Achieving net zero CO_2 emission in the biobased production of reduced platform chemicals using defined co-feeding of methanol

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Abstract

Next generation bioprocesses of a future bio-based economy will rely on a flexible mix of readily available feedstocks. Renewable energy can be used to generate sustainable CO_2-derived substrates. Metabolic engineering already enables the functional implementation of different pathways for the assimilation of C1 substrates in various microorganisms. In addition to feedstocks, the benchmark for all future bioprocesses will be sustainability, including the avoidance of CO_2 emissions. Here we review recent advances in the utilization of C1-compounds from different perspectives, considering both strain- and bioprocess engineering technologies. In particular, we evaluate methanol as co-feed for enabling CO_2 emission-free production of acetyl-CoA-derived compounds. Possible metabolic strategies are analyzed using stoichiometric modeling combined with thermodynamic analysis and prospects on industrial-scale implementation are discussed.

Background

Reaching the defined sustainability goals encourages industry and individuals to find sustainable solutions and reduce greenhouse gas emissions in all sectors. Biotechnological production processes use renewable feedstocks and are expected to drive the transition to a sustainable, biobased economy with a lower or zero carbon footprint. Currently, bioprocesses rely on rather defined feedstocks from the first or second generation, which are rich in sugars as these are the preferred substrates of current industrial microorganisms [1,2]. While based on a renewable feedstock, CO_2 is released during aerobic as well as anaerobic processes, mostly because of a degree of reduction or energy imbalance. Rare exceptions of products with complete carbon conservation and redox, energy balanced pathways are lactate and 3-hydroxypropionic acid.

CO2 emission free production is desirable for several reasons. CO2 emissions have been identified as a main contributor for global warming and climate change, whose social, economic and ecological consequences are both unpredictable and potentially catastrophic. Additionally, with no CO2 emissions, full carbon conservation and thus resource efficiency is achieved, i.e. better use of raw materials and reduction in land use. The latter is further improved using second-generation agricultural feedstock. Next to ecological motivations, there are economic drivers for CO2 emission free processes. Government across the world are pushing for a CO2 free economy and taxes to CO2 emissions have been introduced and are expected to be increased in the future. Thus, a CO2 emissions free bioprocess will also be competitive by no to very low CO2 taxation.

A potential solution for this problematic is the feeding of co-substrates to “boost” energy or reduction equivalents [2]. An electron deficit can be solved by feeding additional electrons and applying non-oxidative pathways. For example, the product yield of ethanol from glucose can be increased by 50% when non-oxidative glycolysis is used in combination with H_2 gas feed [3].

Such high-energy co-substrates can be gained from (electrochemical) CO_2 regeneration from the off-gas stream. Van Winden and coworkers used the off-gas CO_2 to generate formiate that was fed to the bioprocess and increased the glucose-based biomass yield of *Yarrowia lipolytica* by 20% [4]. For the future it is expected that excess renewable energy will be available to generate CO_2-derived reduced carbon substrates at scale. In addition to being a sustainable, renewable source, such feedstock could enable fully CO_2emission-free bioprocesses even without recycling loops or additional measures like explosion protection due to dangerous gas-mixtures.

Here, we explore the opportunities for net-zero CO_2 production of several, reduced compounds by co-feeding methanol as a reduced carbon source. The co-feeding of methanol and sugars has been reported in previous efforts for achieving methanol auxotrophic strains [5-7] ; in this article, we aim to expand the scope by taking in consideration CO2 emissions, as well as carbon and electron efficiency.

Methanol was chosen as it is liquid, well soluble, pH neutral and industrially-relevant hosts have a high tolerance to this alcohol. Renewable methanol (also known as green methanol) can be obtained from several different sources: (1) oxidation of methane from biogas plants using carbon waste streams, (2) reduction of CO2 in industrial waste streams using green H_2 (first commercial plants have been established, for example Carbon Recycling International (CRI) based in Reykjavik, Iceland). (3) potentially from direct electrochemical reduction of CO2 to methanol, although a process suitable for commercial applications is yet to be developed [8,9]

Green methanol as substrate is not yet economically viable when compared to methanol from fossil origin (also known as grey methanol). However, with the rapid developments of green technologies, the production costs will lower. At the same time the pressure from regulators, with increasing tax burden in carbon emissions, may eventually tip the balance in favour of green methanol.

