of solventogenesis utilizes Adh to reduce acetaldehyde and butyraldehyde into ethanol and butanol, respectively. For ethanol synthesis, transposon mutagenesis and enzymatic assay in *C. acetobutylicum* showed the involvement of a specific Ald that does not interact with butyryl-CoA, and a NAD(P)H-dependent Adh [127, 128]. The production of butanol by *C. acetobutylicum* is mainly due to the action of butanol dehydrogenase A and B (BdhA and BdhB), and bifunctional butyraldehyde/butanol dehydrogenase 1 and 2 (AdhE1 and AdhE2) [95]. In *C. carboxidivorans* [54] and *C. ljungdahlii* [62] both *adhE1* and *adhE2* are arranged in tandem and separated by a 200bp gap which contains a putative terminator [62,

111]. This is likely the result of gene duplication [62]. qRT-PCR analysis from *C. carboxidivorans* fed with syngas showed that the two *adhE* showed differential expression, and the more abundant *adhE2* was significantly upregulated over 1000 fold in a time span that coincided with the greatest rate of butanol production [111].

Pyruvate is a central molecule for anabolism and it is predominantly generated from glycolysis during heterotrophic growth. But under autotrophic growth, this four carbon molecule can be synthesized by PFOR and potentially also the pyruvate-formate lyase (PFL). Two variants of PFOR were reported in *C. autoethanogenum*, and transcriptional analysis showed that they were differentially expressed when grown using industrial waste gases (containing CO, CO₂ and H₂) [104]. Unlike PFL from most other microorganisms that only catalyze the lysis of pyruvate into formate and acetyl-CoA, clostridial PFL (*C. kluyveri*, *C. butylicum*, and *C. butyricum*) were reported to readily catalyze the reverse reaction (i.e. pyruvate formation) [129]. Apart from roles in anabolism, pyruvate is also a precursor to other products such as lactic acid and 2,3-butanediol. Small amounts of lactic acid are converted from pyruvate in acetogens, a reaction which is catalyzed by lactate dehydrogenase (Ldh) [104, 118]. Recently, Köpke *et al.* (2011) reported the production of 2mM 2,3-butanediol from acetogenic bacteria (*C. autoethanogenum*, *C. ljungdahlii*, and *C. ragsdalei*) using industrial waste gases (containing CO, CO₂ and H₂) as feedstock [104]. Pyruvate is first converted into α -acetolactate by the enzyme acetolactate synthase, followed by acetolactate decarboxylase which split acetolactate into acetoin and CO₂, before a final reduction of acetoin into 2,3-butanediol by 2,3-butanediol dehydrogenase [104] (Figure 4).

9. Strain improvement and metabolic engineering

The genomes of several solventogenic Clostridia, including gas fermenting species, have been sequenced since 2001 [54, 62, 109, 119, 123, 130], and an array of transcriptomic [100, 116, 121, 131, 132], proteomic [132] and systems analysis [133, 134] are being made increasingly available. However, the generation of stable recombinant Clostridia has been severely hindered by the difficulties encountered introducing foreign DNA into cells and a lack of established genetic tools for this genera of bacteria. In comparison to starch-utilizing Clostridia, very little information is available for metabolic engineering of acetogens. Although this section describes recent advances in the development of genetic tools for mostly sugar-utilizing Clostridia, these techniques are highly relevant and applicable to the closely related acetogenic Clostridia for biofuels or chemical production via gas fermentation.

The ideal microbial catalyst for industrial scale gas fermentation might exhibit the following traits: high product yield and selectivity, low product inhibition, no strain degeneration, asporogenous, prolonged cell viability, strong aero-tolerance, high biomass density and efficient utilization of gas substrates. These can be achieved by directed evolution, random mutagenesis and/or targeted genetic engineering. Traditionally, chemical mutagenesis [135-137] and adaption strategies [138, 139] have been deployed to select for these traits. However, these strategies are limited and often come with the expense of unwanted events.

First attempts of targeted genetic modification of Clostridia were made in the early 1990s by the laboratory of Prof. Terry Papoutsakis [140-142]. While these pioneering efforts relied on use of plasmids for (over)expression of genes in *C. acetobutylicum*, more sophisticated tools were later developed for a range of solventogenic and pathogenic Clostridia.

Antisense RNA (asRNA) has been employed to down-regulate genes. Here, single stranded RNA binds to a complementary target mRNA and prevents translation by hindering ribosome-binding site interactions [143]. For instance, this method has been used to knockdown *ctfB* resulting in production of 30 g/l solvents with significantly suppressed acetone yield in *C. acetobutylicum* ATCC 824 [144, 145].

