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Acetogenic mixotrophy: novel options for yield improvement in biofuels and biochemicals production

Alan G Fast[1], Ellinor D Schmidt[1], Shawn W Jones[2] and Bryan P Tracy[2]

Mass yields of biofuels and chemicals from sugar fermentations are limited by the decarboxylation reactions involved in Embden–Meyerhof–Parnas (EMP) glycolysis. This paper reviews one route to recapture evolved CO_2 using the Wood–Ljungdahl carbon fixation pathway (WLP) in a process called anaerobic, non-photosynthetic (ANP) mixotrophic fermentation. In ANP mixotrophic fermentation, the two molecules of CO_2 and eight electrons produced from glycolysis are used by the WLP to generate three molecules of acetyl-CoA from glucose, rather than the two molecules that are produced by typical fermentation processes. In this review, we define the bounds of ANP mixotrophy, calculate the potential metabolic advantages, and discuss the viability in a number of host organisms. Additionally, we highlight recent accomplishments in the field, including the recent discovery of electron bifurcation in acetogens, and close with recommendations to realize mixotrophic biofuel and biochemical production.

Addresses
[1] Molecular Biotechnology Laboratory, Department of Chemical & Biomolecular Engineering, & the Delaware Biotechnology Institute, University of Delaware, Newark, DE 19711, USA
[2] Elcriton, Inc., 15 Reads Way, New Castle, DE 19720, USA

Corresponding author: Tracy, Bryan P (bryantracy@elcriton.com)

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Introduction

In order to commercialize next-generation biofuels and commodity chemicals at large scale, the combined costs of production must be low enough and provide sufficient co-product revenue to allow bio-based fuels to be sold at prices competitive with the oil-derived fuels they replace. The wholesale price of gasoline has fluctuated between $2.60 and $3.00 in the first half of 2014 (US Energy Information Administration), making it difficult to develop cost-competitive second-generation biofuels. Currently, cellulosic-biofuel production in the US lags behind projections. The previous 2013 cellulosic biofuel mandate was six million gallons, but production only reached 800,000 gallons earlier this year [1]. One of the primary factors affecting the economic viability of next generation biofuel processes is the cost of feedstock and feedstock pretreatment, which can exceed 50% of total operating expenditure (OPEX) [2,3]. One way to mitigate high feedstock costs is by maximizing feedstock conversion to the product of interest. The ability to achieve high mass yields from carbohydrate fermentations, however, is impeded by CO_2 loss during classical Embden–Meyerhof–Parnas (EMP) glycolysis. Because of the decarboxylation of pyruvate when forming acetyl-CoA, one third of hexose carbons cannot be recovered as useful products. In practice, this means that the theoretical maximum mass yields for gasoline replacements range from 33% for 3-methyl-1-butanol to 51% for ethanol [4]. For diesel replacements, the maximum mass recovery from hexose is even lower, with theoretical yields ranging from 29% in the case of farnesene to 35% for ethyl hexadecanoate. Finally, for biocrudes, the theoretical yields are the lowest at 25% for squalene to 30% for hentriacontene.

In response, researchers are investigating biological approaches that minimize or ideally eliminate CO_2 loses and theoretically could result in complete conversion of carbohydrate sources to acetyl-CoA [5–7], a central building block of metabolism. Two distinctly different methods for mitigating carbon loss from glycolysis have been suggested. The first is a recently developed, synthetic pathway, termed non-oxidative glycolysis (NOG), which can theoretically allow for stoichiometric conversion of carbohydrate feedstocks into acetyl-CoA (i.e. 2.5–3 mol of acetyl-CoA per mole of pentose and hexose sugar, respectively) [6]. An engineered NOG pathway was demonstrated both *in vitro* and *in vivo* using *Escherichia coli* as a host (refer to Box 1 for more detail).

A second proposed approach to improve carbon yields from carbohydrate fermentation is to genetically engineer and metabolically enhance acetogenic mixotrophic fermentation [7,8••], whereby CO_2 evolved during EMP glycolysis is reassimilated into biomass and fermentation products by a carbon fixation pathway. Below, we review a specific version of mixotrophic fermentation by defining the bounds of the concept, calculating the potential metabolic advantages, and discussing the viability in a number of host organisms.

Box 1 A synthetic non-oxidative glycolysis (NOG) route

A synthetic non-oxidative glycolysis (NOG) route to avoid the loss of CO_2 through traditional glycolysis has been recently proposed and demonstrated by Bogorad *et al.* [6]. The pathway begins with fructose 6-phosphate, which can be generated through a number of routes including the phosphorylation and isomerization of glucose, at the loss of ATP. A total of three fructose 6-phosphate molecules are irreversibly converted into three acetyl phosphate molecules and three erythorose 4-phosphate molecules. The three acetyl phosphate molecules are then converted into three acetyl-CoA molecules, and the three erythorose 4-phosphate molecules undergo a series of carbon rearrangement reactions to reform two fructose 6-phosphate molecules. Thus no carbon is lost in the generation of acetyl-CoA.

The authors were able to demonstrate the feasibility of the NOG pathway in both an *in vitro* and *in vivo* system using *Escherichia coli* as a host. The *in vitro* system consisted of eight core enzymes that were shown to convert six-carbon, five-carbon, and three-carbon phosphates (i.e. fructose 6-phosphate, ribose 5-phosphate, and glyceraldehyde 3-phosphate) nearly to the maximum theoretical amounts of acetyl phosphate. The *in vivo* NOG pathway was unable to metabolize six-carbon sugars due to dependence of the phosphotransferase system on the glycolysis intermediate, phosphoenolpyruvate (PEP). However, the five-carbon sugar, xylose, was consumed to form 2.2 mol of acetate per mole xylose, which is near the theoretical maximum yield of 2.5 mol for the NOG pathway and is above the theoretical maximum yield of 1.67 mol for EMP glycolysis.

Overview of anaerobic, non-photosynthetic (ANP) mixotrophic fermentation

In this review, we focus on mixotrophy in acetogenic bacteria, which are anaerobic, non-photosynthetic bacteria that concurrently utilize organic and inorganic (i.e. CO_2/H_2, CO, formate, methanol, etc.) substrates for growth and metabolism [7]. This is in contrast to the well-documented mixotrophic growth of photosynthetic cyanobacteria and microalgae [9,10]. As mentioned, the major motivation behind utilizing mixotrophy is to reassimilate the CO_2 and H_2 evolved during glycolysis in order to produce additional acetyl-CoA. While any of the six known natural carbon fixation pathways could be used to re-capture CO_2, as reviewed elsewhere [7], only the bacteria that use the Wood–Ljungdahl carbon fixation pathway (WLP), termed acetogens, are natively capable of performing the stoichiometric conversion of 1 mol of glucose or an assortment of six carbon sugars into 3 mol of acetate. This ability to stoichiometrically convert sugar to acetate is well known [11], and companies are exploring the commercial potential to produce acetic acid and then catalytically convert it into other industrial chemicals. However, the potential to directly convert sugars stoichiometrically to desirable biofuels and longer chain chemicals is far less explored.

