Native and synthetic methanol assimilation in *Saccharomyces cerevisiae*

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Abstract

Microbial fermentation for chemical production is becoming more broadly adopted as an alternative to petrochemical refining. Fermentation typically relies on sugar as a feed-stock. However, one-carbon compounds like methanol are a more sustainable alternative as they do not compete with arable land. This study focused on engineering the capacity for methylotrophy in the yeast *Saccharomyces cerevisiae* through a yeast xylulose monophosphate (XuMP) pathway, a 'hybrid' XuMP pathway, and a bacterial ribulose monophosphate (RuMP) pathway. Through methanol toxicity assays and ^{13}C-methanol-growth phenotypic characterization, the bacterial RuMP pathway was identified as the most promising synthetic pathway for methanol assimilation. When testing higher methanol concentrations, methanol assimilation was also observed in the wild-type strain, as ^{13}C-ethanol was produced from ^{13}C-methanol. These results demonstrate that *S. cerevisiae* has a previously undiscovered native capacity for methanol assimilation and pave the way for further development of both native and synthetic one-carbon assimilation pathways in *S. cerevisiae*.

Introduction

Our current dependence on fossil fuels is not sustainable. Fossil fuel resources are finite, and our dependence on them has resulted in negative environmental impacts. By-products from fossil fuel combustion include a myriad of toxic air pollutants and CO_2, which is the main contributor to climate change. These complex environmental problems call for a global effort to move towards a bio-economy where microbial metabolism is used for the conversion of renewable materials into useful products [1]. Typically, sugars derived from sugarcane or maize are used as feed-stocks for fermentation. However, sugar production is costly and requires arable land that competes with other land uses, such as food production. It is estimated that the sugar cost represents up to 70 % of the total production costs [2], making many biotechnological processes uncompetitive with petrochemical processes.

Growing crops for feed-stock also requires water and fertilizer, which could be instead allocated to grow food to meet the demand of an ever-rising population. Alternatively, lignocellulosic non-food biomass could be used for obtaining sugars, but the process is limited by recalcitrance of the feed-stock and difficulties utilising the large lignin fraction of biomass. One-carbon (C1) metabolites are abundant and inexpensive, and can be obtained from waste resources such as agricultural, municipal or industrial waste, or natural gas [3]. Methanol in-particular is becoming an attractive resource due to its abundance and liquid state, which makes it compatible with existing fermentation, storage, and transportation infrastructure [4]. Methanol can also be obtained inexpensively from methane and carbon dioxide stemming from industrial waste streams [4]. Engineering microorganisms to grow on and convert C1 compounds such as methanol into food, fuels, chemicals, and pharmaceuticals has therefore become a major goal in the fields of synthetic biology and metabolic engineering [3,5].

Organisms capable of growing by metabolising C1 compounds into biomass and energy (methylotrophs), exist in bacterial, archaeal, and yeast species. Recent attempts have been made to produce valuable metabolites from methylotrophic bacteria and to develop the molecular

biology tools to genetically engineer them. For example, *Methylobacterium extorquens* AM1 has been engineered to produce 3-hydroxypropionic acid from methanol [6]. However, methylotrophs currently lack the genetic tools, depth of characterisation, and robust industrial growth necessary for successful metabolic engineering outcomes [3,4,7,8]. An attractive alternative to enable the use of methanol as feed-stock is to engineer synthetic methylotrophy into industrially-robust and well-characterised microorganisms. Model organisms are not only easier to genetically manipulate and optimise, but also provide the opportunity to engineer a non-native central carbon metabolism that has greater genetic and metabolic plasticity. Recent approaches have focused on engineering synthetic methylotrophy in bacteria, such as *Escherichia coli* and *Corynebacterium glutamicum* [9-16]. Incorporation of ^{13}C-methanol into central carbon metabolites and specialty products has been demonstrated in both species; however, growth on methanol still requires yeast extract or additional carbon sources like xylose.