Several homologous recombination methods have been developed for integration or knock-out of genes in a range of sugar-utilizing Clostridia. In early stage, knockout mutants were almost exclusively generated from single crossover events that could revert back to wild-type [146-152], with stable double crossovers only observed in rare cases [153, 154]. For *C. acetobutylicum* [155] and cellulolytic *C. thermocellum* [156] counter selectable markers have been developed to allow more efficient screening for the rare second recombination event.

ClosTron utilizes the specificity of mobile group II intron Ll.*ltrB* from *Lactococcus lactis* to propagate into a specified site in the genome via a RNA-mediated, retro-homing mechanism which can be used to disrupt genes [157]. This technique has initially been developed by InGex and Sigma-Aldrich under the name 'TargeTron™' and successfully adapted to a range of solventogenic and pathogenic Clostridia including *C. acetobutylicum*, *C. difficile*, *C. sporogenes*, *C. perfringens*, and *C. botulinum* [158-160] by the laboratory of Prof. Nigel Minton.

The same laboratory recently also developed another method for integration of DNA into the genome. Termed Allele-Coupled Exchange (ACE), this approach does not employ a counter selective marker to select for the rare second recombination event. Rather, it utilizes the activation or inactivation of gene(s) that result in a selectable phenotype, and asymmetrical homology arms to direct the order of recombination events [161]. Remarkably, the whole genome of phage lambda (48.5kb minus a 6kb region) was successfully inserted into the genome of *C. acetobutylicum* ATCC 824 in three successive steps using this genetic tool. This technique was also demonstrated in *C. difficile* and *C. sporogenes* [161].

For reverse engineering, mainly transposon mutagenesis has been utilized. Earlier efforts of transposon mutagenesis were demonstrated in *C. acetobutylicum* P262 (now: *C. saccharobutylicum* [162]), *C. acetobutylicum* DSM792, *C. acetobutylicum* DSM1732, and *C. beijerinckii* NCIM 8052, but issues with multiple transposon insertions per mutant, and non-random distribution of insertion were reported [163, 164]. Recent developments have seen the successful generation of mono-copy random insertion of transposon *Tn1545* into cellulolytic *C. cellulolyticum* [165] and mariner transposon *Himar1* into pathogenic *C. difficile* [166].

While there is still a lack of some other essential metabolic engineering tools such as efficient inducible promoters, the array of available tools that enabled significant improvements to the ABE process and cellulolytic Clostridia fermentations as summarized in Table 3.

Organism	Genetic modification	Phenotypes/Effects	Ref
Acetogens			
<i>C. ljungdahlii</i>	Plasmid overexpression of butanol biosynthetic genes from <i>C. acetobutylicum</i> (<i>thlA</i> , <i>crt</i> , <i>hbd</i> , <i>bcd</i> , <i>adhE</i> and <i>bdhA</i>)	Produced 2 mM butanol from syngas [62]	
<i>C. autoethanogenum</i>	Plasmid overexpression of butanol biosynthetic genes from <i>C. acetobutylicum</i> (<i>thlA</i> , <i>crt</i> , <i>hbd</i> , <i>bcd</i> , <i>etfA</i> , & <i>etfB</i>)	Produced 26 mM butanol using steel mill gas	
<i>C. autoethanogenum</i>	Plasmid expression of native <i>groES</i> and <i>groEL</i>	Increased alcohol tolerance	[168]
<i>C. aceticum</i>	Plasmid overexpression of acetone operon from <i>C. acetobutylicum</i> (<i>adc</i> , <i>ctfAB</i> , <i>thlA</i>)	Produced up to 140 μ M acetone using gas	[169, 170]
Acidogenesis and Solventogenesis			
<i>C. acetobutylicum</i>	Inactivation of <i>buk</i> and overexpression of <i>aad</i>	Produced same amount of butanol as control but relatively more ethanol, corresponding to a total alcohol tolerance of 21.2 g/l	[171]
<i>C. acetobutylicum</i>	Inactivation of <i>hbd</i> using Clostron	Produced 716 mM ethanol by diverting C4 products	[172]
<i>C. acetobutylicum</i>	Inactivation of <i>ack</i> using Clostron	Reduction in acetate kinase activity by more than 97% resulted in 80% less acetate produced but similar final solvent amount	[173]
<i>C. tyrobutylicum</i>	Inactivation of <i>ack</i> and plasmid overexpression of <i>adhE2</i> from <i>C. acetobutylicum</i>	Produced 216 mM butanol	[174]
<i>C. thermocellum</i>	Inactivation of <i>ldh</i> and <i>pta</i> via homologous recombination	Showed 4 fold increase in ethanol yield (122 mM instead of 28 mM)	[156]
<i>C. cellulolyticum</i>	Inactivation of <i>ldh</i> and <i>mdh</i> (malate dehydrogenase) using Clostron	Generated 8.5 times higher ethanol yield (56.4 mM) than wild type (6.5 mM)	[175]
<i>C. acetobutylicum</i>	Plasmid overexpression of a synthetic acetone operon (<i>adc</i> , <i>ctfA</i> , <i>ctfB</i>) and primary/secondary <i>adh</i> from <i>C. beijerinckii</i> NRRL B593	Produced 85 mM isopropanol	[176]