Acetogens are capable of carbon conservation from glucose to acetate due to the low ATP cost (i.e. <1 mol ATP per mole of acetyl-CoA produced) of the WLP, which allows the excess NADH produced during glycolysis to be used for CO_2 fixation. The other five carbon fixation pathways — namely, the reductive pentose phosphate cycle, the reverse tricarboxylic acid cycle, the 3-hydroxypropionate pathway, the 3-hydroxypropionate/4-hydroxybutyrate pathway, and the dicarboxylate/4-hydroxybutyrate pathway — all have higher ATP costs than the WLP. To generate the ATP required for CO_2 fixation, the organisms with these pathways either utilize photosynthesis or an electron transport chain that oxidizes reducing equivalents using terminal electron acceptors, such as O_2 or oxidized sulfur compounds. Both methods of ATP generation are undesirable for a bioprocess, as they increase the cost and complexity of fermentation. Moreover, the oxidation of reducing equivalents to generate ATP decreases the reducing pool that could otherwise be used for biosynthesis or biochemical production [7]. For these reasons, this review will focus on mixotrophic fermentation using the WLP.

Figure 1 presents the overall concept of ANP mixotrophic fermentation, whereby glycolysis and the WLP are combined to generate pyruvate and acetyl-CoA, respectively, which serve as intermediates for the production of potential biofuels. During glycolysis, 2 mol of pyruvate, 2 mol of ATP, and four electrons are generated per mole of hexose sugar (e.g. glucose). For most biofuel production pathways (discussed below), the two molecules of pyruvate are converted to acetyl-CoA by a pyruvate synthase or pyruvate:ferredoxin oxidoreductase, which results in the release of 2 mol of CO_2 and four additional electrons per mole of glucose. Overall, glycolysis yields 2 mol of acetyl-CoA, 2 mol of ATP, 2 mol of CO_2, and eight electrons from each mole of glucose. Without a mechanism for CO_2 reassimilation, CO_2 is lost, and much of the reducing equivalent pool is oxidized by hydrogenase activity to release H_2.

The WLP consists of two branches: the methyl or eastern branch and the carbonyl or western branch [12]. In the methyl branch, CO_2 is reduced by two electrons to formate by formate dehydrogenase (FDH). An ATP-dependent formate-tetrahydrofolate ligase binds formate to tetrahydrofolate to form 10-formyltetrahydrofolate. Subsequent reduction and dehydration reactions consume an additional four electrons to reduce the 10-formyltetrahydrofolate to 5-methyltetrahydrofolate. In the carbonyl branch, a bifunctional CO dehydrogenase/acetyl-CoA synthase (CODH/ACS) reduces CO_2 to CO, which is condensed with the methyl-group from 5-methyltetrahydrofolate and coenzyme-A to form acetyl-CoA. In summary, glycolysis and the WLP are complementary in that the two molecules of CO_2 and eight electrons generated from glycolysis can be fully utilized by the WLP to produce an additional acetyl-CoA, thereby increasing the acetyl-CoA yield by 50% as compared to standard EMP glycolysis.

Figure 1

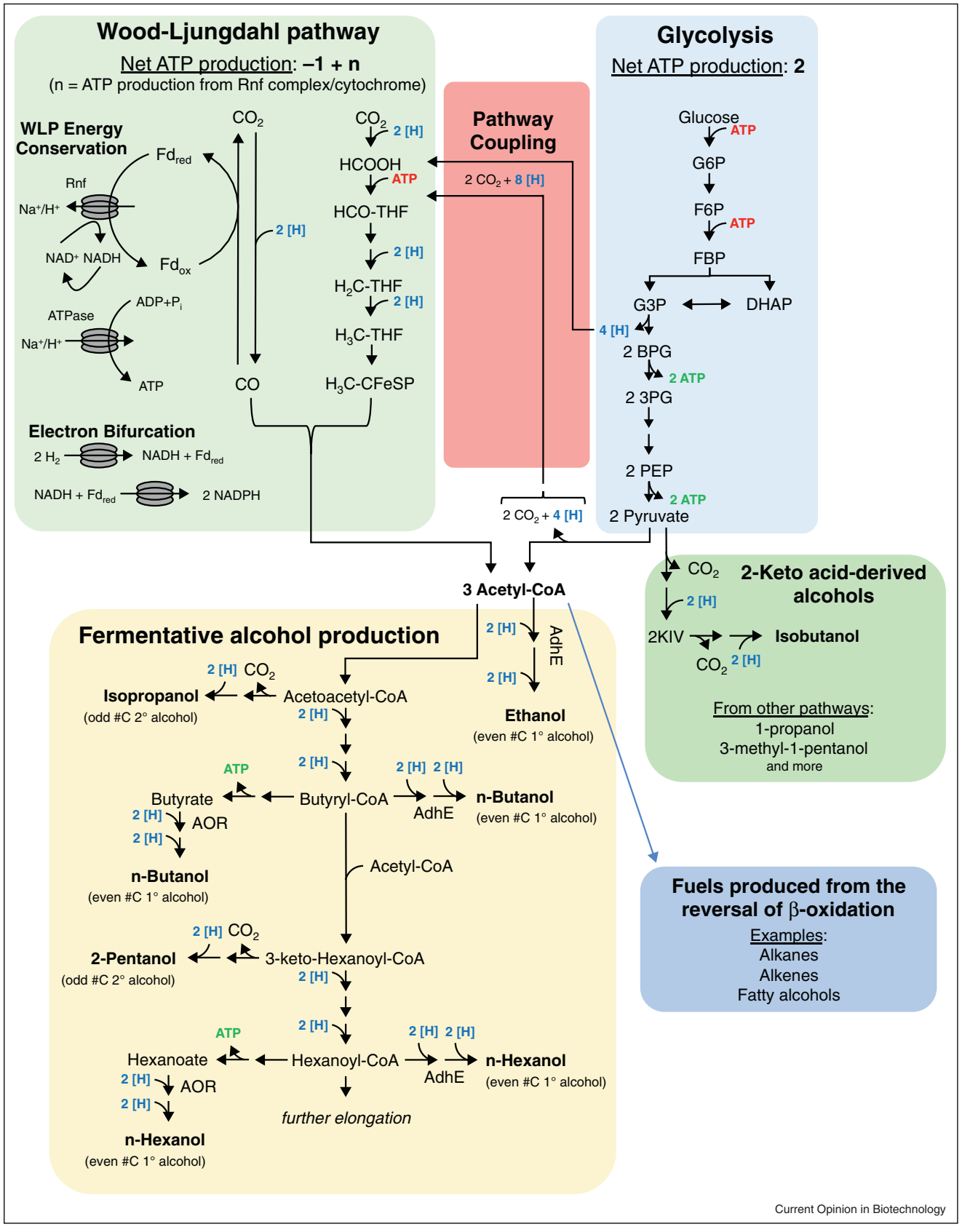
Wood-Ljungdahl pathway
Net ATP production: –1 + n
(n = ATP production from Rnf complex/cytochrome)
WLP Energy Conservation
Rnf
Na+/H+
NAD+ NADH
Fdred
Fdox
ATPase
ADP+Pi
ATP
CO2
2 [H]
CO
HCOOH
ATP
HCO-THF
H2C-THF
H3C-THF
H3C-CFeSP
Electron Bifurcation
2 H2
NADH + Fdred
NADH + Fdred
2 NADPH
Pathway Coupling
2 CO2 + 8 [H]
2 CO2 + 4 [H]
Glycolysis
Net ATP production: 2
Glucose
ATP
G6P
F6P
ATP
FBP
G3P
DHAP
4 [H]
2 BPG
2 ATP
2 3PG
2 PEP
2 ATP
2 Pyruvate
3 Acetyl-CoA
2-Keto acid-derived alcohols
CO2
2 [H]
2KIV
Isobutanol
CO2
2 [H]
From other pathways:
1-propanol
3-methyl-1-pentanol
and more
Fermentative alcohol production
2 [H]
AdhE
Ethanol
(even #C 1° alcohol)
2 [H] CO2
Isopropanol
(odd #C 2° alcohol)
Acetoacetyl-CoA
2 [H]
2 [H]
ATP
Butyrate
2 [H]
AOR
2 [H]
n-Butanol
(even #C 1° alcohol)
Butyryl-CoA
2 [H] 2 [H]
AdhE
n-Butanol
(even #C 1° alcohol)
Acetyl-CoA
2 [H] CO2
2-Pentanol
(odd #C 2° alcohol)
3-keto-Hexanoyl-CoA
2 [H]
2 [H]
ATP
Hexanoate
2 [H]
AOR
2 [H]
n-Hexanol
(even #C 1° alcohol)
Hexanoyl-CoA
2 [H] 2 [H]
AdhE
n-Hexanol
(even #C 1° alcohol)
further elongation
Fuels produced from the reversal of β-oxidation
Examples:
Alkanes
Alkenes
Fatty alcohols
Current Opinion in Biotechnology

