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Anaerobic microbial methanol utilization as a one-carbon feedstock

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Acetogens are anaerobic bacteria of special interest in fighting environmental and economic impacts caused by massive carbon emissions that pollute our Earth's atmosphere. These microbes have the unique ability to convert carbon monoxide, carbon dioxide, or methanol into value-added bioproducts. The gas fermentation technology is already at industrial scale and is making use of special acetogens able to produce ethanol natively. Here, we propose a methanol-based bioconversion process using anaerobic methylotrophic acetogens (*Eubacterium limosum or Eubacterium callanderi*) to produce natively only butyrate or butanol, if genetically modified. Therefore, we depict the crucial fermentation parameters and explain the underlying metabolic pathways to steer these biocatalysts towards sole butyrate production. Additionally, the available genetic toolkits are outlined, and the insights gained via system biology approaches are presented. The concept of the suggested bioprocess is only sustainable if green methanol is used as one-carbon feedstock. The use of black or gray methanol would undoubtedly counteract all efforts towards net-zero CO_2 emissions. To meet tightening climate targets and environmental, social, and governance commitments, stakeholders must evaluate a spectrum of low-carbon technologies. The data presented here indicate that biotechnological fermentations can reduce emissions while remaining commercially competitive, and therefore warrant serious consideration for future industrial deployment and investment.

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A methanol-based bioprocess towards sole butyrate

Methanol (CH_3OH) is a colorless, volatile, flammable liquid and is considered a key liquid hydrogen (H_2) energy carrier [1]. Its classification — often indicated by color or label — depends on the sources of hydrogen and carbon dioxide used in its production [1]. Brown and gray methanol refers to a production based on fossil fuels such as coal and gas. Blue methanol relies on captured CO_2 emissions and H_2 from natural gas (CH_4). E-methanol is synthesized using hydrogen from water electrolysis powered by renewable electricity and CO_2 captured from industrial sources or the atmosphere. Biomethanol follows a similar principle but uses organic waste streams, often derived from biomass or municipal solid waste, as a carbon source. Green methanol refers more broadly to methanol produced with both renewable hydrogen and biogenic CO_2, typically sourced from biomass. In general, the technology used for methanol production is available at an industrial scale, and the overall global demand is currently more than 120 million tons per year [1]. The major part of this demand is currently covered by brown and gray methanol, priced at

around 200 USD/ton. Blue and green methanol is still 4 to 7.5 times more expensive, ranging from 800 to 1500 USD/ton [1]. Bio-methanol produced from municipal solid waste and biomass, such as by ENERKEM, falls within a cost range of 320 to 770 USD/ton, approaching cost competitiveness with fossil methanol as technology advances [1].

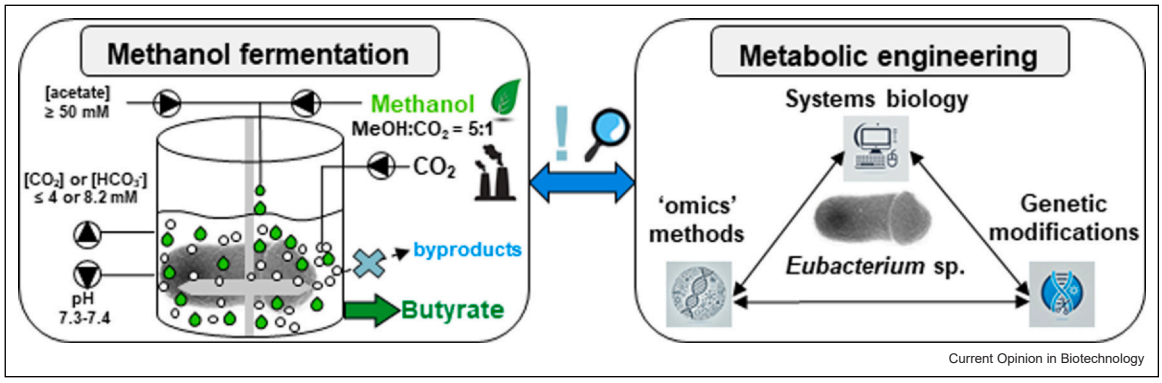
Here, we propose a bioprocess based on methanol as the major feedstock, which is converted anaerobically by bacterial acetogens into butyrate and, in the future, potentially also into butanol (Figure 1). Butyrate and butanol are important industrial platform chemicals and are used for various applications such as solvents, perfumes, and fuel additives. Current prices per ton for butyrate and butanol are approximately 1800 USD/ton and 1400 USD/ton, respectively [2,3]. Using renewable methanol as the feedstock places this bioconversion firmly within environmental, social, and governance (ESG)-aligned strategies while still offering impressive economics. With bio-methanol, gross margins range from 93% to 364%, and even the higher priced blue or green methanol maintains margins of up to 86% (Eq. 1). Although fermenting methanol derived from fossil fuels can boost product value by as much as 640%, prioritizing renewable sources highlights the low-carbon credentials of the process, strengthening its appeal to private and public investors focused on sustainability. Furthermore, conversion to butanol would offer additional market opportunities. By sequestering CO_2 as a co-substrate, this process not only enhances economic returns but also supports circular economy principles, positioning it as a competitive and sustainable alternative to petrochemical routes [4].