<i>C. acetobutylicum</i>	Genome insertion of <i>adh</i> gene from <i>C. beijerinckii</i> NRRL B593 using Allele-Coupled Exchange	Converted acetone into 28 mM isopropanol without affecting the yield of other fermentation products	[161]
Biosynthesis of New Products			
<i>C. cellulolyticum</i>	Plasmid overexpression of <i>kivD</i> , <i>yqhD</i> , <i>alsS</i> , <i>ilvC</i> and <i>ilvD</i>	Produced 8.9 mM isobutanol by diverting 2-ketoacid intermediates	[177]
<i>C. acetobutylicum</i>	Plasmid expression of native <i>ribG</i> operon and mutated PRPP amidotransferase	Produced 70 mg/l riboflavin and 190 mM butanol	[178]
Solvent- and Aero-tolerance			
<i>C. acetobutylicum</i>	Plasmid overexpression of glutathione <i>gshA</i> and <i>gshB</i> from <i>E. coli</i>	Improved aero- and solvent-tolerance	[179]
<i>C. acetobutylicum</i>	Plasmid overexpression of chaperone <i>groESL</i>	Showed 85% decrease in butanol inhibition and 33% increase in solvent yield	[180]
Substrate Utilization			
<i>C. acetobutylicum</i>	Plasmid expression of <i>acsC</i> , <i>acsD</i> and <i>acsE</i> from <i>C. difficile</i>	Increased incorporation of CO ₂ into extracellular products	[99]
<i>C. saccharoperbutylacetonicum</i> strain N1-4	Knockdown hydrogenase <i>hupC</i> expression using siRNA delivered from plasmid	Significantly reduced hydrogen uptake activity to 13% (relative to control strain)	[181]

Table 3. Genetically modified solventogenic Clostridia

In contrast, to date only a limited number of acetogenic Clostridia have been successfully modified. Pioneering work in this area has been undertaken in the laboratory of Prof. Peter Dürre. *C. ljungdahlii*, a species that does not naturally produce butanol, was modified with butanol biosynthetic genes (*thlA*, *hbd*, *crt*, *bcd*, *adhE* and *bdhA*) from *C. acetobutylicum* ATCC 824 resulting in production of up to 2 mM of butanol using synthesis gas as sole energy and carbon source [62]. By delivering a plasmid with acetone biosynthesis genes *ctfA*, *ctfB*, *adc*, and *thlA* in *C. aceticum*, production of up to 140 µM acetone was demonstrated from various gas mixes (80% H₂/20% CO₂ and 67% H₂/33% CO₂) [169, 170]. Recent patent filings by Lanza-Tech describe the production of butanol as main fermentation product and increased alcohol tolerance in genetically engineered acetogens. Up to 26 mM butanol were produced with genetically modified *C. ljungdahlii* and *C. autoethanogenum* using steel mill gas (composition 44% CO, 32% N₂, 22% CO₂, and 2% H₂) as the only source of carbon and energy when the butanol biosynthetic genes *thlA*, *hbd*, *crt*, *bcd*, *etfA*, and *etfB* were heterologously expressed [167]. Overexpression of native *groESL* operon in *C. autoethanogenum* resulted in a strain that displayed higher alcohol tolerance relative to wild-type when challenged with ethanol [168].

