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

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**Summary**

Unlike gaseous C₁ feedstocks for acetogenic bacteria, there has been less attention on liquid C₁ 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⁻¹day⁻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 (H₂) 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 H₂), 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 C₁ 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 C₁ 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 C₁ utilization (Espinoza *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 C₁ feedstocks can be produced efficiently from carbon dioxide (CO₂). Methanol can be...
synthesized renewable via direct hydrogenation of CO₂ 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 CO₂ reduction, and the technology is at pre-commercialization stage (Spurgeon and Kumar, 2018). Therefore, using these liquid C₁ feedstocks may provide another viable production alternative for acetogenic cell factories.

Compared with acetogenic gas fermentation using various combinations of gas substrates of CO₂, CO and H₂, less research has focused on methylotrophic and fermentotrophic growth using acetogens. This is interesting since methanol and formate have the highest energetic efficiency of all C₁ 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 CO₂ demand for the carbonyl branch, without net generation of CO₂ (Lindley et al., 1987; Müller, 2019). Formate, on the other hand, is similar to CO metabolism in that there is excess oxidation to CO₂ 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 CO₂ 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 H₂ and CO co-metabolism or CO₂ and H₂, 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 C₁ 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 C₁.

_Eubacterium limosum_ used methanol and formate to primarily produce biomass, butyrate and acetate, with carbon and electron balances closing within 10% (Fig. 2B). Raw data are provided in Table S1. At a ratio of 0:1, that is, when only formate is provided, CO₂ 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).

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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 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 CO₂ 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.
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 ± 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 ± 1.2% of carbon went to biomass (Fig. 2B). However, formate resulted in high CO₂ 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 H₂/CO₂ 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 pCO₂ and salt stress (Richter et al., 2016; Blasco-Gómez et al., 2019; Klask et al., 2020). Alcohols

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.
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-Navéira et al., 2017; Norman et al., 2019; Klask et al., 2020).

Table S1 shows a maximum butanol titre of 2.0 ± 1.1 mM-C (38 ± 20 mg 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 (Heffeman 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 methylotrrophic 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 methylotrrophic fermentations (maximum specific rate of 220 ± 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 ± 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.

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**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 methanol-to-to-formate in triplicate.