The guiding concept of our analysis is to use a second generation agricultural feedstock containing glucose or xylose together with methanol as a booster, such that full carbon conservation is achieved and the bioprocess reaches net zero CO2 emissions. Theoretically, methanol alone could achieve emission-free production of reduced compounds. Nevertheless, this approach leads to a loss of electrons and thus decreased efficiency. The degree reduction of methanol is higher than the products discussed here. Furthermore, due to the multi-carbon nature of sugars, a lower amount of C-C bonds, which are energetically costly, have to be formed, leading to an increase in energy yields overall.

Following the formulated concept of sustainable metabolic engineering (SME) we apply flux balance analysis to determine the relevant pathway combination(s) and choose substrate mixtures that are resource efficient [10]. In a second step, the putative flux distributions are evaluated with respect to the thermodynamic feasibilities [11,12]

Reduced chemicals with importance for different sectors were chosen as products: polyhydroxybutyric acid (PHB) as a model biopolymer, noreugenin for compounds derived from polyketides, and butyric acid methyl ester (BAME) for products derived from fatty acids. These precursors have applications in food, pharma as well as biofuels, partly after modifications. The product pathways are short and demonstrated functional under various conditions.

Such pathways can be easily introduced in industrial hosts thanks to recently developed powerful genome editing tools like CRISPR-Cas9 and user-friendly cloning tools, creating custom microbial cell factories in a short time [13,14], although not without challenges such as the potential generation of cytotoxic intermediates [15]. For example, engineered yeast variants have successfully synthesized pharmaceutical precursors through complex biosynthetic pathways with numerous genetic edits [16].

Methanol assimilation pathways

Currently, there are four known, natural pathways for assimilation of methanol in methylotrophic bacteria and yeasts: (1) The ribulose monophosphate (RuMP) cycle of β- and γ-proteobacteria [17], (2) the serine cycle [18], (3) the ribulose biphosphate (RuBP) cycle, found in α-proteobacteria [19] and (4) the xylulose monophosphate cycle (XuMP) exclusively found in yeasts [20].

All pathways have a tight link with a glycolytic or TCA cycle intermediate (Figure 1). The RuMP cycle generates fructose-6-phosphate (F6P) via the combination of formaldehyde and ribulose-5-phosphate (Ru5P) by the enzymes hexulose-6-phosphate synthase (HPS) and hexulose-6-phosphate isomerase (PHI) [21-24]. The Serine cycle is fed by formate and produces acetyl coenzyme A (AcCoA) that is interconnected with the ethyl malonyl-CoA cycle and the TCA cycle [18,25].

In addition to the native pathways, several synthetic pathways with specific properties have been developed. The modified serine cycle [26] features a simplified oxidation of formaldehyde to formate using formaldehyde dehydrogenase and prevents the use of the enzyme Hpr in *E.coli*, which converts glyoxylate to glycolate faster than it converts hydroxypyruvate to glycerate. Researchers transaminated glyoxylate with alanine to produce glycine and converted serine to pyruvate using serine dehydratase, thus avoiding hydroxypyruvate as an intermediate. However, there is a high energy cost of 3 ATP molecules per molecule of AcCoA. Another variant is the serine-threonine cycle [27,28], which regenerates glycine using the threonine biosynthesis and cleavage system; here, only one non-native enzyme has to be introduced in *E. coli*. A more energy efficient variant is the homoserine cycle based on two promiscuous formaldehyde-condensing aldolase reactions that form AcCoA from two molecules formaldehyde [29].

The DAS Pathway uses the enzyme dihydroxyacetone synthase from *Pichia angusta* to combine Xu5P and formaldehyde to produce GA3P and DHA [30].