Besides the classical Clostridial butanol pathway (which constitutes genes *thlA*, *crt*, *hbd*, *bcd*, *etfA* and *etfB*; see earlier section), a non-fermentative approach has been described and demonstrated in *E. coli* for branched chain higher alcohol production [182]. This alternative approach requires a combination of highly active amino acid biosynthetic pathway and artificial diversion of 2-keto acid intermediates into alcohols by introduction of two additional genes: broad substrate range 2-keto-acid decarboxylase (*kdc*) which converts 2-keto acids into aldehydes, followed by *Adh* to form alcohols [182]. Engineered strains of *E. coli* have been shown to produce alcohols such as isobutanol, n-butanol, 2-methyl-1-butanol, 3-methyl-1-butanol and 2-phenylethanol via this strategy [182]. For instance, the overexpression of *kivD* (KDC from *Lactococcus lactis*), *adh2*, *ilvA*, and *leuABCD* operon, coupled with deletion of *ilvD* gene and supplementation of L-threonine, increased n-butanol yield to 9 mM while producing 10 mM of 1-propanol [182]. An even more remarkable yield of 300 mM isobutanol was achieved through introduction of *kivD*, *adh2*, *alsS* (from *B. subtilis*), and *ilvCD* into *E. coli* [182]. Like butanol, isobutanol exhibits superior properties as a transportation fuel when compared to ethanol [177]. By applying similar strategy into *C. cellulolyticum*, 8.9 mM isobutanol was produced from cellulose when *kivD*, *yqhD*, *alsS*, *ilvC*, and *ilvD* were overexpressed [177]. This result suggests that such non-fermentative pathway is suitable target for metabolic engineering of acetogens for the biosynthesis of branched chain higher alcohols. Via synthetic biology and metabolic engineering, production of additional potential liquid transportation fuels like farnesene or fatty acid based fuels has successfully been demonstrated in *E. coli* or yeast from sugar [183, 184]. Given the unsolved energetics in acetogens, it is unclear if production of such energy dense liquid fuels could be viable via gas fermentation.

10. Fermentation and product recovery

10.1. Bioreactor design

An optimum gas fermentation system requires efficient mass transfer of gaseous substrates to the culture medium (liquid phase) and microbial catalysts (solid phase). Gas-to-liquid mass transfer has been identified as the rate-limiting step and bottleneck for gas fermentation because of the low aqueous solubility of CO and H₂, respectively at only 77% and 68% of that of oxygen (on molar basis) at 35°C [185]. Hence, a bioreactor design that delivers sufficient gas-to-liquid mass transfer in an energy-efficient manner at commercial scale for gas fermentation represents a significant engineering challenge. A brief overview of reactor configurations reported in gas fermentation operations is given below.

10.2. Continuous Stirred Tank Reactor (CSTR)

In continuous stirred tank reactor (CSTR), gas substrates are continuously fed into the reactor and mechanically sheared by baffled impellers into smaller bubbles, which has greater interfacial surface area for mass transfer [16]. In addition, finer bubbles have a slower rising velocity and a longer retention time in the aqueous medium, resulting in higher gas-to-liq-

uid mass transfer [24]. Fermentation reactions using *C. ljungdahlii* have been successfully maintained in a 2 litre CSTR under autotrophic conditions for more than a month, while achieving peak ethanol level of 6.5 g/l and CO conversion rate of 93% [186]. The production of 49 g/l of ethanol from gas substrates using *C. ljungdahlii* was demonstrated using CSTR [113]. In another example, a 100 litre stirred tank reactor was demonstrated to produce up to 24.57 g/l ethanol, 9.25 g/l isopropanol and 0.47 g/l n-butanol during a 59-day semi-batch gas fermentation using "*C. ragsdalei*" strain P11 as biocatalysts [112]. An improved version of CSTR incorporates microbubble sparger to generate finer bubbles to achieve higher mass transfer coefficient [187]. Although CSTR offers complete mixing and uniform distribution of gas substrates to the microbes, the high power per unit volume required to drive the stirrer are thought to make this approach economically unviable for commercial scale gas fermentation systems [187].

10.3. Bubble column reactor

In contrast to CSTR, gas mixing in bubble column reactor is achievable by gas sparging, without mechanical agitation. This reactor configuration has fewer moving parts, and consequently has a lower associated capital and operational costs while exhibiting good heat and mass transfer efficiencies, making it a good candidate for large scale gas fermentation [17]. However, excessive level of gas inflow for enhanced mixing have been cited as an issue that leads to heterogeneous flow and back-mixing of the gas substrates [16, 17]. *C. carboxidivorans* strain P7 was cultured in a 4 litre bubble column reactor for 20 days using a combination of producer gas and synthetic syngas, generating a peak ethanol concentration of 6 g/l [13].