$$10\ CH_3OH + 2\ CO_2 \rightarrow 3\ CH_3\text{-}(CH_2)_2\text{-}COOH + 8\ H_2O\ \Delta_rG'^{m} = -417{\cdot}3 \pm 62{\cdot}2\ [kJ/mol] \quad (1)$$

Metabolic and bioreactor constraints

In general, acetogens are famous for their remarkable ability to convert C1-substrates such as CO and CO_2 autotrophically into acetate [5]. Only very few acetogens convert C1-substrates into four-carbon-containing products, whereas acetate production is more common, especially in anaerobic methylotrophic acetogens. Indeed, there are quite a few acetogens that have been initially described to use methanol as substrate for growth, including several species of the genera *Acetobacterium*, *Moorella*, and *Sporomusa* [5], but subsequent studies that determine their respective kinetics are missing. The most prominent and well-characterized methylotrophic acetogens are named *Eubacterium limosum* and *Eubacterium callanderi*, which have a long and interesting history (Box 1). These biocatalytic anaerobic microbes have the remarkable ability to convert high amounts of methanol, together with CO_2, exclusively into butyrate (100%) using highly efficient metabolic pathways [6,7]. However, narrow metabolic constraints and specific bioreactor configurations are needed to force the cells into exclusive butyrate production; otherwise, acetate will be their major metabolic product (Figure 1). The respective pathways are depicted in Figure 2 and focus on the required reducing equivalents NADH (reduced nicotinamide adenine dinucleotide) and reduced Ferredoxin (Fd^{2-}). This methanol metabolism is functioning physiologically equally in both *Eubacterium* strains [8], and the key parameters are the CO_2 and acetate concentrations, as well as the pH present in the required growth medium. These parameters are considered to be crucial to steer butyrate production in a pressurized (1 bar) continuous anaerobic fermentation [6,7]. A 5:1 methanol to CO_2 ratio (Eq. 1) was found in recent studies to be optimal for butyrate production [9]. A study investigating batch methanol fermentations of