Table 1

Comparison of ATP, CO_2 evolved or consumed, and acetyl-CoA generation between four different fermentation strategies. Glycolysis refers to EMP glycolysis. WLP refers to Wood–Ljungdahl pathway of carbon fixation. NOG refers to non-oxidative glycolysis. The feedstock for each strategy is identified in the second column. Negative values for ATP and CO_2 imply consumption

	Feedstock	Net ATP	CO_2 evolved	Acetyl-CoA
Glycolysis	Hexose	2	2	2
WLP	$CO_2 + H_2$	Less than 1	−2	1
Mixotrophic	Hexose	1	0	3
NOG	Hexose	−1	0	3

Potential metabolic and economic advantages of ANP mixotrophy for *n*-butanol production

In order to broadly compare the potential benefits of ANP mixotrophic fermentation, we first considered the requirements for acetyl-CoA production from four pathways — namely, EMP glycolysis, the WLP, NOG, and ANP mixotrophic fermentation. The comparison was based upon the net consumption or production of ATP, CO_2, and reduced electron carriers (Table 1). Acetyl-CoA was used as a common metabolite due to its importance in the production of many advanced biofuels of interest (as shown in Figure 1) as well as in many cellular biosynthesis processes. The ideal fuel production pathway would maximize acetyl-CoA generation, minimize CO_2 evolution, and produce excess ATP in order to create and maintain biomass. As shown in Table 1, the ANP mixotrophic fermentation is the only approach that produces acetyl-CoA, does not evolve CO_2, and produces excess ATP. EMP glycolysis on its own generates excess ATP, but CO_2 is evolved. The WLP converts CO_2 to acetyl-CoA, but the pathway is ATP consuming. As reviewed in Box 1, the NOG pathway is a viable approach for complete carbon conversion from sugar to acetyl-CoA, but the pathway consumes one mole of ATP for every mole of hexose sugar consumed. Both the NOG and WLP have a negative ATP yield when producing acetyl-CoA. Therefore, in order to balance ATP requirements, both the NOG and WLP must either produce acetate as a byproduct to generate ATP through substrate-level phosphorylation or, in the case of the acetogens, couple the WLP to energy conserving membrane reactions, as will be discussed.

For a more comprehensive analysis of ANP mixotrophic potential, we quantified outcomes for *n*-butanol production. From here on, we refer to *n*-butanol as butanol, unless a different isomer is being referred to. Butanol is used in this analysis because it is desirable biofuel that complements or even exceeds the potential of ethanol. Butanol exhibits favorable energy density and physical properties for blending into gasoline [13], which may allow for higher than 10% (v/v) gasoline blends and potentially mitigate the United States' ethanol blending wall. Additionally, butanol production highlights the differences in limited and supplemented mixotrophic fermentation, as detailed below.

Accordingly, we created a stoichiometric and energetic model similar to the one described by Fast and Papoutsakis [7] and calculated the potential butanol production with biomass formation under four different culture scenarios: first, glucose only (i.e. heterotrophic), second, gas only (i.e. autotrophic), third, glucose and gases derived directly from glycolysis (i.e. limited mixotrophic), and fourth, glucose with CO_2 evolved from glycolysis and exogenous hydrogen (i.e. supplemented mixotrophic). All calculations were performed using 100 mol of glucose as substrate. Calculations for autotrophic growth using only the WLP were performed assuming 700 mol of H_2 consumption with the necessary CO_2 as the carbon source. In the supplemental mixotrophic case, we provided an additional 600 mol of molecular hydrogen per 100 mol of glucose to the model.

Briefly regarding the model, we considered ATP and acetyl-CoA production according to Eqs. (1) and (4). Eq. (1) describes CO_2 fixation via the WLP. Included in Eq. (1) is an ATP conservation coefficient (n), which accounts for ATP generated through membrane-bound ATPases, driven by cytochrome-created or Rnf-created H^+ or Na^+ membrane gradients. We evaluated outcomes of autotrophic fermentation for the three different ATP conservation coefficients ($n = 0.4$, 0.7, and 1). As previously reviewed [5], n ranges from 0.2 to 0.77 when cultures are grown on CO_2 and H_2 only, but can be higher when grown on CO. Therefore, $n = 1$ may be unrealistic, but represents the alleviation of ATP constraints for biomass formation via the WLP alone.

Eq. (4) represents ATP, acetyl-CoA, CO_2 and reducing equivalent production from glycolysis of hexose sugar to

(Figure 1 Legend) Outline of mixotrophic fermentative pathways leading to biofuel production. Glycolysis generates excess ATP, acetyl-CoA, CO_2 and reducing equivalents, while the WLP reassimilates the CO_2 into additional acetyl-CoA, which is fed into downstream pathways. Importantly, acetate does not need to be generated for ATP production. Advanced biofuels can be generated from a number of pathways, as indicated, with different input molecules. A detailed scheme for fermentative alcohol production of long chain alcohols is depicted. Primary (1°) alcohols with an even number of carbon atoms can be generated from one of two routes. One pathway uses an aldehyde:ferredoxin oxidoreductase (AOR) and results in the production of ATP. The other pathway utilizes the bifunctional aldehyde/alcohol dehydrogenase (AdhE) and does not produce ATP. Abbreviations of the different metabolites: G6P, glucose 6-phosphate; F6P, fructose 6-phosphate; FBP, fructose 1,6-bisphosphate; G3P, glyceraldehyde 3-phosphate; DHAP, dihydroxyacetone phosphate; BPG, 1,3-bisphosphoglycerate; 3PG, glycerate 3-phosphate; PEP, phosphoenolpyruvate; 2KIV, 2-ketoisovalerate.

