microbial biotechnology

Brief Report

High methanol-to-formate ratios induce butanol production in *Eubacterium limosum*

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Summary

Unlike gaseous C_1 feedstocks for acetogenic bacteria, there has been less attention on liquid C_1 feedstocks, despite benefits in terms of energy efficiency, mass transfer and integration within existing fermentation infrastructure. Here, we present growth of *Eubacterium limosum* ATCC8486 using methanol and formate as substrates, finding evidence for the first time of native butanol production. We varied ratios of methanol-to-formate in batch serum bottle fermentations, showing butyrate is the major product (maximum specific rate 220 ± 23 mmol-C gDCW^{-1}day^{-1}). Increasing this ratio showed methanol is the key feedstock driving the product spectrum towards more reduced products, such as butanol (maximum titre 2.0 ± 1.1 mM-C). However, both substrates are required for a high growth rate (maximum 0.19 ± 0.011 h^{-1}) and cell density (maximum 1.2 ± 0.043 gDCW l^{-1}), with formate being the preferred substrate. In fact, formate and methanol are consumed in two distinct growth phases – growth

phase 1, on predominately formate and growth phase 2 on methanol, which must balance. Because the second growth varied according to the first growth on formate, this suggests butanol production is due to overflow metabolism, similar to 2,3-butanediol production in other acetogens. However, further research is required to confirm the butanol production pathway in *E. limosum*, particularly given, unlike other substrates, methanol likely results in mostly NADH generation, not reduced ferredoxin.

Introduction

Acetogens have long been suggested as cell factories for useful products because of their ability to close the carbon cycle using reducing equivalents from renewable feedstocks such as hydrogen (H_2) as in gas fermentation (Ljungdahl, 2009), or electrons in microbial electrosynthesis systems (Nevin *et al.*, 2010), without necessarily relying on arable land for the carbonaceous feedstock. However, unlike utilization of waste synthesis gas (syngas, a mixture of carbon monoxide (CO) and H_2), as has been commercialized by LanzaTech (Köpke and Simpson, 2020), recent technoeconomic assessment for microbial electrosynthesis suggests this approach is not currently viable (Wood *et al.*, 2021).

Much less attention has been given to liquid C_1 feedstocks (i.e. liquid chemicals containing one carbon atom per molecular unit). Examples such as methanol and formate could have transformational impacts on biotechnology as they are completely miscible in water, overcoming key mass-transfer limitations in gas fermentations (Cotton *et al.*, 2020). Being liquid, they avoid many of the transportation issues presented with gaseous C_1 substrates and are more compatible with existing fermentation infrastructure. Unsurprisingly, there have been recent efforts to engineer conventional model organisms such as *S. cerevisiae* and *E. coli* for liquid C_1 utilization (Espinosa *et al.*, 2020; Keller *et al.*, 2020; Kim *et al.*, 2020). However, some acetogens have a native assimilation pathway, which would overcome the need for genetic engineering or building new gas fermentation facilities.

Importantly, liquid C_1 feedstocks can be produced efficiently from carbon dioxide (CO_2). Methanol can be

synthesized renewably via direct hydrogenation of CO_2 for as little as US\$560/t (Hank *et al.*, 2018) and is expected to reach parity with fossil fuel-derived methanol by 2032 (Detz *et al.*, 2018; Hank *et al.*, 2018). Formate can be synthesized through electrochemical CO_2 reduction, and the technology is at pre-commercialization stage (Spurgeon and Kumar, 2018). Therefore, using these liquid C_1 feedstocks may provide another viable production alternative for acetogenic cell factories.

Compared with acetogenic gas fermentation using various combinations of gas substrates of CO_2, CO and H_2, less research has focused on methylotrophic and formatotrophic growth using acetogens. This is interesting since methanol and formate have the highest energetic efficiency of all C_1 electron donors (Claassens *et al.*, 2019; Cotton *et al.*, 2020). Theoretically, using more reduced substrates should allow synthesis of more reduced products than the typical spectrum produced by acetogens when grown on syngas, i.e. acetate and ethanol.