Figure 1

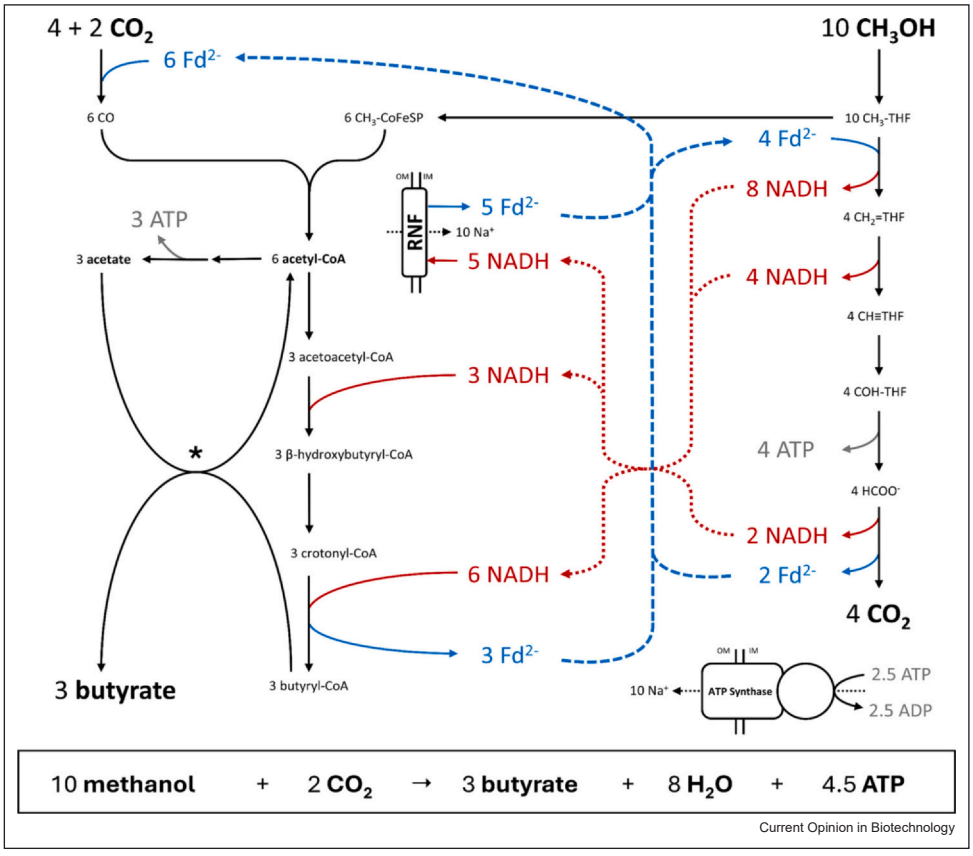


Methanol-based anaerobic bioprocess for sole butyrate production by methylotrophic anaerobic acetogens. The graph illustrates key parameters and constraints required to steer *E. limosum* or *E. callanderi* towards exclusive butyrate production in a methanol-based bioprocess. To improve performance and broaden the product spectrum—e.g., butanol—metabolic engineering strategies, including genetic modification, systems biology, and multi-omics approaches, are already established and actively being pursued in these strains.

Box 1 A brief history of *E. limosum*.

Eubacterium limosum and the closely related *Eubacterium callanderi* share a flustered history, including several reclassifications and contradicting information about methanol metabolism. *Eubacterium limosum* was isolated in 1935 and published as '*Bacteroides limosus*' [15]. Shortly after, in 1938, Prévot proposed the genus *Eubacterium* and deposited the strain in the ATCC strain collection. In the beginning of the 1980s, this strain, which was accepted as type strain in 1983, had fallen into oblivion, however, experienced a resurgence within the last ten years as its genome has been sequenced [16] and was used as a platform acetogen to establish various metabolic engineering tools [17–22]. However, the methanol metabolism, for which *E. limosum* is well known, was elucidated in the 1980s with the strains RF and B2 (both not publicly available), which were isolated shortly after another in 1981 and 1983, respectively [10,23]. The first acetogen described to utilize methanol was *Butyribacterium methylotrophicum* isolated in 1980 [24]. The decision to use *Butyribacterium* as a genus name instead of *Eubacterium* was based on the identification of atypical spore formation, although other features were similar to those described for *E. limosum*. Later studies could not confirm spore formation, and genome comparisons revealed that this strain belongs to the species *E. callanderi*, which was isolated six years after *B. methylotrophicum* [8]. Interestingly, *E. limosum* KIST612, also recently reclassified to be *E. callanderi* KIST612 and often used for genetic engineering approaches and physiology studies, shares 99.9% sequence similarity with *B. methylotrophicum* [8]. Hence, it was proposed to reclassify *B. methylotrophicum* to *E. callanderi*. Noteworthy, the characterization of the *E. callanderi* type strain, isolated in 1986, was puzzling, as it was reported that the strain is unable to convert methanol [25]. Recently, this finding was refuted as it was shown that methanol consumption is similar to other *E. limosum* and *E. callanderi* strains [8]. Despite the flustered history, all strains that belong rather to the species *E. limosum* or *E. callanderi* are capable of converting methanol to produce butyrate.