acetyl-CoA. Cell production was defined according to Eq. (2), and approximately 5% of carbon flux was directed to biomass formation, as previously discussed by Fast and Papoutsakis [7]. Reducing equivalent production from hydrogen was modeled according to Eq. (3). Finally, butanol production was modeled according to Eq. (5).

$$2CO_2 + 4H_2 + ATP \rightarrow \text{Acetyl-CoA} + 2H_2O + n\text{ATP} \quad \text{(WLP)} \tag{1}$$

$$^1/_2\ \text{Acetyl-CoA} + (M_{cell}/Y_{ATP})\text{ATP} + 0.3\text{NAD(P)H} \rightarrow \text{C-mole Biomass}(CH_{2.08}O_{0.53}N_{0.24})\ \text{(Biomass Production)} \tag{2}$$

$$H_2 + \text{NAD(P)}^+ \leftrightarrow \text{NAD(P)H} + H^+\ \text{(Hydrogenase)} \tag{3}$$

$$1\text{Hexose} \rightarrow 2\text{Acetyl-CoA} + 2CO_2 + 2\text{ATP} + 4\text{NAD(P)H}\ \text{(EMP Glycolysis)} \tag{4}$$

$$2\text{Acetyl-CoA} + 4\text{NAD(P)H} \rightarrow \text{Butanol}\ \text{(Butanol Production)} \tag{5}$$

Results shown in Table 2 suggest that supplemented mixotrophic fermentation has the highest carbon efficiency and butanol mass yield. It is important to note that when producing highly reduced molecules such as butanol the limited mixotrophic pathway without H_2 supplementation does not generate excess reducing equivalents, thus the evolved CO_2 cannot be fixed via the WLP. Accordingly, the outcomes from hexose alone and hexose + gas evolved are identical. For the case of autotrophic growth (i.e. H_2 and CO_2 alone) using the WLP, increasing values of the ATP conservation coefficient, n, result in improved butanol yields. This outcome is expected because the previous model [7] showed that autotrophic butanol production via the WLP is an ATP-limited process. Therefore, since n is a measurement of the number of ATP molecules that can be generated per CO_2 consumed via ATPase ion pumping and the associated ion gradient generated by the either cytochromes or an Rnf complex in native acetogens, increasing n results in greater butanol production. Even at high values for n (i.e. 1) that are not observed in acetogens growing on H_2/CO_2 [5], acetate still must be formed to generate ATP for biomass production. Mixotrophic fermentation alleviates the ATP constraint due to the large amount of ATP produced by glycolysis and, depending upon the amount of biomass generated, can actually result in an excess of ATP, as shown in Table 2. In the presence of exogenous hydrogen, all carbon from hexose can theoretically be converted into butanol resulting in a near 100% carbon efficiency. This results in a mass yield of 58.3% on glucose, which is a 53% increase over what is possible using EMP glycolysis alone. Using a conservative estimate of sugar cost being 50% of total advanced biofuel OPEX, a 53% increase in mass yield decreases total OPEX by more than 17%. If one assumes sugar cost to be upwards of 75% of total OPEX, which has been reported in some cases [3], this mass yield benefit would reduce total OPEX by more than 25%.

The benefits of limited ANP mixotrophy beyond butanol production

As shown in Table 2, the limited mixotrophic production of butanol provides little benefit over the heterotrophic

Table 2

Stoichiometric calculations to determine the maximum yield of *n*-butanol using glycolysis, the WLP, and ANP mixotrophic fermentation

Substrate or metabolite	Heterotrophic	Autotrophic			Limited mixotrophic	Supplemented mixotrophic
	Hexose	Gas (0.4 ATP)	Gas (0.7 ATP)	Gas (1 ATP)	Hexose + gas evolved	Hexose + gas evolved + H_2
ATP cons.	N/A	0.4	0.7	1	0.4	0.4
Substrate						
CO_2	200.0	−320.7	−282.0	−249.7	193.0	−11.2
H_2	21.0	−700.0	−700.0	−700.0	0.0	−600.0
Hexose	−100.0	0.0	0.0	0.0	−100.0	−100.0
Metabolite						
Cell	30.0	8.0	7.0	6.2	30.2	35.3
ATP	80.0	0.0	0.0	0.0	77.2	0.0
Acetate	0.0	128.3	70.5	25.2	0.0	4.5
n-BuOH	92.5	14.0	33.5	48.7	94.2	141.7
Output						
Carbon effic.	61.7%	17.5%	47.5%	78.0%	76.0%	94.5%
n-BuOH mass yield	38.1%	7.4%	20.0%	32.8%	38.8%	58.3%
Hydrogen effic.[a]	N/A	2.0%	4.8%	7.0%	N/A	8.2%

[a] Hydrogen efficiency is calculated as the ratio of butanol produced per mole hydrogen for autotrophic cases (mol butanol/mol H_2) and as additional moles butanol produced per mole hydrogen for the supplemented mixotrophic case ((mol $\text{butanol}_{\text{supp mixo}}$ − mol $\text{butanol}_{\text{hetero}}$)/mol H_2).

case because all of the electrons from glucose are consumed in the production of butanol, leaving no excess electrons for CO_2 fixation. However, limited mixotrophic fermentation provides a tangible yield improvement over heterotrophic fermentation when considering the production of less reduced chemicals, such as organic acids as well as alcohols with lower electron requirements. To demonstrate this feature of limited mixotrophic fermentation, we modeled the production of ethanol, acetate, butyrate, isopropanol, and 2-pentanol using Eqs. (6)–(10) for both heterotrophic and limited mixotrophic conditions.

$$\text{Acetyl-CoA} + 2\text{NAD(P)H} \rightarrow \text{Ethanol} \quad \text{(Ethanol Production)} \tag{6}$$

$$\text{Acetyl-CoA} \rightarrow \text{ATP} + \text{Acetate} \quad \text{(Acetate Production)} \tag{7}$$

$$2\text{Acetyl-CoA} + 2\text{NAD(P)H} \rightarrow \text{ATP} + \text{Butyrate} \quad \text{(Butyrate Production)} \tag{8}$$

$$2\text{Acetyl-CoA} + 1\text{NAD(P)H} \rightarrow \text{Isopropanol} + \text{CO}_2 \quad \text{(Isopropanol Production)} \tag{9}$$

$$3\text{Acetyl-CoA} + 3\text{NAD(P)H} \rightarrow \text{2-Pentanol} + \text{CO}_2 \quad \text{(2-Pentanol Production)} \tag{10}$$

A comparison between the potential mass yields from heterotrophic fermentation and mixotrophic fermentation are shown in Table 3. Like butanol, ethanol production from glucose is not improved by limited mixotrophic fermentation, as nearly all the reducing equivalents produced from glycolysis are used to reduce acetyl-CoA to ethanol. In contrast, the production of carboxylic acids such acetic and butyric acid provides a large excess of electrons that can be used for fixing CO_2 and generating additional product. Consequently, the mass yields of acetate and butyrate can be improved in limited mixotrophic fermentation by 51% and 22% over heterotrophic fermentation, respectively. The secondary alcohols, isopropanol and 2-pentanol, are produced through a decarboxylation reaction, rather than a stepwise reduction of the carboxyl-group using NAD(P)H. Because of the lower NAD(P)H requirement, isopropanol and 2-pentanol show improved yields under the limited mixotrophic condition of 35% and 22%, respectively. Ultimately, the potential benefit of limited mixotrophic fermentation is directly correlated to the ratio of NAD(P)H to acetyl-CoA required to produce a given chemical. Ethanol and butanol, which have a 2:1 NAD(P)H to acetyl-CoA ratio, show little improvement in the limited mixotrophic case, whereas the other molecules shown in Table 3 all have lower NAD(P)H to acetyl-CoA ratios that allow the excess reducing equivalents to be used for CO_2 fixation.