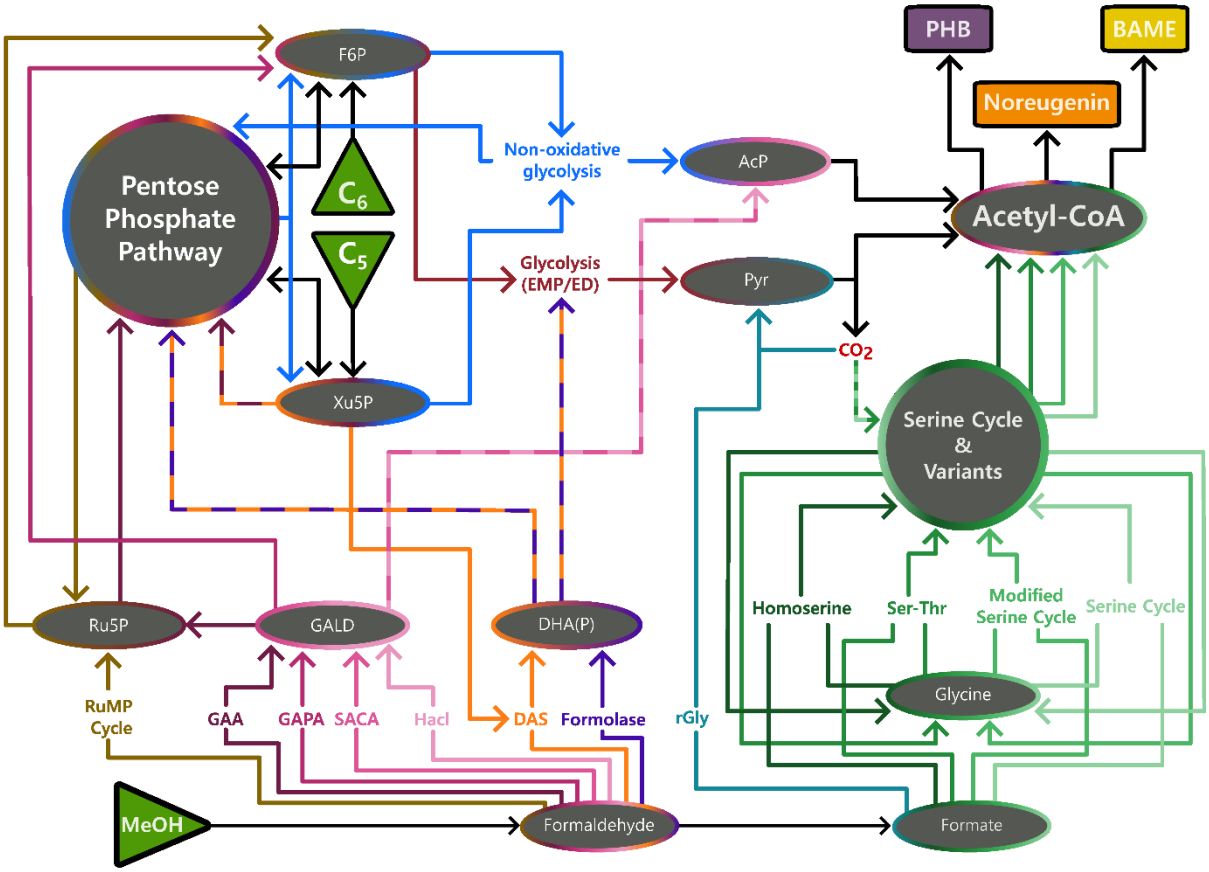

Figure 1: Schematic overview of selected methanol assimilation pathways. The green and grey triangles represent the chosen substrates, key intermediates and metabolic cycles, respectively. Multi-colored lines indicate identical steps in different pathways. Details for all reactions and metabolites of the pathways can be found in the supplementary information (S1). A complete graphical network representation is available in S2. AcP: Acetyl-Phosphate, DHA(P): Dihydroxyacetone (phosphate), F6P: Fructose-6-phosphate, GALD: Glycolaldehyde, MeOH: Methanol, Ru5P: Ribulose-5-phosphate, Pyr: Pyruvate, Xu5P: Xylulose-5-phosphate.

So far, the pathways described are cyclic and require formaldehyde acceptor regeneration, which can be challenging [31]. To avoid recycling, several linear pathways have been developed. The reductive glycine pathway (rGly) was designed [32,33] for the incorporation of formate and/or CO_2. The pathway is a combination of the tetrahydrofolate system, the glycine cleavage system, and the serine hydroxymethyltransferase and deaminase. This

combination allows for a higher biomass yield, all reactions are thermodynamically feasible and there were no hurdles for the heterologous expression. Here, two ATP are required for the assimilation of formate (resp. methanol).