Figure 2



Schematic overview of methanol conversion to butyrate via the Wood–Ljungdahl pathway in *Eubacterium limosum* centered around electron flow. Stoichiometry is based on the overview given for *E. callanderi* KIST612 with the assumption of a bifurcating methylene-tetrahydrofolate (THF) reductase (MTHFR) [12]. The conversion of butyryl-CoA to butyrate is catalyzed by a CoA-transferase* [14]. Electron carriers NADH and Fd^{2-} are displayed in their reduced state. Methanol enters the Wood–Ljungdahl pathway (WLP) as methyl-THF (CH_3-THF) by transferring its methyl group to THF catalyzed by the methyltransferase system. In a series of oxidation steps, methyl-THF is oxidized to CO_2, releasing electrons that are transferred to the electron carriers NADH and Fd^{2-}. Electrons of NADH can be transferred to Fd via the *R*hodobacter *N*itrogen *F*ixation (RNF) complex, exporting Na^+ across the cell membrane. The required Na^+ potential to drive this electron transfer is achieved by ATP hydrolysis by the ATP synthase. Fd^{2-} is required for the reduction of CO_2 to CO in the carbonyl branch of the WLP and for the oxidation of methyl-THF (CH_3-THF) to methylene-THF (CH_2=THF). CO of the carbonyl branch is coupled to the methyl group of the methyl branch of the WLP to form the central intermediate acetyl-CoA. In a series of further reductions, acetyl-CoA is converted to butyrate with the electrons of NADH generated during methanol oxidation. Energy is conserved in the form of ATP during methanol oxidation in the methyl-branch and during acetate production. With the assumption of a bifurcating MTHFR, 1.5 mol ATP can be produced per mol butyrate.

E. callanderi (DSM 3468) showed that acetate production or its consumption is dependent on the initial CO_2 and acetate concentrations at the start of the batch process [6]. Thus, CO_2 levels below 14 mM and acetate around 50 mM resulted in stable acetate levels and a sole butyrate production (Figure 1). However, lower acetate (10 mM) levels with the same CO_2 amount led to net acetate production and omitted butyrate production. In contrast, limited CO_2 (4 mM) levels in the fermentation medium with higher acetate (50 mM) even enhanced acetate consumption and yielded only butyrate as a product. Growth on methanol and CO_2 without additional acetate showed an initial lag period of 3 to 5 days, underscoring the essential role of CO_2 as a co-substrate during methanol fermentation [6]. Notably, Genthner and coworkers showed that *E. limosum* (DSM 20543) is unable to grow on methanol alone if only N_2 is present in the gas phase, suggesting that CO_2 derived solely from methanol oxidation is insufficient to support growth under these conditions [10]. Complementary findings for *E. limosum* B2 highlight how different forms of CO_2, specifically hydrogen carbonate (HCO_3^-), influence its metabolism [7]. Optimal growth occurs at a neutral pH (7.3–7.4), where CO_2 serves as an essential co-substrate. Under these conditions, dissolved CO_2 equilibrates with water to form bicarbonate (HCO_3^-), which is the primary inorganic carbon species taken up and metabolized by the microbes. High HCO_3^- levels promote acetate production, while lower HCO_3^- levels favor butyrate synthesis, and if HCO_3^- is limited below 0.5 g/L (8.2 mM), butyrate is produced exclusively (Eq. 1) [7]. Eq. 2 represents the carbon and oxygen balance for methanol and CO_2 conversion into acetate and butyrate under nonlimited conditions. The necessity to limit HCO_3^- is also reflected by the free Gibbs energy ($\Delta_rG'^m$) given for both overall reaction schemes (see Eqs. 1 and 2). Under nonlimited conditions, cells are producing acetate because it is thermodynamically much more favorable compared to sole butyrate production [4].

$$10\ CH_3OH + 2\ CO_2 \rightarrow 4{\cdot}5\ CH_3\text{-}COOH + 0{\cdot}75\ CH_3\text{-}(CH_2)_2\text{-}COOH + 3{\cdot}5\ H_2O\ \ \Delta_rG'^m = -856{\cdot}5 \pm 59{\cdot}9\ [kJ/mol] \quad (2)$$