10.4. Immobilized cell column reactor

One of the key challenges of gas fermentation is cell density. Immobilization of microbes through crosslinking or adsorption to insoluble biosupport materials and the subsequent packing within the column offers a range of benefits [14]. These include high cell densities, plug flow operation, high mass transfer rate via direct contact between microbe and gas, reduction of retention time, and operation without mechanical agitation [14, 16]. However, channelling issues may arise when the microbe overgrows and completely fill the interstitial space. Due to limitations in column dimensions and packing, this reactor configuration lacks flexibility to operate in various gas fermentation conditions [14, 16].

10.5. Trickle-bed reactor

Trickle-bed reactor is a gas- or liquid- continuous reactor consisting of packed bed, which liquid culture trickles down through packing media containing suspended or immobilized cells [16, 24, 187]. The gas substrate is delivered either co-currently or counter-currently to the liquid flow, and no mechanical agitation is required [187]. In this reactor format, low gas and liquid flow rates are typically applied, generating relatively low pressure drops [187]. Trickle-bed reactor was found to exhibit excellent gas conversion rates and higher productivities than CSTR and bubble column reactor [15].

11. Gas fermentation parameters

11.1. Gas composition

The gas composition and its impurities can have an impact on the productivity of the gas fermentation process. Greater molar ratio of H₂:CO allows greater efficiency in the conversion of the carbon from CO into products such as ethanol, because reducing equivalents are generated from oxidation of H₂ (rather than CO). However, CO is also a known inhibitor of hydrogenase which can affect utilization of H₂ during fermentation. In *B. methylotrophicum*, H₂ utilization was inhibited until CO was exhausted [108]. When CO is consumed, acetogens are able to grow using CO₂ and H₂. Common impurities from biomass gasification or other waste gases are tar, ash, char, ethane, ethylene, acetylene, H₂S, NH₃ and NO [17, 22, 24, 188]. These have been shown to cause cell dormancy, inhibition of hydrogen uptake, low cell growth and shift between acidogenesis and solventogenesis in acetogens [13, 188]. For instance, NH₃ from the feed gas readily convert into NH₄⁺ in the culture media and these ions were recently shown to inhibit hydrogenase and cell growth of acetogen "*C. ragsdalei*" [189]. A number of strategies to mitigate the impact of such impurities have been proposed, for example installing 0.025 mm filters, or the use of gas scrubbers or cyclones, and improvement in gasification efficiency and scavenging for contaminants in the gas stream using agents such as potassium permanganate, sodium hydroxide or sodium hypochlorite [24, 190-192]. H₂S does not have a negative effect on acetogens such as *C. ljungdahlii* up to 5.2% (v/v) [193].

11.2. Substrate pressure

The partial pressure of syngas components have a major influence on microbial growth and product profiles because the enzymes involved are sensitive to substrate exposure [194]. Due to the low solubility of CO and H₂ in water, the growth of dense bacterial cell cultures can face mass transfer limitations, so increasing the partial pressure of gaseous substrates can help alleviate this problem. For instance, studies in which the CO partial pressure (P_{CO}) increased from 0.35 to 2.0 atm showed that this resulted in a 440% increase in maximum cell density, a significant increase in ethanol productivity and a decrease in acetate production in *C. carboxidivorans* strain P7 [195]. In another study involving *C. ljungdahlii*, the increase of P_{CO} from 0.8 to 1.8 atm had a positive effect on ethanol production, and the microbe did not exhibit any substrate inhibition at high P_{CO} [196]. In less CO-tolerant microorganisms, the effect of increasing P_{CO} partial pressure range from non-appreciable in the case of *Rhodospirillum rubrum* [197], to negative impact on doubling time of *Peptostreptococcus productus* (now: *Blautia product*) [194] and *Eubacterium limosum* [198]. Similar to CO, the increase in partial pressure of H₂ (pH₂) to 1700 mbar enhanced acetate productivity of *A. woodii* to 7.4g acetate/l/day [199].