Other synthetic linear pathways are based on non-natural reactions. (1) The formolase pathway uses the computationally designed enzyme formolase, with catalyzes the carboligation of three formaldehyde molecules into one DHA molecule [34]. This pathway requires only five steps that are thermodynamically feasible. (2) The Synthetic AcCoA pathway (SACA) [35] produces AcCoA in three steps. First, two formaldehyde molecules are condensed to glycolaldehyde (GALD) by an engineered glycolaldehyde synthase. Then GALD reacts to acetylphosphate (AcP) using a phosphoketolase and in the last step, AcP is transformed to AcCoA. The authors claim this pathway to have 100% carbon conservations and a very high Max-min Optimized Driving-Force (MDF) of 26.9 kJ/mol. (3) The HACL pathway [36] is based on the enzyme 2-hydroxyacyl CoA lyase (HACL), which catalyses the conversion of formaldehyde with formyl-CoA to glycolyl-CoA. In combination with an acyl-CoA reductase, different compounds are produced, such as AcP or glycolate. (4) The GAA and GAPA pathways [37,38] uses a modified aldolase reactions and glyceraldehyde synthetase to convert two molecules of formaldehyde into one molecule of glyceraldehyde. Further details of the different methanol degradation pathways stoichiometries can be found in the comprehensive review of [39] and the supplementary materials.

Sugar metabolization pathways

For the catabolism of C5 and C6 sugars several different glycolytic pathways were analyzed. Firstly, we included the Embden-Meyerhof-Parnas (EMP) pathway and the Entner-Doudoroff due to their ubiquitousness amongst living organisms which also show a high ATP-yield (2 resp. 1 ATP per glucose) [40]. However, the final product of these two pathways is pyruvate. The conversion to acetyl-CoA, the precursor of the analyzed products leads to the release of CO_2, leading to a significant loss (33%) in carbon efficiency.

While this could be mitigated using previously discussed methanol assimilating pathways fixating CO2 (for example the serine cycle or the rGly pathway) we included the non-oxidative glycolysis introduced by Bogorad and colleagues [41]. This pathway avoids the release of CO2, which ensures that all carbon is captured in the product as potentially not all CO2 can be captured by CO2 fixation pathways. The non-oxidative pathway uses the enzyme phosphoketolase from *Bifidobacterium adolescentis*, which transforms one C6 molecule into three molecules of acetyl-phosphate (AcP). AcP is later converted to acetyl-CoA thanks to the enzyme phosphate acetyltranspherase from *Bacillus subtilis*. Clearly, without oxidation there is an energetic cost, one ATP is required for the formation of three AcP molecules. Nevertheless, the implementation of the non-oxidative glycolysis enabled higher yield conversions for various products, including ethanol or farnesene [42].

Stoichiometric and thermodynamic analysis

Most of the above pathways have been discussed and analyzed in earlier reviews [43,44], with focus on precursor and biomass synthesis as well as energy efficiency [31] [45]. A combination with sugar-based feedstocks and product pathways however, is not yet available.

Here, we perform a stoichiometric and thermodynamic analysis under comparable constraints for all putative combinations and couple the analysis to product synthesis (Figure 2). Therefore a stoichiometric model with reactions from the central carbon metabolism was taken and extended with selected methanol degradation and assimilation pathways. For methanol assimilation, we included known native pathways such as the RuMP cycle and the serine cycle as well as currently discussed synthetic pathways such as the homoserine and the reductive glycine pathway [45-47] (see Figure 1). Furthermore selected product pathways and non-oxidative glycolysis were included. Details on the networks and applied constraints can be found in the supplementary information and via github.