The yeast species *Saccharomyces cerevisiae* also has potential for engineering synthetic methylotrophy as it can enable distinct advantages over organisms such as *E. coli* for use in industrial fermentation. For example, yeasts can correctly express, fold, and post-translationally modify eukaryotic proteins, are not susceptible to phage contamination, have a greater tolerance to low pH concentrations and solvents like methanol, and have organelles that can be co-opted for localization of specialized metabolism [17-19]. The engineering of synthetic methylotrophic metabolism in *S. cerevisiae* could therefore provide several benefits to methanol-fed bioprocesses. Here, we designed, built, and tested three different methanol-assimilation pathways in *S. cerevisiae* along with five pathway sub-variations. Using methanol-dependent growth assays and ^{13}C-methanol tracer studies, we identified the most promising pathways for future development of methylotrophy as well as bottlenecks associated with formaldehyde assimilation in *S. cerevisiae*. Importantly, when testing higher (2 %) methanol concentrations, we also identified for the first time the native capacity of *S. cerevisiae* to assimilate methanol through central carbon metabolism.

Results

Engineering strategy

After evaluating the different methanol assimilation pathways available in nature, three metabolic pathways were designed for engineering synthetic methylotrophy in *S. cerevisiae*. The first pathway design (coloured red in Fig. 1) was based on the xylulose monophosphate (XuMP) pathway in the methylotrophic yeast *Pichia pastoris* (*Komagataella phaffii*). Here, methanol assimilation is targeted to the peroxisome and starts with the oxidation of methanol to formaldehyde and hydrogen peroxide by an FAD-dependent alcohol oxidase (Aox1p). Hydrogen peroxide is detoxified by a catalase (Cat1p) in the peroxisome while formaldehyde is converted to glyceraldehyde-3-phosphate by dihydroxyacetone synthase (Das1p), which uses xylulose-5-phosphate as a co-substrate. Glyceraldehyde-3-phosphate can then be incorporated into central carbon metabolism to produce biomass. A potential challenge involved in this pathway is the need for peroxisome proliferation as it occurs in *P. pastoris* when grown on methanol as the sole carbon source [20]. The second major pathway was termed a 'hybrid' because it links the *P. pastoris* XuMP pathway with an NAD-dependent methanol dehydrogenase (Mdh) from *Bacillus stearothermophilus*, which oxidises methanol to formaldehyde but does not produce hydrogen peroxide. Formaldehyde is then converted to glyceraldehyde-3-phosphate by a cytosolically localised Das1p from *P. pastoris*. Utilising Mdh has the advantage of not requiring oxygen for the reaction and not producing hydrogen peroxide, unlike Aox1p, and generating energy in the form of NADH. The third pathway was based on the bacterial ribulose monophosphate (RuMP) pathway, where Mdh oxidises methanol to formaldehyde while hexulose-6-phosphate synthase (Hps) uses ribulose-5-phosphate as a co-substrate to convert formaldehyde to hexulose-6-phosphate, which is then converted to fructose-6-phosphate by 6-phospho-3-hexuloisomerase (Phi) from *Bacillus methanolicus*. Fructose-6-phosphate can then enter glycolysis to produce biomass precursors.