11.3. Medium formulation

Although acetogens are able of utilizing CO and CO₂/H₂ as carbon and energy source, other constituents such as vitamins, trace metal elements, minerals and reducing agents are also required for maintenance of high metabolic activity [16, 113]. Studies indicated that formation of ethanol in solventogenic Clostridia is non-growth associated and limitation of growth

by reducing availability of carbon-, nitrogen- and phosphate- nutrients shift the balance from acidogenesis to solventogenesis [113, 200, 201]. Optimization of medium formulation for *C. ljungdahlii* through reduction of B-vitamin concentrations and elimination of yeast extract significantly enhanced the final ethanol yield to 48 g/l in a CSTR with cell recycling (23 g/l without cell recycling) [113]. Another study by Klasson *et al.* showed that the replacement of yeast extract with cellobiose not only increased maximum cell concentration, but also enhanced ethanol yield by 4-fold [14]. Media formulation for *C. autoethanogenum* was investigated using Plackett-Burman and central composite designs, but only low ethanol yield was recorded overall [202]. In an attempt to reduce the cost of fermentation medium and improve process economics, 0.5 g/l of cotton seed extract without other nutrient supplementation was shown to be a superior medium for *C. carboxidivorans* strain P7 in producing ethanol from syngas fermentation [203]. A recent study showed that increasing concentrations of trace metal ions such as Ni^{2+} , Zn^{2+} , SeO_4^- , WO_4^- , Fe^{2+} and elimination of Cu^{2+} from medium improved enzymatic activities (FDH, CODH, and hydrogenase), growth and ethanol production in "*C. ragsdalei*" under autotrophic conditions [107].

A low redox potential is necessary for strict anaerobes to grow, hence reducing agents such as sodium thioglycolate, ascorbic acid, methyl viologen, benzyl viologen, titanium (III)-citrate, potassium ferricyanide, cysteine-HCl and sodium sulfide are commonly added to fermentation medium [14, 16, 204]. Furthermore, the addition of reducing agent directs the electron and carbon flow towards solventogenesis by enhancing the availability of reducing equivalents to form NADH for alcohol production [16, 205]. Excessive addition of reducing agents can cause slower microbial growth due to reduced ATP formation from acetogenesis so it is important to determine the optimum concentration of reducing agents [14, 16]. The sulfur containing gases (e.g. H_2S) present in syngas are toxic to chemical catalysts but can be beneficial for microbial catalysts by reducing medium redox potential, stimulate redox sensitive enzymes such as CODH, and promote alcohol formation [206, 207].

11.4. Medium pH

Like other organisms, acetogens have a limited range of pH for optimal growth so the pH of the fermentation medium needs to be closely controlled. The extracellular pH directly influences the intracellular pH, membrane potential, proton motive force, and consequently substrate utilization and product profile [208, 209]. In most studies, lowering pH medium divert carbon and electron flow from cell and acid formation towards alcohol production [113, 209-211]. By applying this knowledge, Gaddy and Clausen performed a two-stage CSTR syngas fermentation systems using *C. ljungdahlii* where they set the first reactor at pH 5 to promote cell growth, and pH 4 - 4.5 in the second reactor to induce ethanol production [212]. One recent study with *C. ljungdahlii* showed conflicting results in which cell density and ethanol production were both higher at pH 6.8 when compared to pH 5.5 [213].

11.5. Temperature

The optimum temperature for mesophilic acetogens are between 30-40°C, while thermophilic acetogens grow best between 55 and 58°C. The fermentation temperature not only affects

substrate utilization, growth rate and membrane lipid composition of the acetogens, but also gas substrate availability because gas solubility increases with decreasing temperature [24, 211]. "*C. ragsdalei*" was reported to produce more ethanol at 32°C than at the optimum growth temperature of 37°C [211].

12. Cell separation and product recovery

To retain high cell densities in reactor, microbes can be grown as biofilm attached to carrier material. Planktonic cells can be retained in the fermentation broth by installing solid/liquid separators such as membranous ultra-filtration units, spiral wound filtration systems, hollow fibres, cell-recycling membranes and centrifuges [214-216]. The concentrations of solvents from gas fermentation rarely exceed 6% [w/v] so a cost- and energy- efficient product recovery process is required. Furthermore, acetogens also exhibit low resistance towards solvents like ethanol [217, 218] and butanol [219, 220] so an *in situ*/online product recovery system can enhance solvent productivity by decreasing solvent concentrations (and hence toxicity) in the fermentation broth. Distillation has been the traditional method of product recovery but the associated high energy costs have led to the development of alternative methods such as liquid-liquid extraction, pervaporation, perstraction, and gas stripping [24, 221].

12.1. Liquid-liquid extraction

In liquid-liquid extraction, a water-insoluble organic extractant is mixed with the fermentation broth [222]. Because solvents are more soluble in the organic phase than in the aqueous phase, they get selectively concentrated in the extractant. Although this technique does not remove water or nutrients from the fermentation broth, some gaseous substrates might be removed because CO and H₂ have much higher solubility in organic solvents than water [222, 223]. Oleyl alcohol has been the extractant of choice due to its relatively non-toxicity [224].