The challenge is to translate these insights into a continuous fermentation process having a constant methanol feeding rate adjusted to the growth rate of microbes while keeping the critical range of CO_2/HCO_3^- concentrations. Already determined methanol consumption rates and growth rates are listed in Table 1. The methanol:CO_2 ratio needs to be kept at 5:1 throughout a continuous fermentation process. Keeping that low CO_2 concentrations could be technically difficult as bioreactors are usually kept anaerobically using N_2 as stripping gas, which also strips CO_2 out of the bioreactor.

The physiological details of anaerobic methylotrophic methanol consumption by acetogens have been explored by the group of Volker Müller using *Acetobacterium woodii* and *E. callanderi* KIST612 [11,12]. In short, methanol enters the methyl-branch of the Wood–Ljungdahl pathway due to the action of a methyltransferase enzyme complex as methyltetrahydrofolate (CH_3-THF). A certain amount is oxidized by the enzymes of that methyl-branch towards CO_2, providing NADH and ATP. Another part of the methanol is reduced and drives an important reaction of the Wood–Ljungdahl pathway, which is catalyzed by the enzyme complex carbon monoxide dehydrogenase/acetyl-CoA synthase. This complex enables several reactions by fusing the methyl group with CO, resulting in the formation of the central intermediate acetyl-CoA. This serves as a key building block in various biosynthetic pathways for the synthesis of cellular components such as lipids, proteins, and

Table 1

Maximum growth rate and methanol consumption rates of acetogens.

Strain	Maximum growth rate	MeOH consumption rate	Condition	Reference
A. woodii mutant	n.d.	0.3 mM h^{-1}	bottle scale	[26]
C. luticellarii DSM 29923	n.d.	0.66 ± 0.03 g[a] L^{-1}[a]d^{-1} (0.85 mM h^{-1})	bottle scale	[27]
Eubacterium limosum DSM 20543^T (ATCC 8486)	0.07 h^{-1}	6.06 mM h^{-1}	bottle scale	[8]
Eubacterium callanderi DSM 3662^T	0.06 h^{-1}	6.55 mM h^{-1}	bottle scale	[8]
Eubacterium callanderi DSM 3468	0.07 h^{-1}	6.01 mM h^{-1}	bottle scale	[8]
Eubacterium callanderi KIST612	0.07 h^{-1}	4.90 mM h^{-1}	bottle scale	[8]
Eubacterium maltosivorans DSM 20517	0.02 h^{-1}	2.01 mM h^{-1}	bottle scale	[8]
Eubacterium limosum ATCC 8486 (DSM 20543^T)	specific growth rate of 0.017 h^{-1}	75 ± 7.0 mmol[a]$gDCW^{-1}$[a]d^{-1} (1 mM h^{-1})	chemostats	[28]
Eubacterium limosum B2	0.075 h^{-1}	72 mmol[a]gDCW–1[a]d^{-1}	chemostats	[29]
E. callanderi DSM 3468	~0.025h^{-1}[a]	~ 0.8 mM $^{h-1}$[a]	bottle scale	[24]
E. callanderi DSM 3468	~0.02h^{-1}[a]	~ 1.2 mM h^{-1}[a]	bottle scale	[6]

[a] Rates were calculated based on the provided figures in the respective publications.

nucleic acids. In *A. woodii*, acetyl-CoA is mainly converted into acetate, which is excreted as a metabolic end product [9]. In *E. callanderi*, acetyl-CoA is further converted into acetate and butyrate, while reducing equivalents are balanced via the RNF complex [13]. The previously mentioned requirement for acetate arises from its role in ATP formation and in the conversion of butyryl-CoA to butyrate, during which the CoA moiety is transferred to acetate, forming acetyl-CoA (Figure 1). This reaction is catalyzed by a butyryl-CoA:acetate CoA-transferase, whose physiological activity was proven by Oh and coworkers [14]. Since a constant level of acetate is required to drive this enzyme, a downstream product separation is needed to purify the butyrate.