Viability of ANP mixotrophy in acetogenic bacteria

A major concern in the implementation of ANP mixotrophic fermentation is how the consumption of sugars will affect carbon fixation via the WLP and vice versa. One concern is that carbon catabolite repression (CCR), a regulatory mechanism by which bacteria repress the consumption of secondary carbon sources in the presence of a preferred carbon or energy source [14], may cause WLP genes to be downregulated in the presence of sugars. A separate concern is that the kinetics of the WLP and glycolysis will be incompatible in a way that impedes the complete consumption of CO_2 by the WLP. Below, we review some of the questions surrounding CCR and pathway kinetics. Additionally, we discuss recent accomplishments in our understanding of energy conservation and electron bifurcation mechanisms that are essential for acetogenic growth, and presumably also for mixotrophic growth.

Differential regulation and carbon catabolite repression

For 70 years, there has been evidence for co-utilization of the glycolysis and the WLP which was first observed in a *Moorella thermoacetica* (f. *Clostridium thermoaceticum*) culture that could produce 2.5 mol of acetate per mole of glucose [11]. While there are limited data on the co-utilization of syngas mixtures alongside carbohydrates, it appears that differential regulation of the WLP genes in response to carbohydrate carbon sources varies among species and culture conditions. In some cases, catabolite

Table 3

Potential mass yields of different chemicals and biofuels from heterotrophic and mixotrophic fermentations

	Potential yield from heterotrophic and mixotrophic fermentation			
	Heterotrophic (mol/100 mol glucose)	Limited mixotrophic (mol/100 mol glucose)	Yield increase (%)	NAD(P)H to acetyl-CoA ratio
Ethanol	185.0	188.4	2%	2
n-Butanol	92.5	94.2	2%	2
Acetate	185.0	279.9	51%	0
Butyrate	92.5	112.6	22%	1
Isopropanol	92.5	124.8	35%	0.5
2-Pentanol	61.7	75.1	22%	1

repression of the WLP genes is clearly observed in the presence of carbohydrate sources. In an early study by Braun and Gottschalk, the ability of *C. aceticum* to consume CO_2/H_2 was tested in both the presence and absence of sugars [8••]. While *C. aceticum* readily consumes H_2/CO_2 in the absence of fructose, only small amounts of H_2/CO_2 consumption were observed in the presence of fructose. It was also shown that cell extracts of mixotrophically grown *C. aceticum* had much lower hydrogenase activity than extracts from *C. aceticum* grown on H_2/CO_2. Though Braun and Gottschalk only tested for the downregulation of hydrogenase activity, it is possible that other WLP genes were also repressed in the presence of fructose. Hexose-dependent hydrogenase regulation was also recently shown to occur in *M. thermoacetica*. *M. thermoacetica* cell extracts from glucose-grown cells were tested for hydrogenase activity, and it was found that both the NADP-dependent hydrogenase and the electron bifurcating hydrogenase were downregulated 100-fold and 3-fold, respectively, compared to H_2/CO_2-grown cell extracts [15••]. Cultures of *Eubacterium limosum* when consuming mixtures of glucose and methanol — which is metabolized through the WLP like other one-carbon substrates — exhibit a diauxic growth profile that is characteristic of CCR. In one study, *E. limosum* was inoculated into media containing an equimolar carbon mixture of glucose and methanol (50 mM methanol and 8.3 mM glucose) [16]. During early stages of culture, only glucose is consumed; however, once glucose is depleted to 6 mM, the cells begin to consume methanol and glucose simultaneously during a short period of mixotrophy. Interestingly, once glucose is completely depleted, *E. limosum* undergoes a lag phase for ~12 h before resuming the consumption of methanol. The precise mechanism that governs the diauxic growth of *E. limosum* has not yet been investigated.

Despite the evidence for the downregulation of WLP genes in the presence of sugars, many organisms seem to be able to use gases and carbohydrates simultaneously without any signs of WLP inhibition. The aforementioned Braun and Gottshalk study on *C. aceticum*, for instance, also tested the ability of *Acetobacterium woodii* to grow on CO_2/H_2 in the presence of fructose, glucose, and lactate [8••]. In all three cases, *A. woodii* readily consumed both the gaseous and organic substrates, resulting in the production of higher yields of acetate than could be produced using the organic substrates alone. Furthermore, they measured the hydrogenase activity of *A. woodii* and found that it was not differentially regulated in the presence of fructose, in contrast to the hydrogenase of *C. aceticum*. *Butyribacterium methylotrophicum*, a close relative of *E. limosum*, has been shown to co-utilize 1-carbon substrates and sugars. One study showed that a CO-adapted strain of *B. methylotrophicum* could consume both glucose and CO/CO_2 simultaneously [17]. However, due to saturation of the ferredoxin pool with electrons from CO and pyruvate:ferredoxin oxidoreductase, pyruvate overflow and lactate production were observed. The addition of the artificial electron carrier neutral red alleviated the over-reduction of the ferredoxin pool, allowing for redirection of carbon flux from pyruvate and lactate to acetate and butyrate.

Since the publication of the *Clostridium ljungdahlii* genome sequence [18], a number of studies have examined the transcriptomic response of *C. ljungdahlii* to the presence of carbohydrates. An RNA-seq based study from Tan *et al.* collected transcriptomic data on *C. ljungdahlii* cells grown autotrophically on a CO/CO_2 mixture as well as *C. ljungdahlii* grown on 5 g/L fructose [19•]. Their results showed that a number of carbon fixation genes were upregulated under autotrophic growth conditions, including genes encoding both the methyl-branch and carbonyl-branch of the WLP, which suggests that the WLP may be repressed in the presence of sugar [19•]. In a separate study from Nagarajan *et al.*, total RNA was collected from *C. ljungdahlii* cells grown heterotrophically on fructose and autotrophically on H_2/CO_2. In this case, transcriptomic analysis via RNA-seq showed no differential expression of WLP genes. The authors postulated that the disparate finding was due to a different gas composition for their autotrophic study (CO/CO_2 for the study showing differential regulation and H_2/CO_2 for the study that showed similar expression levels for both autotrophic and chemotrophic growth). However, both transcriptomic studies found no significant up or down regulation of the Rnf genes between the autotrophic and heterotrophic conditions. Another study that established the importance of the ATP-conserving Rnf complex in the autotrophic growth of *C. ljungdahlii*, which will be discussed below, showed through quantitative reverse transcription PCR (qRT-PCR) that transcription from the Rnf operon was significantly higher in cells grown on H_2/CO_2 as compared to transcription in cells grown on fructose [20]. Given the conflicting transcriptional analyses, it is clear that further experiments need to be conducted to clearly establish evidence for catabolite repression and that more omics tools need to be applied to elucidate the mechanisms involved. Furthermore, the inclusion of mixotrophic growth conditions in future studies would provide additional insight as to whether downregulation of the WLP — if it occurs — is triggered by the absence of gases or the presence of sugars.