12.2. Perstraction

Liquid-liquid extraction is associated with several problems including toxicity to the microbes, formation of emulsion, and the accumulation of microbes at the extractant and fermentation broth interphase [222]. In an attempt to remediate these problems, perstraction was developed and this technique employs membrane to separate the extractant from the fermentation broth. This physical barrier prevent direct contact between the microbe and the toxicity of extractant, but it can also limit the rate of solvent extraction and is susceptible to fouling [219, 221]

12.3. Pervaporation

In a product recovery technique termed pervaporation, a membrane that directly comes in contact with fermentation broth is used to selectively remove volatile compounds such as ethanol and butanol [219, 222]. The volatile compounds diffuse through the membrane as

vapour and are then collected by condensation. To facilitate volatilization of permeates into vapour, a partial pressure difference across the membrane is usually maintained by applying a vacuum or inert gas (e.g. N₂) across the permeate side of the membrane [219]. Polydimethylsiloxane (PDMS) is the current material of choice for the membrane, but other materials such as poly(1-trimethylsilyl-1-propyne) (PTMSP), hydrophobic zeolite membranes, and composite membranes have also been investigated [225].

12.4. Gas stripping

Gas stripping is an attractive product recovery method for gas fermentation because the exit gas stream from the bioreactor can be used for *in situ*/online product recovery [219]. Following product recovery via condensation, the effluent and gas can be recycled back into the bioreactor. In sugar-based fermentation using *C. beijerinckii* mutant strain BA101, *in situ* gas stripping was shown to improve ABE productivity by 200%, complete substrate utilization and also complete acid conversion into solvents, when compared to non-integrated process [226].

13. Commercialization

The growing commercial interests in using gas fermentation as a platform for biofuels production is evident in the recent spike in patent filings within the field [105]. A 2009 report compared mass and energy conversion efficiencies from a process engineering standpoint between enzymatic hydrolysis fermentation of lignocellulose, syngas fermentation and FTP [227]. The authors concluded that while syngas fermentation offers a range of advantages such as low pretreatment requirement and low energy requirement for bioconversion, the technology is severely limited by low ethanol productivity [227]. Another report documented the techno-economic analysis of gas fermentation and concluded that the selling price of ethanol using this technology would still be significantly higher than gasoline in 2009 [228]. In contrast, Griffin and Schultz recently compared the production of ethanol from CO-rich gas using thermo-chemical route and biological gas fermentation route [22]. The authors concluded that gas fermentation offers superior fuel yield per volume of biomass feed, carbon conversion to fuel, energy efficiency and lower carbon emissions relative to the thermo-chemical approach to bioethanol production.

Ethanol and butanol are the most attractive fuel products from current gas fermentation but other by-products such as 2,3-butanediol, acetic acid and butyric acid are also valuable commodities that have the potential to provide significant additional revenue streams, setting off costs for biofuel production. 2,3-butanediol is a high value commodity which can be used to synthesize chemical products such as 1,3-butanediol, methyl ethyl ketone, and gamma butyrolactone, with a combined potential market value of \$43 billion [104]. Acetic acid is an important precursor for synthesis of polymers while butyric acid can be used as a flavouring agent in the food industry [229, 230]. With the development of advanced genetic

tools for expansion of product range, the industry might witness an increasing emphasis on the production of high-value commodities in addition to biofuels.

Several companies are actively engaged in the development of the gas fermentation technology and some are approaching commercialization. Bioengineering Resources Inc (BRI) founded by Prof. James Gaddy of University of Arkansas, Fayetteville, an early pioneer in the investigation of gas fermentation at scale, was the first company to explore the potential of gas fermentation for industrial bioethanol production. BRI was acquired by chemical company INEOS and rebranded as INEOS Bio (www.ineosbio.com). A pilot-scale facility in Arkansas has been operated since 2003 using several isolates of *C. ljungdahlii* [231] and is building a US\$130 million commercial facility in Florida with its joint venture partner New Planet Energy Florida [232]. The commercial facility is expected to start operation in the second quarter of 2012 and is aiming to generate 8 million gallon of cellulosic ethanol per annum and 6 MW of power to the local communities [232]. INEOS Bio also announced design of a second plant, the Seal Sands Biorefinery in Teeside, UK [233].