Genetic engineering toolkits for *E. limosum* and *E. callanderi*

The genetic toolkits for both *Eubacterium* species have expanded significantly in recent years. The first replicating plasmids and selectable markers were reported for the *E. limosum* type strain (ATCC 8486) in 2018 [30], followed by an inducible promoter and a plasmid-based CRISPR-Cas9 system for genome engineering in 2019 [31]. Similar tools were reported soon after for the KIST612 strain [32], and fluorescent reporters compatible with anaerobic conditions based on the FAST (Fluorescence-Activating and absorption-Shifting Tag) system have also been developed [33].

Two research groups reported a dramatically expanded set of foundational tools, including new antibiotic resistance markers, well-characterized libraries of promoters and terminators, and more efficient electrocompetent cell preparation and transformation procedures [34,35]. This work also revealed that *E. limosum* is sufficiently aerotolerant to permit electroporation on the benchtop (outside an anaerobic chamber) with only modest loss of efficiency [34]. *E. limosum* also appears to have a more efficient homologous recombination machinery than other acetogens, permitting the generation of knockouts using linear PCR products comprising 1 KB homology arms and an antibiotic resistance cassette [18]. Integration of large genetic constructs (> 10 KB) was recently reported by adapting the Chassis-Independent Recombinase-Assisted Genome Engineering (CRAGE) workflow [36]. Here, a landing pad (LP) containing Cre recombinase and orthogonal *lox* sites is integrated into the genome. Subsequent transformation of the cargo DNA flanked by the same *lox* sites results in highly efficient integration into the LP. [15].

Two methodologies have recently been developed for making precise genome modifications without the incorporation of selectable markers. The first uses an inducible counter-selectable marker based on a toxin-antitoxin system and a non-replicating integrative plasmid carrying the desired mutation and homology arms [16]. The second is a recombineering approach that uses a highly efficient RecT recombinase derived from an *E. limosum* phage to integrate the desired mutations encoded on a DNA oligonucleotide with short (40 bp) overhangs, with Cas9 counterselection [22]. The former is attractive because it can be targeted anywhere in the genome, whereas the latter requires a nearby PAM site to be mutated. The advantage of the latter is encoding of homology arms and desired mutations on oligonucleotides, which avoids tedious cloning, hastening the process and allowing for the use of degenerate libraries to multiplex mutations. Finally, a CRISPRi system has been developed for *E. limosum* and was recently used in a landmark study to examine the effects of genome-wide targeted downregulation on growth, identifying several novel determinants of autotrophic growth rate [17].

Collectively, these developments will facilitate the development of an acetogenic methanol-based bioprocess by enabling metabolic engineering efforts aimed at enhancing methanol conversion rates and extending the pathway to butanol. Indeed, recombinant butanol formation at very low levels has already been achieved via plasmid-based expression of *adhE1* originating from *Clostridium autoethanogenum* [14] or *adhE2* from *Clostridium acetobutylicum* [33], both encoding a bifunctional acetaldehyde/alcohol dehydrogenase (ADH) for converting butyryl-CoA to butanol. The production metrics still require improvement, and the high-efficiency tools developed in the last few years could speed this effort. For example, recombineering could be used for high-throughput screening of ribosome-binding sites to balance levels of pathway enzymes to maximize pathway flux. Facile genome integration could provide stable expression of heterologous genes, and/or exploration of alternative pathway variants, such as the use of aldehyde ferredoxin oxidoreductase to convert butyrate to butyraldehyde, followed by ADH for further reduction to butanol. Regarding methanol metabolism, an intriguing approach recently proposed would be to examine alternative Wood–Ljungdahl pathway genes from different acetogens with different co-factor specificity, potentially resulting in higher ATP yield and/or more optimal distribution of thermodynamic driving force across the pathway [37]. Overall, the advanced tools now available for these strains will speed these metabolic engineering endeavors.