Kinetics

To realize the successful implementation of mixotrophic fermentation, both uptake rates of sugar as well as gases need to be carefully considered. Because autotrophic growth rates vastly differ among organisms, we will focus on a few select organisms that have been studied in more detail. As an example, for *C. ljungdahlii*, Nagarajan *et al.* reported an experimentally determined uptake rate of fructose of 1.9 mmol/gDW/h, which agreed with the

proposed metabolic model [21••]. Assuming this consumption of fructose, 3.8 mmol/gDW/h of CO_2 is released during fermentation. The model was constrained with a maximum CO_2 uptake rate of 10 mmol/gDW/h, which yielded feasible growth rates and would be sufficient to uptake all of the generated CO_2 from fructose during glycolysis. However, the paper only predicted an uptake rate for autotrophic growth based on the model and failed to confirm this experimentally [21••]. Additionally, the metabolic model from Nagarajan *et al.* was constrained at an uptake rate for CO of 20 mmol/gDW/h, whereas others reported the maximum specific uptake rate of CO as 34.4 mmol/gDW/h for *C. ljungdahlii* grown on a mixture of $CO/CO_2/H_2$ [22]. This shows that the gaseous uptake rates may yet be higher than the model suggested and certainly seem to be higher than the production rate during glycolysis. However, none of these reported rates have been confirmed in a mixotrophic fermentation. For *A. woodii*, the Braun and Gottschalk study showed the simultaneous consumption of both organic substrates (fructose, glucose and lactate) and of H_2/CO_2, with significantly larger molar amounts of gas being consumed than organic substrates [8••]. Interestingly, different amounts of H_2/CO_2 were consumed, depending on the co-utilized organic substrate, with the gas uptake rate being higher when co-utilized with glucose than when co-utilized with either fructose or lactate. Accordingly, it would be interesting to investigate whether catabolite repression and/or transcriptional regulation of gas consumption is dependent on the specific carbohydrate source, and how it varies among the acetogens that are more commonly studied today.

ATP conservation in acetogens

In addition to gene regulation and substrate utilization kinetics, the yield of ATP during autotrophic and ANP mixotrophic fermentation likely has a tremendous effect on product yields [7]. The reactions of the WLP do not produce the net ATP required for biosynthesis through substrate level phosphorylation. Instead, autotrophically grown acetogens couple the most exergonic reduction reactions of the WLP to the translocation of protons or Na^+ ions across the cell membrane, a process that has been reviewed elsewhere [23]. The resulting H^+ or Na^+ membrane gradient is captured by membrane-bound ATPases. Thus ATP allows for gluconeogenesis and the formation of fermentation products other than acetate. The mechanism for ion translocation varies among acetogens. While *C. ljungdahlii*, *C. carboxidivorans*, and *A. woodii* all use an Rnf complex for energy conservation, *A. woodii* uses a Na^+-dependent Rnf complex, whereas *C. ljungdahlii* — and potentially other clostridial acetogens such as *C. carboxidivorans* and *C. autoethanologenum* — uses a H^+-dependent Rnf complex [20]. Some acetogens, such as *M. thermoacetica*, use cytochromes or quinones instead of an Rnf complex to couple electron transfer to the creation of trans-membrane H^+ gradients. Because *A. woodii* uses a Na^+-dependent Rnf complex, the effect of removing the Rnf was studied without the need for genetic manipulation by the omission of Na^+ from the medium [24]. The recent development of genetic tools for *C. ljungdahlii* has allowed for the study of H^+ Rnf knockouts [20]. In one report, Tremblay *et al.* disrupted the *rnf* operon in *C. ljungdahlii* by a single-crossover integration, which removed genes for two subunits of the Rnf complex. The disruption of the *rnf* operon resulted in complete inhibition of autotrophic growth. Moreover, the Rnf-negative mutant showed a decreased growth rate on fructose alone [20], indicating that Rnf-based energy conservation is important for both heterotrophic and autotrophic metabolism. The relationship between Rnf complex functionality and heterotrophic growth rate was also observed in the early studies of the Na^+-dependent Rnf complex from *A. woodii* [24]. Expression and activity of energy conservation machinery under mixotrophic growth remains to be studied, but presumably, its expression would have a positive effect on growth and ATP production

Aldehyde:ferredoxin oxidoreductase (AOR)

Alcohol-producing, acetogenic clostridia often contain aldehyde:ferredoxin oxidoreductase (AOR) enzymes that have been shown *in vitro* to use electrons from reduced ferredoxin to produce aldehydes from non-activated carboxylic acid species [25]. The reduction of unactivated carboxylic acids has been demonstrated for a number of organic acid substrates, including acetate, propionate, and butyrate, as well as higher acids [26]. It has been suggested that this reaction provides a means for acetogenic bacteria to produce alcohols while also generating ATP from the production of carboxylic acid species [18]; however, due to the high reducing potential required to directly reduce carboxylic acids, it is still unclear how significant a role the AOR reaction plays *in vivo*. Perez *et al.* investigated the ability of both *C. ljungdahlii* and *Clostridium ragsdalei* to reduce short-chain carboxylic acids to their corresponding alcohols [27]. They demonstrated the reduction of *n*-butyric, propionic, *n*-valeric, *n*-caproic and isobutryic acid by *C. ljungdahlii* and the reduction of propionic acid and *n*-butyric acid by *C. ragsdalei*. While energetic calculations show that either the carboxykinase/phosphotranscarboxylase pathway or the AOR pathway could be responsible for acid reduction, no experimental analysis has tested this definitively.

Electron bifurcation for energy conservation

Reduced ferredoxin is required to drive many steps in acetogenic metabolism, including the reduction of CO_2 to CO, the synthesis of pyruvate from acetyl-CoA, and the proposed direct reduction of acetate to acetaldehyde via aldehyde:ferredoxin oxidoreductase (AOR). In addition, the aforementioned Rnf complexes of *A. woodii* and *C. ljungdahlii* require high-energy electrons from reduced ferredoxin ($E^{o\prime} \sim -500$ mV) to drive ion translocation