Eubacterium limosum is one of the few known acetogens to metabolize methanol to produce butyrate, *via* direct condensation of acetyl-CoA (Shin *et al.*, 2019). Methanol as a sole substrate in the acetogen Wood-Ljungdahl Pathway (WLP) results in partial oxidation to generate reducing equivalents and satisfy CO_2 demand for the carbonyl branch, without net generation of CO_2 (Lindley *et al.*, 1987; Müller, 2019). Formate, on the other hand, is similar to CO metabolism in that there is excess oxidation to CO_2 in order to generate the required number of reducing equivalents as shown in Fig. 1.

By itself, methanol is a poor substrate, and a more oxidized co-substrate, such as CO_2 or formate is required to maintain sustained growth (Lebloas *et al.*, 1996). This allows a lever to control fermentations, with certain substrate ratios known to have higher specificity towards butyrate (Kerby and Zeikus, 1987; Lebloas *et al.*, 1994; Pacaud *et al.*, 1986). This is similar to that seen with H_2 and CO co-metabolism or CO_2 and H_2, leading to higher specificity towards more reduced products (Liew *et al.*, 2016; Jack *et al.*, 2019; Heffernan *et al.*, 2020), due to regulation at the thermodynamic and metabolite level (Mahamkali *et al.*, 2020). The *Eubacterium limosum* genome shows a native butanol production pathway, a more reduced product than butyrate (Song and Cho, 2015); however, this has yet to be seen experimentally. It is noted, a recent investigation showed non-native butanol and acetone production from methanol with recombinant *Eubacterium limosum* (Flaiz *et al.*, 2021). Unlike butyrate, butanol has a sizeable chemical market and holds promise for use as a drop-in fuel (Wang *et al.*, 2014; Wood *et al.*, 2021). In this study, we investigated C_1 liquid growth in *Eubacterium limosum*, and strategies to improve specificity for reduced products, obtaining the first evidence of native butanol production from methanol.

Results and Discussion

Growth profile

Eubacterium limosum ATCC 8486 (*E. limosum)* was cultivated anaerobically at 37°C in a phosphate-buffered medium modified from Valgepea *et al.* (2017), as detailed in the Text S1. A series of batch tests were undertaken in triplicate for seven methanol-to-formate substrate ratios between 0:1 and 10:1. Substrate ratios of 3:1 and 5:1 resulted in the highest maximum growth rates (Fig. 2A) (up to a maximum of 0.19 ± 0.011 h^{-1}), which are amongst the highest reported for acetogen growth on C_1.

Eubacterium limosum used methanol and formate to primarily produce biomass, butyrate and acetate, with carbon and electron balances closing within 10% (Fig. 2 B). Raw data are provided in Table S1. At a ratio of 0:1, that is, when only formate is provided, CO_2 is produced, which was not measured or accounted for in the balance (Table 1, Eq. 5). At substrate ratios of 2:1 and below, both methanol and formate were completely consumed (data not shown), and hence the culture was carbon limited under those conditions. Above this, methanol was not completely consumed. Consequently, if we consider the average methanol-to-formate uptake ratio across the entire growth, they diverged from the substrate ratio above 2:1 (Fig. 2C). This is not likely related to product inhibition, given butyrate is below the previously reported limit of 20 g l^{-1} (Lindley *et al.*, 1987), but rather depletion of the ATP pool (Lebloas *et al.*, 1996). Excess methanol has not previously been observed as, to our knowledge, only substrate ratios below 3:1 methanol-to-formate have been tested.