System Biology for *E. limosum* and *E. callanderi*

The advent of 'omics' technologies has revolutionized the study of bacterial metabolism, providing an extensive dataset on molecular components such as

metabolites, transcripts, and proteins. These advancements have enabled a Systems Biology approach, offering a holistic framework to uncover the mechanisms underlying specific phenotypes and cellular functions. A significant challenge in this field, however, is the construction of reliable networks that integrate diverse omics data into a unified metabolic framework, with the metabolic network serving as a chassis. Decades of research on the biochemical characterization of metabolic reactions, including enzymatic activity assays, genome sequencing, transcriptomics, and proteomics, have facilitated the development of genome-scale models (GSMs) of metabolism. These models mathematically represent the metabolic reactions occurring within cells and enable the calculation of reaction fluxes [38,39].

In the context of autotrophic growth, a Systems Biology analysis was performed on *E. limosum* ATCC 8486 [30]. This analysis combined GSM to RNA sequencing (RNA-Seq) to study both the transcriptome and the translatome by ribosome profiling (Ribo-Seq). Ribo-Seq, which sequences ribosome-protected mRNA fragments during translation, provides insights into the translation efficiency of individual mRNAs. Compared to proteomics, which often faces challenges such as the difficulty of extracting membrane-bound proteins, Ribo-Seq offers greater accuracy [40]. The study of Song et al. on *E. limosum* revealed the critical role of the 5′ untranslated region (5′UTR) in translation efficiency. The findings suggest that the 5′UTR may regulate translation under specific conditions, such as autotrophy, where energy resources are limited. Interestingly, while transcript levels for the carbonyl branch of the WLP and energy conservation systems (e.g. the RNF complex and ATPase) were elevated under autotrophic conditions, translation efficiency was reduced. This Systems Biology approach uncovered hidden regulatory mechanisms influencing metabolic responses [30].

Similarly, a Systems Biology study on *E. callanderi* KIST612 investigated metabolic fluxes and proteomic changes under methylotrophic and autotrophic conditions [41]. Despite comparable proteomic fold changes for most WLP enzymes and energy conservation systems across both conditions, significant phenotypic differences were observed. Specifically, growth rates and H_2 consumption rates were 4- and 2.7-fold higher under methylotrophic conditions. These phenotypic differences could not be fully explained by variations in central carbon metabolism or energy conservation protein abundance, highlighting the need for a broader perspective that integrates transcriptomics, proteomics, fluxomics, and other omics data to comprehensively elucidate metabolic regulation [41].

More recently, a Systems Biology approach was employed using chemostat cultures to analyze the heterotrophic and methylotrophic metabolism of *E. limosum* B2 [42]. This study combined metabolic flux analysis with the absolute quantification of mRNA and proteins. By doing so, it enabled genome-scale characterization of ribosome-binding site strengths as well as the strengths of all promoters under heterotrophic and methylotrophic conditions, providing highly valuable parts for metabolic engineering studies. Additionally, k_{app} values (*in vivo* turnover numbers) for most central metabolic enzymes were determined, offering key insights into potential rate-limiting steps under heterotrophic and autotrophic conditions. Notably, the methyltransferase complex was identified as the primary bottleneck in methanol assimilation by *E. limosum* B2.

Conclusion

Both methylotrophic acetogens, *E. limosum* and *E. callanderi*, show potential for butyrate and recombinant butanol production using methanol as a primary substrate. While sole butyrate production already seems feasible in a scaled-up fermentative process, sufficient butanol production needs further research. Despite the well-elucidated physiology of both *Eubacterium* species and reports of both native and recombinant butanol production, achieving higher yields remains a challenge. However, combining the discussed state-of-the-art genetic engineering tools, system biology approaches, including available omics data, and a fine-tuned fermentation process might generate an efficient butanol-producing biocatalyst.

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CRediT authorship contribution statement

CSL: Writing – review & editing, Writing – original draft, Table conceptualization, Data curation. **MF:** Writing – review & editing, Writing – original draft, Figure conceptualization, Data curation. **BW:** Writing – review & editing, Writing – original draft, Data curation. **PS:** Writing – review & editing, Writing – original draft, Data curation. **FRB:** Writing – review & editing, Writing – original draft, Validation, Supervision, Project administration, Investigation, Formal analysis, Conceptualization.

Data Availability

No data were used for the research described in the article.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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- • of special interest
- •• of outstanding interest

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