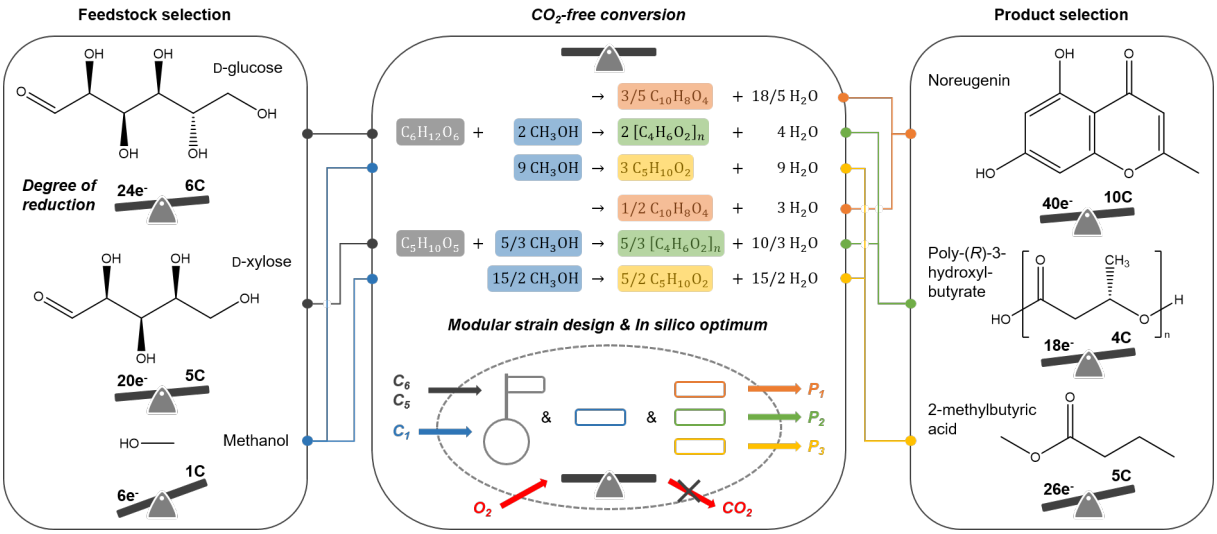

Figure 2: Reaction design for the CO_2 emission-free conversion of mixtures of glucose or xylose and methanol for production of selected AcCoA-derived compounds. With optimal chemical conversion, the depicted maximum molar product yields from the C6 or C5 sugars can be achieved, while the imbalance in the degree of reduction is resolved by appropriate addition of methanol. For an optimal biochemical conversion, the proper choice of the methanol assimilation pathway is key to meet additional co-factor and thermodynamic conditions.

In a first step, all combinations were tested for maximal product yield using parsimonious flux balance analysis (pFBA) [48], constraining CO_2 emissions to zero and allowing free substrate mixtures of glucose or xylose in combination with methanol. For thermodynamic analysis, the values for Max-min Driving Force (MDF) were computed for each pathway combination achieving 100% carbon conservation in the previous analysis. The Max-min Driving Force (MDF) is a measure of the thermodynamic favorability of a pathway under physiological conditions[49]. It represents the smallest value of -ΔG' among all pathway reactions, achieved by optimizing metabolite concentrations to maximize the favorability of each reaction (-ΔG' as positive as possible). For MDF calculations,the following assumptions on substrate concentrations were made: *i)* for methanol, a high concentration of 1 mol/L was allowed. Methanol is a water-free substrate, allowing for addition without increasing the aqueous volume in a fed-batch or comparable setting; *ii*) for xylose and glucose, low residual

concentrations were enforced (10 mmol/L), and *iii*) as the process should enable high product concentrations, a minimal concentration of 100 mmol/L was enforced. The combined stoichiometric and thermodynamic analysis under putative process conditions can deliver further insights for metabolic engineering [50,51].

In general, the pathways stoichiometries yield different energetic and thermodynamic efficiencies. The RuMP Cycle shows the highest ATP yield, with one ATP produced per pyruvate molecule vs. 2 and 7 ATP consumed by the serine cycle and the RuBP cycle. On the other hand, when using methanol as only substrate, the serine cycle can produce acetyl-CoA without carbon loss. Further described C1 assimilation pathways, RuBP and XuMP cycle were not included in the analysis. RuBP has a low energetic efficiency [31,52] [53] while the XuMP cycle “wastes” electrons by using an alcohol oxidase in the first step [54].

Fine chemical for food and pharma with short product pathway: Noreugenin

The biosynthesis of the malonyl-CoA-derived polyketide noreugenin has been demonstrated experimentally feasible in *C. glutamicum* [55]. The calculations show that different combinations enable CO_2emission-free production of noreugenin and the production is thermodynamically favoured for many combinations. The best two scenarios for the two substrate combinations are summarized in Table 1.