We generally observed a first growth phase on formate, followed by growth on methanol (Fig. 3A). During the formate growth phase, cells produced biomass and a mix of acetate and butyrate, resulting in an increase in pH (Fig. 3A and B). After formate was exhausted at around 24 h, methanol was the sole substrate, yielding biomass, butyrate and a lowering of pH. The most productive substrate ratio, 5:1, achieved a butyrate production of 3.0 ± 0.79 g l^{-1} day^{-1} (190 ± 55 mmol-C gDCW^{-1} day^{-1}), after formate was exhausted (Fig. 3B). In terms of product spectrum, increasing the substrate ratio increased the flux to more reduced products such as butyrate, butanol and hexanoate (Figs 2 and 3). This is the first, albeit small, evidence of native butanol production in a methylotrophic fermentation to our knowledge. Higher substrate ratios also led to more methanol consumption, yet cell densities did not increase and so more carbon ended up as products (Fig. 2B).
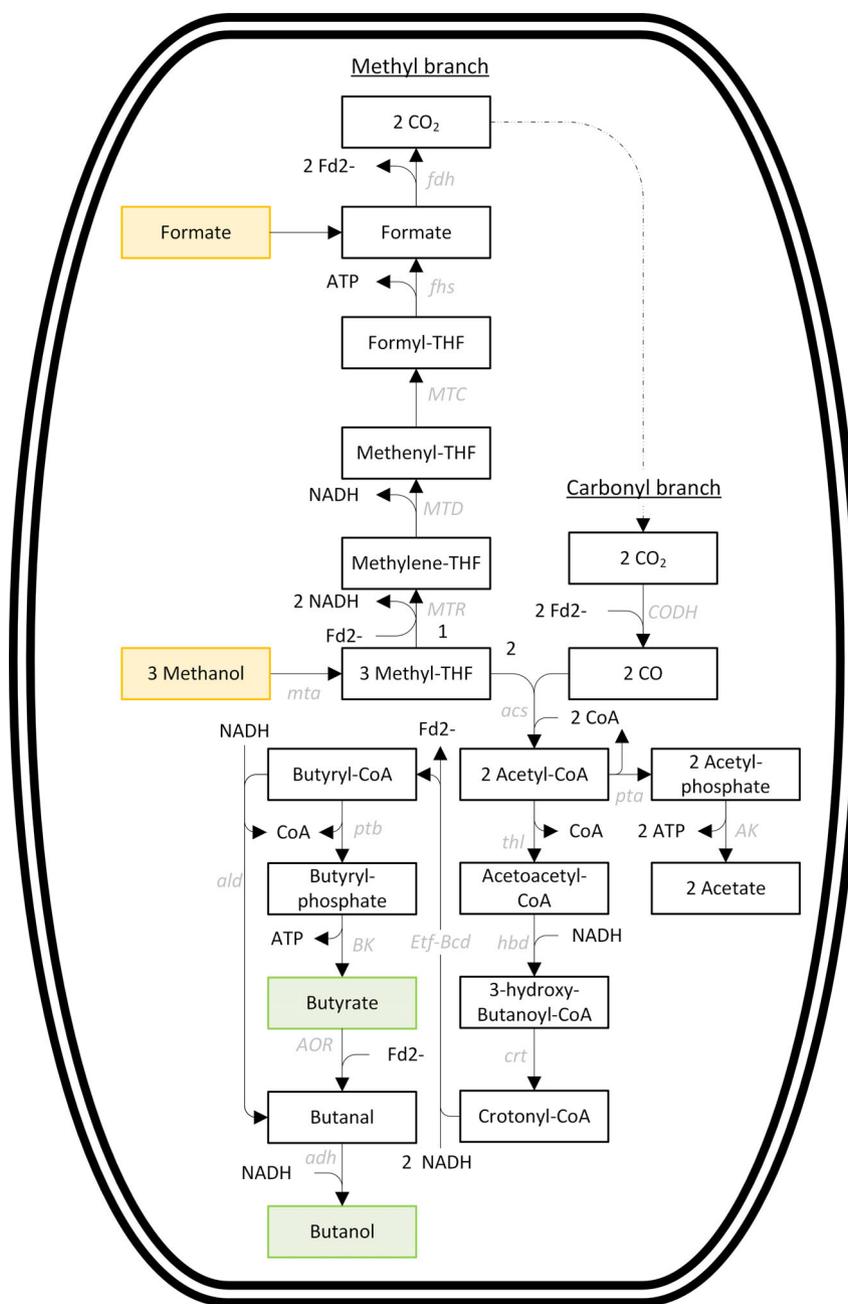
Fig. 1. Wood-Ljungdahl pathway in model acetogen, *E. limosum*, for carbon fixation, showing entry points for other C$_1$ substrates. Only the pathway from methanol and formate to butyrate is balanced and complete. Adapted from Bengelsdorf *et al.* (2013); Jeong *et al.* (2015); Song *et al.* (2017, 2018); Kremp *et al.* (2018) assuming Rnf translocates 4 Na$^+$ ions using reduced ferredoxin, with ATPase importing 4 Na$^+$ ions to generate 1 ATP. *E. limosum* contains a NADH and ferredoxin-dependent hydrogenase. Electron bifurcation is also likely for energy conservation in reduction of crotonyl-CoA to butyryl-CoA and methylene-THF to methyl-THF, coupling NADH oxidation with reduction of ferredoxin (Jeong *et al.*, 2015). Abbreviations: ald, aldehyde dehydrogenase; ACS, acetyl-CoA synthase; adh, alcohol dehydrogenase; AK, acetate kinase; AOR, aldehyde:ferredoxin oxidoreductase; Etf-Bcd, butyryl-CoA dehydrogenase; CODH, CO dehydrogenase; crt, crotonase; fdh, formate dehydrogenase; fhs, formyl-THF synthetase; hbd, 3-hydroxybutyryl-CoA dehydrogenase; MTA, methanol-dependent methyltransferase; MTC, methenyl-THF cylcohydrolase; MTD, methylene-THF dehydrogenase; MTR, methyltransferase/methylene-THF reductase; pta, phosphotransacetylase; ptb, phosphotransbutyrylase (Song *et al.*, 2017) and therefore a butyrate kinase is assumed; THF, tetrahydrofolate; thl, thiolase.