across the cell membrane [20,28••]. To generate the low-potential reduced ferredoxin required for the WLP, acetogens have evolved unique systems for producing highly reduced electron carriers from less reduced sources. When cells are grown with H_2 as an electron source, the redox potential of H_2 ($E^{o\prime}$ = −414 mM) is not sufficiently low to reduce ferredoxin directly. Therefore, electron bifurcation — a recently discovered mechanism [29••] in which an 'uphill,' endergonic reduction is coupled to a 'downhill,' exergonic reduction — is required to generate the reduced ferredoxin necessary for CO_2 fixation and ATP generation via the Rnf complex. Electron bifurcating hydrogenases catalyze the oxidation of two molecules of H_2 to form Fd_{red} and NADH ($E^{o\prime}$ = −320 mV). Because the reduction of NADH with H_2 is exergonic, the excess energy is used to enable the unfavorable reduction of Fd_{red} from H_2. NAD-specific electron-bifurcating hydrogenases were first isolated from *M. thermoacetica* and *A. woodii* [30,31]; *Clostridium autoethanogenum* was shown to contain an NADP-specific electron-bifurcating hydrogenase [32]. The NADP-specific hydrogenase from *C. autoethanogenum* forms a complex with formate dehydrogenase, allowing for the reduction of two molecules of CO_2 to formate using high-energy electrons from Fd_{red} and low-energy electrons from NADPH. The same genes are also found in *C. ljungdahlii*. Furthermore, *A. woodii* was recently discovered to contain a hydrogenase which forms a complex with formate dehydrogenase as well [33]. Considering the many hydrogenase genes contained within acetogens, we can postulate that many more reactions proceeding in an electron-bifurcating manner will be discovered in the future. Electron bifurcation serves a second purpose in acetogens to correct cofactor imbalances that arise during mixotrophic growth. In *M. thermoacetica* for instance, two of the CO_2 reduction reactions in the WLP require the use of a NADPH cofactor, but glycolysis exclusively produces reducing equivalents in the form of NADH and Fd_{red}. To adjust the available co-factor pool, *M. thermoacetica* uses an electron bifurcating NADH-dependent reduced ferredoxin:$NADP^+$ oxidoreductase (NfnAB), which reduces two molecules of $NADP^+$ using NADH and Fd_{red} [15••]. By transferring electrons between reduced co-factors, *M. thermoacetica* enables the simultaneous utilization of glycolysis and the WLP. The same NfnAB genes are present in *C. ljungdahlii*, although they are expressed as a fusion gene previously annotated as glutamate synthase, and are predicted to have similar function for interconversion between NADPH and NADH during autotrophic growth [21••]. The role of electron bifurcation in biofuel production has yet to be explored. But since electron-bifurcation plays such a large role in the conservation, conversion and efficient utilization of reducing power, electron-bifurcation will likely play a larger role for understanding electron fluxes involved in the production of more reduced products. Because of the fact that the ANP mixotrophic model for butanol production requires electron supplementation in the form of H_2 to achieve the desired yields, electron bifurcation mechanisms could be used to shift electrons from Fd_{red} and NADPH to NADH, which is the most important cofactor required for alcohol fermentation. As more data becomes available regarding electron bifurcation in acetogens, metabolic engineers will potentially be able to leverage the energy conservation and cofactor balancing utility of electron bifurcation to achieve higher product yields in acetogen fermentation processes.

Potential applications of ANP mixotrophic fermentation for fuel production

The improved carbon yields from ANP mixotrophic fermentation could be applied to a number of biofuel processes. The most studied approaches to produce various advanced biofuels include: first, 2-keto acid pathways for branched alcohol synthesis, second, fermentative alcohol production, third, alkane/alkene production via long-chain fatty acid biosynthesis and reverse β-oxidation, and fourth, isoprenoid-based molecules. Several recent reviews provide excellent detail and updates on the pathway development [34,35]. The ANP mixotrophic concept is realistically limited to obligate anaerobic organisms, given the oxygen sensitivity of the WLP. Additionally, ANP mixotrophy is best suited for the production of chemicals that require minimal ATP to generate. Although ATP excess is a benefit of ANP mixotrophy, production of fatty acids and isoprenoids require large amounts of ATP. For example, C_{16} palmitic acid production from acetyl-CoA requires seven ATP. Therefore, we exclude further discussion on fatty acid biosynthesis and isoprenoid production, as both are better suited to be coupled to oxidative respiration. Otherwise, they would require significant production of undesirable byproducts such as acetate in order to generate ATP via substrate level phosphorylation. The 2-keto acid, reverse β-oxidation, and fermentative alcohol pathways can theoretically be integrated into the ANP mixotrophic concept, and as we detail below, hold the potential to produce a huge diversity of straight and branched chain, primary and secondary alcohols that serve as drop in gasoline blending agents or feedstocks for jet-fuel production.

2-Keto acid-derived alcohols

This non-fermentative pathway utilizes the amino acid biosynthetic pathway and diverts 2-keto acid intermediates into a variety of alcohols, including short-chain alcohols such as butanol as well as longer chain, branched alcohols, like 3-methyl-butanol and 3-methyl-1-pentanol [36]. Among all the potential fuels from this pathway, isobutanol has received the most attention. The 2-keto acid intermediate for isobutanol is 2-ketoisovalerate, produced in the valine biosynthesis pathway. To form 2-ketoisovalerate, two pyruvate molecules are combined by an acetolactate synthase (*alsS*) to produce 2-acetolactate and one molecule of CO_2. 2-acetolactate is in turn

converted to 2,3-dihydroxyisovalerate by a ketol-acid reductoisomerase (*ilvC*), which consumes an NADPH molecule, and finally, a dihydroxy-acid dehydratase (*ilvD*) converts 2,3-dihydroxyisovalerate to 2-ketoisovlerate and produces water. Isobutanol is then produced from 2-ketoisovalerate in a two-step process. First, 2-ketoisovalerate undergoes a decarboxylation reaction to produce isobutanal and then an alcohol dehydrogenase reduces the isobutanal into isobutanol. The overall process, from pyruvate to isobutanol, consumes four electrons (one NADPH and one NADH) and produces two molecules of CO_2. Even though 2-keto acid pathways do not proceed through acetyl-CoA, two CO_2 molecules are still generated and could be recaptured by ANP mixotrophic fermentation.

Fermentative alcohol production

As previously mentioned, ethanol fermentation is the dominant biofuel production process of today, but longer chain alcohols (e.g. butanol) are arguably better gasoline blending alcohols and could potentially alleviate the ethanol blending wall in the United States. Whereas ethanol is produced in a two-step reduction of acetyl-CoA to acetaldehyde and acetaldehyde to ethanol, four carbon and longer chain alcohol production begins with the condensation of acetyl-CoA molecules by thiolase to form acetoacetyl-CoA (as shown in Figure 1). Acetoacetyl-CoA is reduced, dehydrated, and reduced again to form butyryl-CoA, which can be converted into butanol by a bifunctional aldehyde/alcohol dehydrogenase (AdhE) or can undergo additional chain elongation by thiolase. In case of AdhE activity, the high-energy thioesterase bond (i.e. -CoA) is not utilized to generate ATP. Alternatively, it is hypothesized that ATP can be generated en route to butanol by first converting butyryl-CoA to butyrate through substrate level phosphorylation. Butyrate is then oxidized to butyryaldehyde by the activity of an AOR enzyme, and subsequently reduced to butanol by the activity of an alcohol dehydrogenase. Butanol production via AOR activity has not been experimentally demonstrated, but it is suggested that AOR plays an important role in ATP generation for acetogenic alcohol fermentation [18]. This AOR pathway could also be used to produce ethanol (from acetate) or any other even-numbered primary alcohol from the corresponding organic acid. Odd-chain length, secondary alcohols can also be produced from thiolase condensation products (e.g. acetoacetyl-CoA). In the case of acetoacetyl-CoA, the CoA can be transferred to acetate or butyrate. The resulting acetoacetate can then undergo a decarboxylation and secondary alcohol dehydrogenation reaction to create isopropanol. An NADPH-dependent primary-secondary alcohol dehydrogenase (CaADH) has been described in *C. autoethanogenum* [37], and Köpke *et al.* found *in vitro* that this enzyme is capable of converting acetone to isopropanol and butanone to 2-butanol [38], suggesting the potential to produce secondary C3 and C4 alcohols. In addition, whole-cell biocatalysis using *C. autoethanogenum*, *C. ljungdahlii*, and *C. ragsdalei* was shown to convert exogenous acetone to isopropanol, even though these organisms do not synthesize acetone or isopropanol naturally. Recently, *C. ljungdahlii* was engineered to produce butyrate from gases with carbon yields between 42% and 68% [39]. In theory, this pathway could be modified to produce butanol, rather than butyrate. However, the ATP limitations inherent in CO_2 fixation via the WLP may require the use of mixotrophic fermentation to achieve high butanol yields.