Following the MDF calculations [56] when using glucose and methanol, the thermodynamically less favourable reactions are the carbon rearrangements required for the functioning of the non-oxidative glycolysis (Table 1). Still, with glucose and linear synthetic pathways for methanol, like GAPA and SACA being the highest yielding combinations, the MDF is as high as 7 kJ/mol. When using xylose and methanol, there is an increase in MDF: Implementing the combination of NOG with either the SACA or the formolase pathway an MDF of 10.59 kJ/mol is obtained. Experimentally, the functional implementation of NOG in combination with the SACA pathway to utilize methanol and xylose for noreugenin synthesis requires the expression of six heterologous genes from various sources. With this configuration, a focus must be put on the balanced gene expression to avoid intracellular

accumulation of the cytotoxic pathway intermediate formaldehyde generated by the methanol dehydrogenase.

Table 1: Top two pathway combinations per product and sugar substrate based on theoretical FBA and MDF calculations. The criteria used to select the top performers, in order of importance, were(1) MDF score, (2) electron efficiency (how many electrons from the substrates are present in the products) and (3) number of required non-native steps to be engineered. DAS: Dihydroxyacetone synthase, GAA: Glycolaldehyde assimilation, GAPA: Glycolaldehyde-allose 6-phosphate, NOG: Non-oxidative glycolysis, SACA: Synthetic acetyl-CoA. All reactions and respective enzyme abbreviations are found in the supplementary information S1. Further simulation results are summarized in S3.

Product	Substrates	Pathway Combination	MDF (kJ, mol)	Limiting reactions	Non-native steps	e-eff (%)	$Cmol_{MEOH}$, $Cmol_{Sugar}$
PHB	Glucose + MeOH	NOG + DAS Pathway	4.35	PTA, ACAT, PHBB, PHBC	7	77	0.48
		NOG + GAPA[1] Pathway	4.35	PTA, ACAT, PHBB, PHBC	7	77	0.48
	Xylose + MeOH	NOG + DAS Pathway	4.35	PTA, ACAT, PHBB, PHBC	7	76	0.51
		NOG + GAPA[1] Pathway	4.28	PTA, ACAT, PHBB, PHBC	8	76	0.51
PHB w/enzyme coupling	Glucose + MeOH	NOG + GAPA[1] Pathway	5.60	PTA, ACAT_PHBB, PHBC	7	77	0.48
		NOG + GAA[1] Pathway	5.60	PTA, ACAT_PHBB, PHBC	8	77	0.48
	Xylose + MeOH	NOG+ GAPA[1] Pathway	5.60	PTA, ACAT_PHBB, PHBC	7	76	0.51
		NOG + GAA[1] Pathway	5.60	PTA, ACAT_PHBB, PHBC	8	76	0.51
Noreugenin	Glucose + MeOH	NOG + GAPA[1] Pathway	7.00	PGI, RPI, RPE, TKT1, TAL	5	60	0.67
		NOG + SACA[2] Pathway	7.00	PGI, RPI, RPE, TKT1, TAL	6	60	0.67
	Xylose + MeOH	NOG + SACA[1] Pathway	10.59	RPI, RPE, TKT1, TAL	6	59	0.70
		NOG + GAA[1] Pathway	9.47	RPE, MDH, GALS, TALB_F178Y	6	59	0.70
BAME	Glucose + MeOH	NOG + GAPA[1] Pathway	7.00	PGI, RPI, RPE, TKT1, TAL	4	63	5.25
		NOG + GAA[1] Pathway	7.00	PGI, RPI, RPE, TKT1, TAL	5	63	5.25
	Xylose + MeOH	NOG + SACA[2] Pathway	10.59	RPI, RPE, TKT1, TAL	5	63	4.63
		NOG + GAA[1] Pathway	9.47	RPE, MDH, GALS, TALB_F178Y	5	63	4.63

[1] GAA und GAPA pathways have not been demonstrated *in vivo* [37,38]

[2] SACA pathway has been demonstrated *in vivo*, although with low substrate affinities [35]

Bulk chemical, the bioplastic model molecule PHB:

In contrast to noreugenin, the PHB production pathway also requires a reduction equivalent (NADH or NADPH) for the first step [57]. This reaction has been described as putative bottleneck in several studies [58]. This is also apparent from the MDF calculations, with a MDF of 4.35 kJ/mol in the best case. This picture changes when assuming a metabolic channel for acetoacetyl-CoA, i.e. coupling of the enzymes β-ketothiolase and acetoacetyl-CoA reductase, as shown for a similar pathway by [59]. Now a higher MDF can be obtained, and there are pathway dependencies. With glucose and methanol, several combinations yield an MDF of 5.6 kJ/mol (Table 1). PTA, ACAT, PHBB, PHBC are the limiting reaction steps. Comparable results are obtained for xylose and methanol as substrates. In contrast to noreugenin the bottleneck is co-factor dependent, i.e. the NAD/NADH ratio limits the PHB synthesis pathway and MDF. Stoichiometrically and thermodynamically, the best combination include NOG for sugar degradation and C1 assimilation via DAS, GAA or GAPA depending on the used sugar or assumption on the product pathway channelling (Table 1).

The heterologous expression of seven genes for the efficient C1-assimilation via DAS and glucose-utilization via NOG is technically no problem. During strain construction, attention should be kept on the expression of the dihydroxyacetone synthase gene and a sufficient regeneration of Xu5P as formaldehyde-acceptor for a rapid assimilation of cytotoxic formaldehyde.

Biofuel, Fatty-acid methyl ester

Short chain fatty acids like butyric acid or derivates such as BAME have a degree of reduction that fits in the range between methanol and sugar. Nevertheless, also for short chain, methanol becomes the dominating substrate with a ratio around 5:1 ($Cmol_{MeOH}$:$Cmol_{Sugar}$). The pathway is thermodynamically feasible with an MDF of up to 10.59 kJ/mol for a combination of SACA and NOG. Although the natural RuMP pathway in

combination with the xylose phosphate ketolase has a lower driving force (4.76 kJ/mol), we chose to discuss the implementation of this combination as RuMP is currently the best established methanol assimilation route. The thermodynamic bottlenecks are reactions of the pentose-phosphate pathway as well as methanol dehydrogenase (Table 1). The functional implementation of the RuMP-pathway (in *E. coli*) was performed several times, and in principal requires the expression of only three genes, i.e., a methanol dehydrogenase, a 3-hexulose 6-phosphate synthase and a 6-phospho 3-hexuloisomerase. However, with this genetic setup, incorporation of methanol-derived carbon into central carbon metabolites is possible, but growth on methanol alone is not possible [60,61]. One of the main reasons is the delicate fine-tuning of relevant enzymatic activities to avoid metabolite depletion and ensure sufficient regeneration of ribulose-5-phosphate as acceptor of formaldehyde. Important steps towards engineered methylotrophy in *E. coli* were made by using FBA to identify methanol-dependent *E. coli* variants with high potential for adaptive laboratory evolution towards this goal [62]. In parallel, the conversion of *E. coli* to a synthetic methylotroph growing solely on methanol could be achieved [63] by following the proposed strategy by Keller et al., [62]. Starting point was a methanol-dependent strain with an incomplete RuMP cycle, which was subjected to laboratory evolution to efficiently use methanol as sole carbon source. Completion of the RuMP cycle, applying kinetic modeling and targeted engineering finally yielded a methylotrophic *E. coli* variant, which exhibited a doubling time of 8 hours and reaching a final OD_{600} of 1.9 (medium containing 400 mM methanol, starting OD_{600} of 0.1). Recent advances toward the generation of synthetic methylotrophs have been reviewed recently [64].

The stoichiometric and thermodynamic results demonstrate the potential of using methanol as carbon and electron source, enabling CO_2 free production with potentially high conversion rates, based on the high thermodynamic driving forces that can be achieved. Clearly, when implementing several heterologous pathways, significant fine-tuning will be required to achieve the metabolic optimum.

Challenges and Perspectives

Reaching net zero CO_2 emission in bioprocesses is challenging and requires significant rerouting of metabolism. Nevertheless, recent advances in synthetic biology, but also engineering to obtain novel carbon feedstocks will allow to reduce and in the best case eliminate CO_2 emissions. While here a specific collection of products has been discussed, there are many further products. Challenges to tackle remain in the product pathways, as many have a CO_2 producing reaction and reassimilation will require cellular energy and inevitably also lead to losses. Here computational enzyme engineering could help to design new pathways [65] that have no decarboxylation step.