Carbon and redox balances

Compared with Fig. 3A, which shows a substrate ratio of 3:1 and no co-consumption, at high substrate ratios of at least 5:1, there was combined growth on both methanol and formate together, implying co-consumption (Fig. 3B). However, the resulting co-consumption uptake ratio was *ca.* 1:1, much lower than the available substrate ratio of
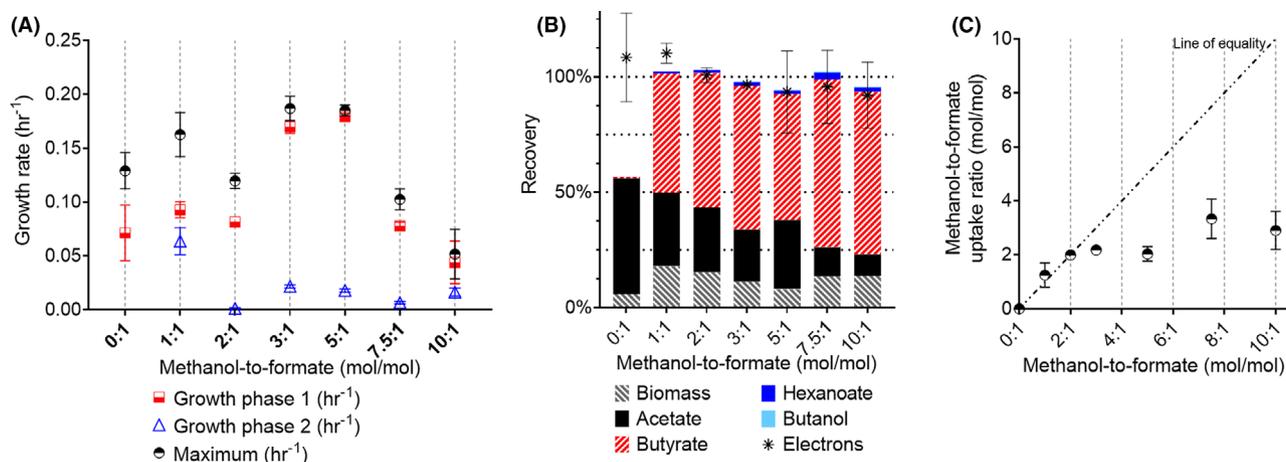
Fig. 2. Investigation of optimum substrate ratio of methanol:formate. (A) Growth rate showing an optimum around 5:1. (B) Product spectrum, carbon and electron recoveries, showing reduced products are favoured at higher ratios. (C) Overall uptake ratios averaged across batch fermentations for different initial substrate ratios of methanol-to-formate. Error bars indicate standard deviation. Optical density (OD) measurements were taken at 600 nm via a UV-Vis spectrophotometer (Thermo Fisher Scientific Genesys 10S UV-Vis Spectrophotometer, USA). pH measurements were taken with B-712 LAQUAtwin Compact pH metre. A biomass formula of $C_4H_7O_2N_{0.6}$ and 0.32 gDCW l^{-1} OD^{-1} (data not shown) used to convert OD to molar cell concentrations. Liquid samples were analysed by high-performance liquid chromatography (using an Agilent 1200 HPLC System with Phenomenex Rezex RHM-Monosaccharide H+ column (7.8 × 300 mm, PN: OOH-0132-KO) and guard column (Phenomenex SecurityGuard Carbo-H, PN: AJO-4490). Analytes were eluted isocratically with 4 mM H_2SO_4 at 0.6 ml min^{-1} for 48 min and column oven temperature of 65°C. 30 μl of sample was injected and monitored using UV/Vis detector (210 nm) and RID at positive polarity and 40°C).