Founded in 2006, Coskata Inc. (www.coskata.com) is a US-based company that has reported achieving ethanol yields of 100 gallons per dry ton of wood biomass in a semi-commercial facility in Pennsylvania [234]. The company licensed several microbial strains from the University of Oklahoma [235], which has filed patents and journal publications for acetogens such as "*C. ragsdalei*" [211, 236, 237] and *C. carboxidivorans* [55, 112]. A patent documenting a new ethanologenic species, "*C. coskatii*" was also recently filed by Coskata [238]. Backed by a conditional US\$250 million loan guarantee from the US Department of Agriculture (USDA), Coskata has announced that it is planning to build a commercial plant with the capacity to produce 55 million gallon fuel grade ethanol per annum in Alabama [234, 239]. While the initial strategy saw biomass as feedstock, the company recently announced its first commercial plant will be switched to 100% natural gas as feedstock [240]. A planned IPO with the aim to tap into private investors to finance the plant was put on hold [241]. In 2012, Coskata and INEOS Bio were involved in a trade secret dispute which culminated in a settlement that see INEOS Bio receiving US\$2.5 million cash payment, shares and right to receive 2.5% of future ethanol royalties from Coskata [242].

LanzaTech is a NZ/US based company that has developed a gas fermentation technology to utilize industrial off-gases from steel making and other sources, as well as syngas produced from biomass as feedstocks. The company has reported the development of a proprietary Clostridial biocatalyst that is able to convert the CO-rich waste gas with minimal gas conditioning into bioethanol and the platform chemical 2,3-butanediol. The use of industrial off-gases as feedstock not only helps to reduce the carbon footprint of the steel-making operations but also allows the production of valuable commodities without the costs associated with feedstock gasification. The company has estimated that up to 30 billion gallon of bioethanol per year can be produced from the CO-rich off gases produced through steel manufacturers globally [243]. Founded in 2005, LanzaTech has successfully demonstrated bioethanol production at a pilot plant at BlueScope Steel in Glenbrook, NZ, since 2008 and the company has recently started operating its 100,000 gallon bioethanol per year demonstration facility in Shanghai, China, using waste gas collected from an ad-

jacent steel mill plant owned by its partner Baosteel Group [243, 244]. LanzaTech is planning to build a commercial facility with the capacity to produce 50 million gallon of bioethanol per annum in China by 2013 [243]. The recent acquisition of a biorefinery facility developed by the US-based gasification technology company Range Fuels in Georgia, and a milestone signing of its first commercial customer, Concord Enviro Systems (India), highlighted LanzaTech's intention to utilize MSW and lignocellulosic waste as feedstocks for biofuel and chemical production [243, 244].

14. Conclusion

One of the fundamental factors that govern the environmental and economical sustainability of biofuel production is feedstock. Through gasification, a spectrum of renewable non-food feedstock such as agricultural wastes, dedicated energy crops, forest residues, and MSW can be converted into syngas. This article presents a detailed examination of gas fermentation technology in capturing the carbon and energy from syngas and produce biofuels and chemicals. In comparison to indirect fermentation of lignocellulose via enzymatic hydrolysis, and thermo-chemical FTP, gas fermentation offers several advantages such as good product yield and selectivity, operation in ambient conditions, high tolerance to gas impurities, and elimination of expensive pre-treatment steps and costly enzymes. Furthermore, some industries such as steel mill, natural gas steam reforming, oil refining and chemical production generate large volumes of CO-rich off-gas. Gas fermentation can access these existing feedstocks and generate valuable products from these while reducing carbon emissions. Pivotal to gas fermentation is acetogens such as *C. ljungdahlii*, *C. carboxidivorans*, "*C. ragsdalei*" and *C. autoethanogenum*, which are able to metabolize CO, and CO₂/H₂ into a range of products such as ethanol, butanol, isopropanol, acetone, 2,3-butanediol, acetic acid and butyric acid. Sustained effort in studying the physiology and biochemistry using advanced molecular techniques such as genomics, transcriptomics, proteomics, metabolomics and systems biology are essential to further the understanding of these microbes. Furthermore, recent advances in Clostridial genetic tools offer endless opportunities to engineer strains that have improved product yield, substrate utilization, no strain degeneration, and synthesis of new products.

The main challenges associated with commercialization of gas fermentation have been identified as gas-to-liquid mass transfer limitation, product yield, substrate utilization efficiency, low biomass density and product recovery. Further development of bioreactor is necessary to improve the availability of gas substrates and maintain high cell density for higher productivity. Improvement in integrated product recovery technology is also essential to lower the costs of product recovery and alleviate product inhibition. Gas fermentation appears to be mature enough for commercialization since several companies have already demonstrated their technologies at pilot scale and are moving towards commercialization in the near future.

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