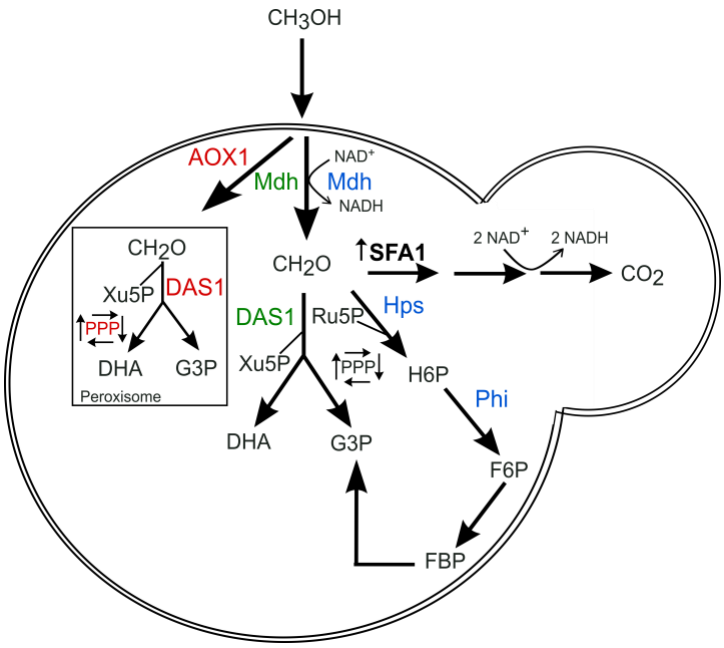


Fig. 1. Methanol assimilation pathways engineered in *S. cerevisiae*. Three different metabolic pathways were engineered for methanol assimilation where methanol is oxidised to formaldehyde and later assimilated to glyceraldehyde-3-phosphate or fructose-6-phosphate. The first yeast XuMP pathway (red) is targeted to the peroxisome. The second 'hybrid' XuMP pathway (green) occurs in the cytosol. The third bacterial RuMP pathway (blue) is also targeted to the cytosol and converts formaldehyde to fructose-6-phosphate. Over-expression of the native formaldehyde detoxification enzyme Sfa1p is shown in bold. AOX1, alcohol oxidase 1; DAS1, dihydroxyacetone synthase; PPP, pentose phosphate pathway; Mdh, methanol dehydrogenase; Hps, hexulose-6-phosphate synthase; Phi, phospho-3-hexuloisomerase; SFA1, S-(hydroxymethyl) glutathione dehydrogenase; Xu5P, xylulose-5-phosphate; Ru5P, ribulose-5-phosphate.

Benchmarking synthetic pathways for methanol assimilation potential

The three pathways, yeast XuMP, 'hybrid' XuMP, and bacterial RuMP, were expressed from low-copy centromeric plasmids in *S. cerevisiae* and tested by spotting serial 10-fold dilutions of the strains onto solid minimal Yeast Nitrogen Base (YNB) medium with 1 %, 2 %, or 4 % methanol alongside an empty vector control (CEN.PK113-5D; Fig. 2). These spot assays are a high-throughput method for assaying growth, with colony size and spot-density comparisons used to identify growth differences. To determine if the growth differences were methanol-specific, strains were also spotted onto solid minimal Yeast Nitrogen Base (YNB) medium with

2 % glucose, YNB medium with no added carbon source (1 x YNB) or 2 % methanol with 0.1 % yeast extract (Fig. 2). The yeast XuMP pathway (*P. pastoris AOX1* and *DAS1*) resulted in a subtle growth increase on 1 % methanol medium compared to the empty vector control, but this was not visible at 2 % methanol. The 'hybrid' XuMP pathway with bacterial *mdh* and *P. pastoris DAS1* expression had increased growth on 1 % and 2 % methanol relative to the control strain. The same was observed in the bacterial RuMP pathway with *mdh*, *hps*, and *phi*. Growth was still observed on 1 x YNB medium with no added carbon source, indicating there are medium components that enable the observed background growth. Strain-specific growth differences were far more pronounced on YNB-methanol media without yeast-extract.