Table 1. Thermodynamic information for methylotrophic and formatotrophic acetogen growth, excluding biomass. All ΔG values are calculated for reactants and products in the aqueous phase according to (Flamholz *et al.*, 2012) using eQuilibrator.

	$\Delta G'_m$ (kJ/mol) [25°C, pH 6]			
Reaction	kJ/mol	kJ/mol-e$^-$	mol-C	Eq.
Methanol + formic acid → acetic acid + H_2O	−66.0 ± 6.9	−8.25 ± 0.86	2	1
3 methanol + formic acid → n-butyric acid + 3 H_2O	−141.7 ± 18.8	−7.09 ± 0.94	4	2
5 methanol + formic acid → n-hexanoic acid + 5 H_2O	−209.0 ± 29.9	−6.53 ± 0.93	6	3
4 methanol → n-butanol + 3 H_2O	−133.6 ± 23.9	−5.57 ± 1.0	4	4
4 formic acid → acetic acid + 2 H_2O + 2 CO_2	−116.3 ± 16.0	−14.5 ± 2.0	4	5

5:1. This would suggest acetate be the main product (Table 1, Eq. 1); however, butyrate was observed in significant amounts. Later, in the second growth phase, we found even higher butyrate specificity despite the significantly slower growth rate (Fig. 2A, Fig. 3B). At this point, the fermentation is unbalanced because an oxidized co-substrate is required for methylotrophic growth (Lebloas *et al.*, 1996) – the exception to this being butanol production (Table 1, Eq. 4).

Whilst butanol has been demonstrated here, significant specificity remains elusive, and so there must be an accumulation of reducing equivalents which cells balance elsewhere. This is also supported by the electron balance, which tends to decrease with increasing substrate ratio (Fig. 2B). We hypothesize the reducing equivalent balance may be through an internal storage mechanism balancing between these two growth phases, as suggested previously for growth under CO_2 limitations (Loubiere and Lindley, 1991). Furthermore, we noted differences in growth rate for each of the substrate ratios in the second phase of growth, despite only methanol being present, suggesting a difference in metabolism triggered by the first phase of growth (Fig. 2A). The data suggest that butanol production is a product of overflow metabolism, similar to 2,3-butanediol production in other acetogens (Köpke *et al.*, 2011).