Furthermore, products like long-chain fatty acids are so highly reduced, that a feedstock with even more electrons than methanol will be required. Putative, anaerobic methane degradation pathways were described [66] that could enable CO_2 emission-free production for highly reduced products. Alternatively, the sugar substrate needs to be replaced by a more reduced feedstock. One possibility is the use of glycerol, with a reduction degree of 4.67 e/C-atom, as a substitute for glucose or xylose.

On the other end, less reduced products could be produced with cofeeding of higher oxidized substrates like formiate or even CO_2 as proposed by [67]. To implement these alternative modules for substrate assimilation and product synthesis with balanced expression of required enzymes, a large number of strain variants needs to be constructed and tested. For this purpose, automation technologies for rational strain construction [68] and rapid phenotyping of entire libraries [69] are being established at a rapid pace in various labs.

Next to the challenges in designing the host, process design will have to provide well-defined multiple-substrate feeding to enforce a defined uptake. These processes will be challenging to monitor and control, especially at large scale with mixing inhomogeneities. Here, the distribution of oxygen and the two carbon sources could lead to a rapidly changing environment for the single microorganisms [70]. How highly engineered organisms react to

changing substrate mixtures needs to be analyzed. Adaptive Laboratory Evolution (ALE) will play a crucial role in several steps of strain design and scale-up [71,72].

Another challenge is the performance of the de novo reactions of synthetic pathways like SACA or formolase, which suffer from low formaldehyde affinity (Km=165 mM for GALS and Km=51 mM for ACPS) [35] or low catalytic efficiency (4.7 $M^{-1}s^{-1}$ for formolase) [34]. Also, the GAA and GAPA pathways have only been proved *in vitro* [37,38]. While these pathways rank among the best performers in our theoretical simulation, further efforts in enzyme and metabolic engineering are required. Currently validated methanol utilization pathways include the RumP cycle and the rGly pathway [33,63]. However, based on the simulations, only the RumP cycle could support combined use of sugars and methanol with no CO2 emissions, albeit with lower MDF scores, as discussed previously (for information about the performance of the pathway combinations not shown in Table 1 please refer to the information provided in Supplementary Information 3)

Supplementary Information

All codes are available on a github repository: https://github.com/sawahl/net_zero_c1.git. The repository also includes the supplementary files.

Credit Author Statement

CAV: Formal analysis, Software, Visualization, original draft.

SN: Visualization, review & editing

JM: original draft

SAW: Conceptualization; Formal analysis; Supervision; original draft, review & editing;

Conflict of Interest

The authors declare no conflict of interest.

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Highlights:

- Recent synthetic biology approaches, combining enzyme engineering and pathway design allow for many methanol assimilation routes
- Several, reduced products can be synthesized from a mixture of sugar and methanol as substrates
- Combined stoichiometric and thermodynamic analysis point to most promising pathways
- For several products zero CO_2 production can be achieved theoretically

Annotated papers:

van Winden WA, Mans R, Breestraat S, Verlinden RAJ, Mielgo-Gmez I, de Hulster EAF, de Bruijn HMCJ, Noorman HJ: Towards closed carbon loop fermentations: Cofeeding of Yarrowia lipolytica with glucose and formic acid. Biotechnology and Bioengineering 2022, 119:2142--2151

* The work experimentally demonstrates the potential of offgas carbon (CO2) recycling by electrochemical reduction. The approach is general and could increase the carbon yield for many processes.

Chen FYH, Jung H-W, Tsuei C-Y, Liao JC: Converting Escherichia coli to a Synthetic Methylotroph Growing Solely on Methanol. Cell 2020, 182:933-946.e914.

** Impressive engineering work to obtain a fully methylotrophic E. coli strain. The application of genetic engineering, modeling and extensive laboratory evolution enabled growth, at about half the growth rate of natural, methylotrophic organisms. The work highlights the challenging aspects of engineering metabolism to a new substrate and how modeling and laboratory evolution can be applied to de-bottleneck the pathway.

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589 reactions and pathways. Such tools are crucial for the rapid comparison of putative
590 engineering strategies.

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594 formaldehyde detoxification, assimilation via natural and synthetic pathways, as well as
595 formaldehyde metabolic regulation.

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599 * This article provides a comparison based on Flux Balance Anaylsis (FBA) of the yields of
600 several natural and synthetic pathways for the production of biomass, pyruvate and acetyl-
601 CoA , using methanol and formate as substrates.