Fatty acid biosynthesis through reverse β-oxidation

Fatty acid biosynthesis by the sequential elongation of acyl-ACP (acyl carrier protein) is impractical using anaerobic fermentation due to the ATP-dependence of chain elongation [40]. The recently engineered reversal of the β-oxidation cycle [41•], which directly condenses acetyl-CoA with acyl-CoA, has been shown to produce a large variety of long-chain species (C_4–C_{18}) without the need for ATP-dependent carboxylation of acetyl-CoA to malonyl-CoA. Therefore, reverse β-oxidation is a more promising method for the anaerobic production of fatty substrates using ANP mixotrophic fermentation. The reverse β-oxidation cycle consists of four steps that are quite similar to the chain elongation steps detailed for fermentative alcohol production. First, a thiolase condensation (*yqeF*, *fadA*) combines acetyl-CoA and (C_n)-acyl-CoA, producing β-ketoacyl-CoA. Hydoxyacyl-CoA dehydrogenase (*fadB*) reduces β-ketoacyl-CoA to Trans-β-hydroxyacyl-CoA, which is then dehydrated by enoyl-CoA hydratase (*fadB*) to form trans-Δ^2-enoyl-CoA. Finally, enoyl-CoA reductase (*ydiO*) reduces the enoyl-CoA to (C_{n+2})-acyl-CoA. With every turn of the reverse β-oxidation cycle, the acyl-CoA chain is lengthened by two carbons. The pathway intermediates can each be converted into potentially useful biomolecules with the addition of alcohol/aldehyde dehydrogenases and acyl-CoA thioesterases, most significantly, long chain alcohols (C4–C10) and long chain fatty acids (C10–C18).

Studies needed to advance the concept

As discussed, one of the major challenges facing the realization of ANP mixotrophic fermentation is the lack of data on how acetogenic organisms regulate metabolism in the presence of gaseous and carbohydrate substrates. With the rise of 'omics' analysis, the collection large datasets on the transcriptional, translational, and metabolic flux changes that occur under differing acetogenic culture conditions will become simpler and more cost effective. Additionally, computational techniques, such as the recent construction of a genome-scale model of *C. ljungdahlii* [20], are going to play a larger role in the identification of gene-targets for engineering. Still, knowledge surrounding many of the underlying mechanisms that enable acetogenic fermentation — including ion translocation, electron bifurcation, and the role of

Table 4

Potential host organisms for ANP mixotrophic processes. This list of acetogens, while by no means exhaustive, provides some examples for good hosts for ANP mixotrophic fermentations

Name	Carbon sources					Native fermentation products	Genome available	Genetic tools
	H_2/CO_2	CO/CO_2	C5 sugars	C6 sugars	Methanol			
A.woodii	X	X		X	X	Acetate, ethanol	Yes	Yes [46•]
B. methylotrophicum	X	X	X	X	X	Acetate, ethanol, butyrate, butanol, lactate	No	No
C. aceticum	X	X	X	X		Acetate	No	Yes [45]
C. carboxidivorans P7T	X	X	X	X		Acetate, ethanol, butyrate, butanol, hexanol	Yes[a]	No
C. ljungdahlii	X	X	X	X		Acetate, ethanol, 2,3-butanediol, lactate	Yes	Yes [19•,42]
C. autoethanogenum	X	X	X	X		Acetate, ethanol, 2,3-butanediol, lactate	Yes	Yes [43]
E. limosum	X	X	X	X	X	Acetate, butyrate, caproate, lactate	Yes	No
M. thermoacetica	X	X	X	X	X	Acetate	Yes	Yes [50]

[a] The genome sequence for *C. carboxidivorans* exists only as a draft.

AOR in alcohol production — are not fully understood, and to a large degree, a better understanding of these mechanisms can only be achieved through enzyme purification, characterization, and other basic science tools. One certainty in the development of ANP mixotrophic fermentation is the need for genetic engineering techniques. Given that few acetogens produce biofuels of interest as sole fermentation products (Table 4), any process will require the ability to add new biosynthesis pathways to and knockout competing reactions from any acetogenic host.

In the past two decades, genetic tools for clostridia species have considerably improved, as recently reviewed [42]. Advancements include broad host range multi-copy plasmid gene expression, dual plasmid gene expression, antisense RNA down-regulation of genes, homologous recombination based gene disruption and deletion, iterative gene disruption/deletion, transposon based gene disruption, Group II Intron based gene disruption [43], negative selection systems for chromosomal manipulations [7,44], and RecT based recombineering strategies [45]. However, the majority of these genetic engineering technologies has been developed and demonstrated only in solventogenic, pathogenic, cellulolytic, acidogenic and/or thermophilic strains of clostridia. The available tools for clostridial acetogens are far more limited, and as indicated in Table 4, have only been demonstrated in four strains, *C. ljundahlii* [18,20,46•], *C. autoethanogenum* [47,48], *C. aceticum* [49] and *A. woodii* [50,51]. The most advanced genetic tools for an acetogen have been demonstrated in *C. ljundahlii*. Recently the Lovley lab developed a robust electroporation protocol for introducing replicating plasmids and suicide vectors into *C. ljundahlii* [46•]. They demonstrated the function of four unique replicative origins for plasmid propagation, and they deleted several genes in the chromosome via double-crossover homologous recombination with suicide vectors. Specifically, they deleted the *adhE1* and *adhE2* genes individually and in combination to demonstrate the ability to redirect carbon and electron flow in this organism via gene disruptions, and also to determine which gene is most responsible for ethanol production. As a result, they witnessed that *adhE1*, but not *adhE2*, diminished ethanol production, and they could partially restore the wild-type phenotype through complementation. More recently, the same lab also demonstrated the utility of a lactose-inducible gene expression system in *C. ljungdahlii* [52]. Briefly, they adapted a lactose-inducible promoter and its divergent regulator (*bgaR*) from *C. perfringens* that was previously demonstrated to maintain tight gene control in *C. perfringens* [53]. They demonstrated the ability to control gene expression with a *gusA* reporter gene, and then showed the ability to affect carbon flux and increase ethanol production by inducing the over-expression of AdhE1.

Given the recent increase in interest, it is likely a short matter of time until more advanced genetic tools available for solventogenic and pathogenic clostridia are adapted to acetogens. Through the coupling of 'omics' datasets, directed genetic engineering studies and consorted commitments by academia and industry, we believe that ANP mixotrophy can have a tremendous positive impact on industrial biofuel and renewable chemical production.

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