This assessment does not consider reaction kinetics. In fact, the kinase enzyme in *E. limosum* is known to have almost 40% higher specificity for butyrate over acetate (Lindley *et al.*, 1987). However, it does illustrate the importance of how substrate ratios can manipulate uptake ratios and hence overall fermentation balance. To improve specificity, methanol uptake must be improved relative to formate uptake, particularly during co-consumption.
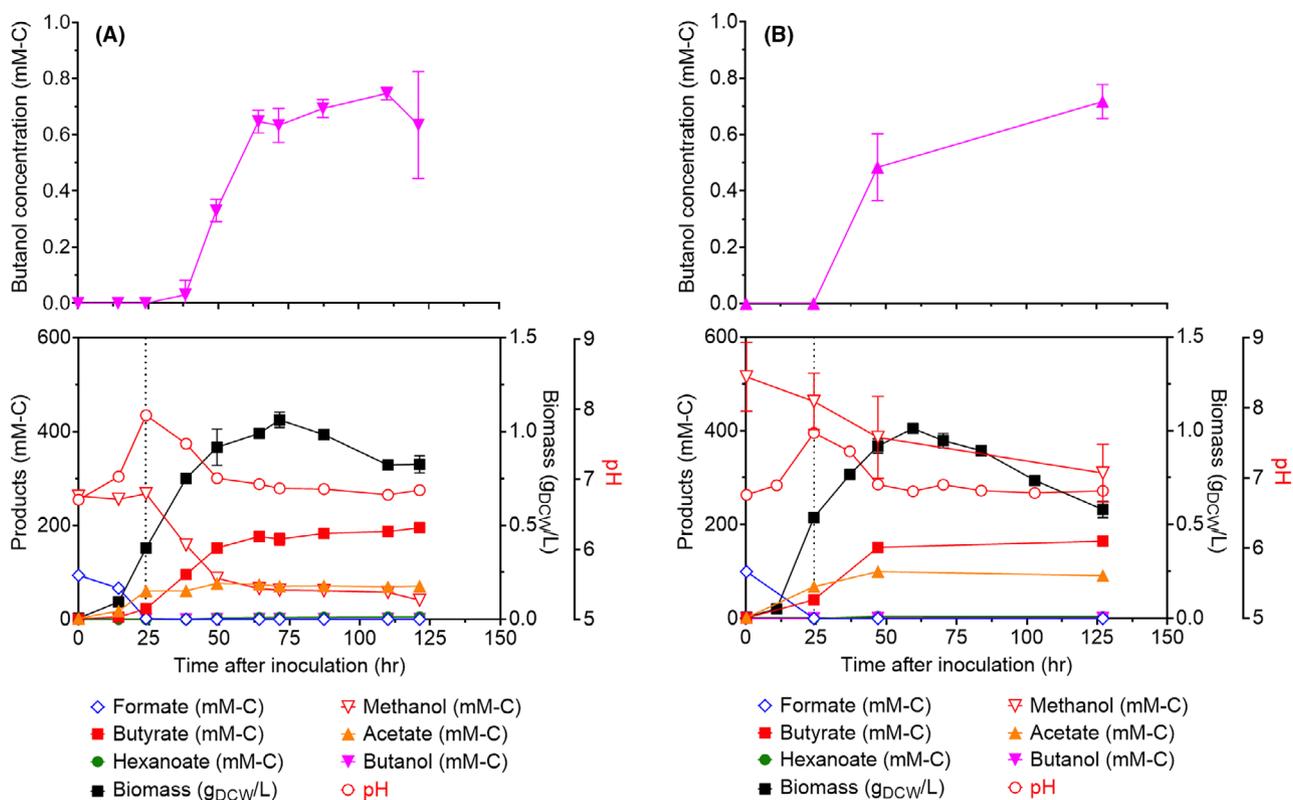
Fig. 3. Time series data, showing butanol (top panel) is favoured during late exponential growth for (A) substrate ratio of 3:1 methanol-to-formate and (B) substrate ratio of 5:1 methanol-to-formate. Vertical dashed line indicates the transition between growth phases after formate is consumed. Error bars indicate standard deviation.

Methanol in the Wood-Ljungdahl pathway

Methanol provides six reducing equivalents per C-mol whilst formate provides two. As such, it is not surprising that methanol is the key substrate to drive the product spectrum towards more reduced, longer carbon chain products. There are however several key obstacles to achieving high product specificity. If methanol is consumed faster than formate (or as the sole substrate), this means the methyl branch of the WLP must reverse in order to balance both branches of the WLP and generate acetyl-CoA (Fig. 1), which is thermodynamically challenging (Kremp *et al.*, 2018). Interestingly, this is however when we noted the highest instantaneous specific rate of 220 ± 23 mmol-C gDCW^{-1} day^{-1} butyrate production (immediately after formate was exhausted), (Fig. 3A). One possible explanation is due to the high reducing equivalent and ATP yield from reversing this pathway (Fig. 1). Unfortunately, this may also be the cause of a relatively high carbon flux to biomass observed in methylotrophic fermentation – here about $12 \pm 2.4\%$ (Fig. 2B) compared with 5% typical for acetogen gas fermentations (Heffernan *et al.*, 2020). Using formate as a sole substrate, only $6.0 \pm 1.2\%$ of carbon

went to biomass (Fig. 2B). However, formate resulted in high CO_2 production and acetate was the major product (Table 1, Fig. 2B). Methanol is useful to obtain better value products, which also resulted in higher growth rates observed here of up to 0.19 ± 0.011 h^{-1}, compared with *ca.* 0.05 h^{-1} (range 0.02 to 0.33 h^{-1}) and acetate as the main product for H_2/CO_2 fermentations (Takors *et al.*, 2018). Imposing a nutrient limitation, which has previously been used as a strategy to redirect reducing equivalents to products (e.g. alcohols Phillips *et al.*, 2015; Fernández-Naveira *et al.*, 2017; Norman *et al.*, 2019; Klask *et al.*, 2020) rather than biomass, may reduce this carbon biomass flux, which will be important to achieve economic viability.

Butanol formation

Acetogens can natively hydrogenate carboxylates to alcohols during solventogenesis (Richter *et al.*, 2016). Indeed, this agrees with our observation that butanol production was during late growth phases (Fig. 3). Researchers have attributed alcohol production to electron consumption, low pH, low pCO$_2$ and salt stress (Richter *et al.*, 2016; Blasco-Gómez *et al.*, 2019; Klask *et al.*, 2020). Alcohols

have a higher reducing equivalent requirement than carboxylates, and so are favoured when carbon uptake is maintained but flux to biomass is reduced, as we observed for higher substrate ratios (Fig. 2B). This is similar to observations during nutrient limitations which favour alcohol production by maintaining a given substrate uptake rate whilst reducing biomass production (Phillips *et al.*, 2015; Fernández-Naveira *et al.*, 2017; Norman *et al.*, 2019; Klask *et al.*, 2020).

Table S1 shows a maximum butanol titre of 2.0 $\pm$ 1.1 mM-C (38 $\pm$ 20mg l^{-1}), for a methanol-to-formate substrate ratio of 7.5:1. High methanol-to-formate substrate ratios (e.g. 7.5:1) also produced the highest titre of hexanoate, in fact ca. 5 times more than butanol (mol-C basis) (Table S1). Hexanoate production has been previously reported for *E. limosum* as a way to consume excess reducing equivalents (Song *et al.*, 2018), and therefore, despite the ratio of produced butanol-to-hexanoate increasing with methanol-to-formate substrate ratio (Table S1), additional conditions could be required to target butanol. We expect a higher butanol selectivity would be achieved at a lower pH, particularly since intracellular pH is 0.5 to 1 unit/s higher than the culturing conditions (Lindley *et al.*, 1987). In fact, typical syngas fermentations producing ethanol are run at pH 5 (Heffernan *et al.*, 2020).

Historically, in a closely related strain to *E. limosum*, *Butyribacterium methylotrophicum*, butanol production was suspected via aldehyde dehydrogenase (*ald*) (Grethlein *et al.*, 1991). A recent investigation showed butanol production from methanol with recombinant *E. limosum* strains by introducing the bifunctional acetaldehyde/alcohol dehydrogenase from *Clostridium acetobutylicum* (Flaiz *et al.*, 2021). However, it is now well accepted that alcohols are predominately formed via the promiscuous aldehyde:ferredoxin oxidoreductase (*AOR*) enzyme in acetogens (Diender *et al.*, 2016; Richter *et al.*, 2016; Liew *et al.*, 2017; Valgepea *et al.*, 2017; Greene *et al.*, 2019). *AOR* activity is important to regenerate ferredoxin and maintain redox balance to control metabolic homeostasis (Mahamkali *et al.*, 2020). Further research is required to confirm this metabolism hypothesis in *E. limosum* for butanol production, particularly given methanol likely results in NADH generation. Proteomics on recently renamed *E. callanderi* (formerly *E. limosum* KIST612) did not observe a significant change in expression of *AOR* or *adh* (Kim *et al.*, 2021); however, this may not be the case for a high methanol-to-formate ratio, given we observed butanol production.

Conclusion

Here, we present the first evidence of native butanol production by *E. limosum* ATCC8486 during methylotrophic growth. We varied substrate ratios, showing methanol is the key feedstock driving the product spectrum towards more reduced products such as butyrate and butanol. Whilst increasing the substrate ratio does increase the uptake ratio, a limit is reached around 1:1 when formate is the co-substrate. Butyrate is the major product during methylotrophic fermentations (maximum specific rate of 220 $\pm$ 23 mmol-C gDCW^{-1} day^{-1}). The imbalance between substrate and uptake ratios necessitated biphasic growth whereby methanol is the sole substrate during late growth phases, coinciding with a maximum observed butyrate to butanol conversion (maximum titre of 2.0 $\pm$ 1.1 mM-C). The growth rate during the first of the two phases is *ca.* 0.19 h^{-1} compared with the second phase of ca. 0.02 h^{-1}, indicating both substrates are required for a high growth rate and cell density, and formate is the preferred substrate. Increasing methanol uptake relative to formate is needed to improve product specificity; however, this also results in high fluxes to biomass. We suggest butanol production, as first shown here, may offer a path to reducing biomass flux, and thus improving overall process economics.

Conflict of interest

None declared.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Text S1. Growth conditions.

Table S1. Mean and standard deviation titer for investigated substrate ratios of methanolto-to-formate in triplicate.