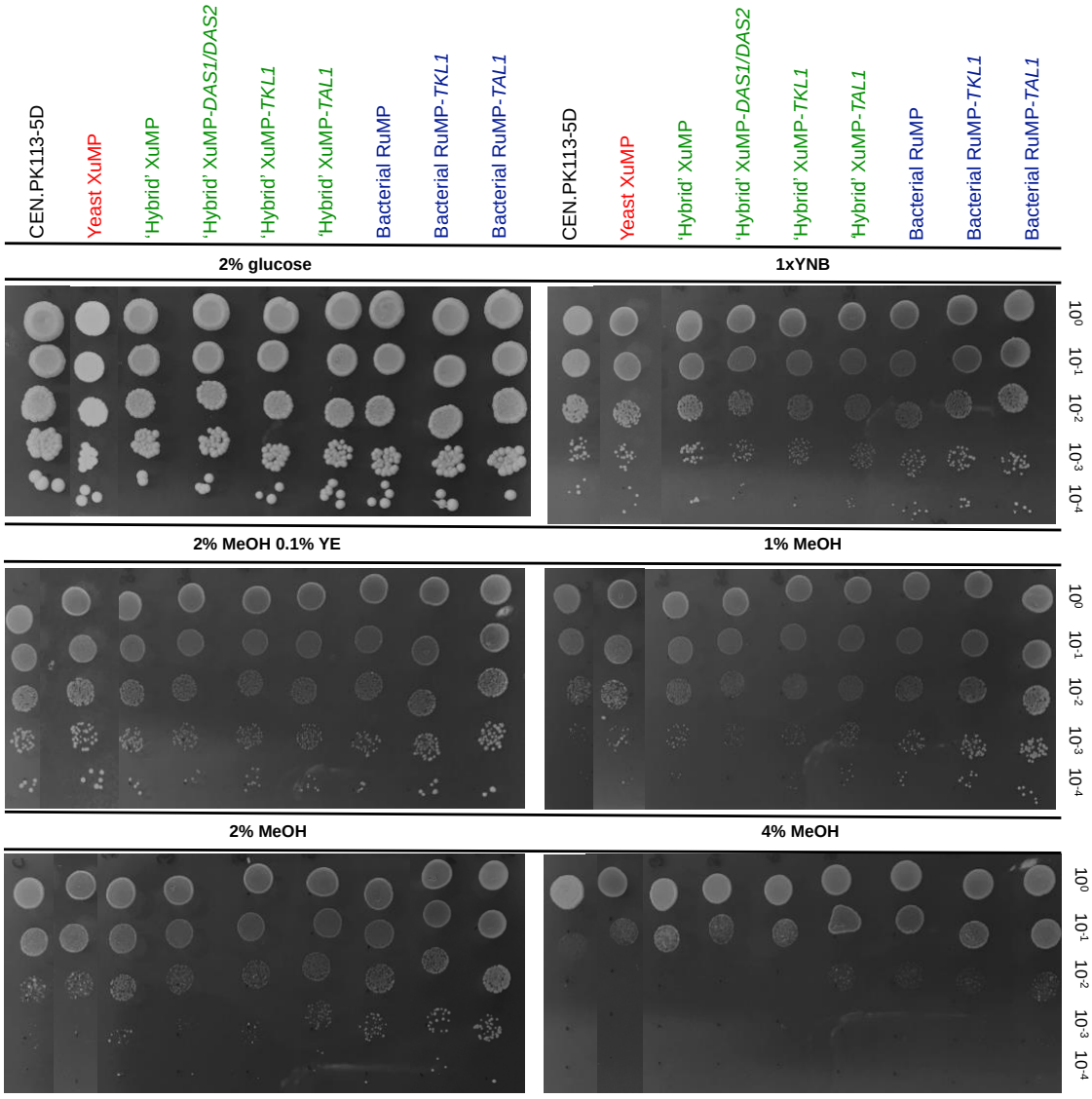


Fig. 2. Spot-assays on different carbon sources of *S. cerevisiae* strains expressing different methanol assimilation pathways. Growth of serially 10-fold diluted strains on solid YNB medium with

indicated carbon sources. The empty vector control is highlighted in black and the yeast XuMP strain is highlighted in red. The 'hybrid' XuMP strain and variations thereof are in green. Lastly, the bacterial RuMP strain and variations thereof are shown in blue. Yeast Nitrogen Base (YNB), Yeast Extract (YE), Methanol (MeOH). Images were taken after incubating at 30 ºC for 5 days. The Yeast XuMP assays are from different plates but following the same methodology and with an empty vector control.

Pentose phosphate pathway engineering to improve methanol assimilation

Following initial growth tests on methanol, the 'hybrid' XuMP and bacterial RuMP strains were selected to improve methanol assimilation through rational engineering. Both strains had improved growth on solid minimal medium with 2 % methanol compared to the empty vector control; however, this growth was reduced at 4 % methanol (Fig. 2). This reduction in growth might have been due to either methanol toxicity or a toxic build-up of formaldehyde in the cell. Formaldehyde assimilation has been suggested as a likely bottleneck in methylotrophic metabolism as high flux is needed both for regeneration of the co-substrates of the assimilation enzymes (xylulose-5-phosphate or ribulose-5-phosphate), and for generation of biomass precursors [21]. In *P. pastoris*, for example, three molecules of methanol are needed to yield just one molecule of glyceraldehyde-3-phosphate [22]. Likewise, rapid assimilation of formaldehyde is needed to overcome the unfavourable kinetics of Mdh and drive its reaction forward from methanol to formaldehyde [16]. Formaldehyde assimilation flux can be increased by (i) improving formaldehyde assimilation enzyme expression and kinetics or (ii) increasing pentose phosphate pathway carbon fluxes for rapid regeneration of xylulose-5-phosphate or ribulose-5-phosphate. For the first approach, it is known that *P. pastoris* has two enzymatically active dihydroxyacetone synthases, Das1p and Das2p [23]. The 'hybrid' XuMP strain contained Das1p and Aox1p, thus the second variant (*DAS2*) was expressed in a new 'hybrid' XuMP *DAS1/DAS2* strain. However, growth on methanol was not improved when assayed in solid media with different methanol concentrations (Fig. 2). It was also possible that increased formaldehyde assimilation could be achieved by increasing the concentration of Das1p in the cell. Integrating multiple copies of a gene into the repeated Ty1 delta sites of the *S. cerevisiae*

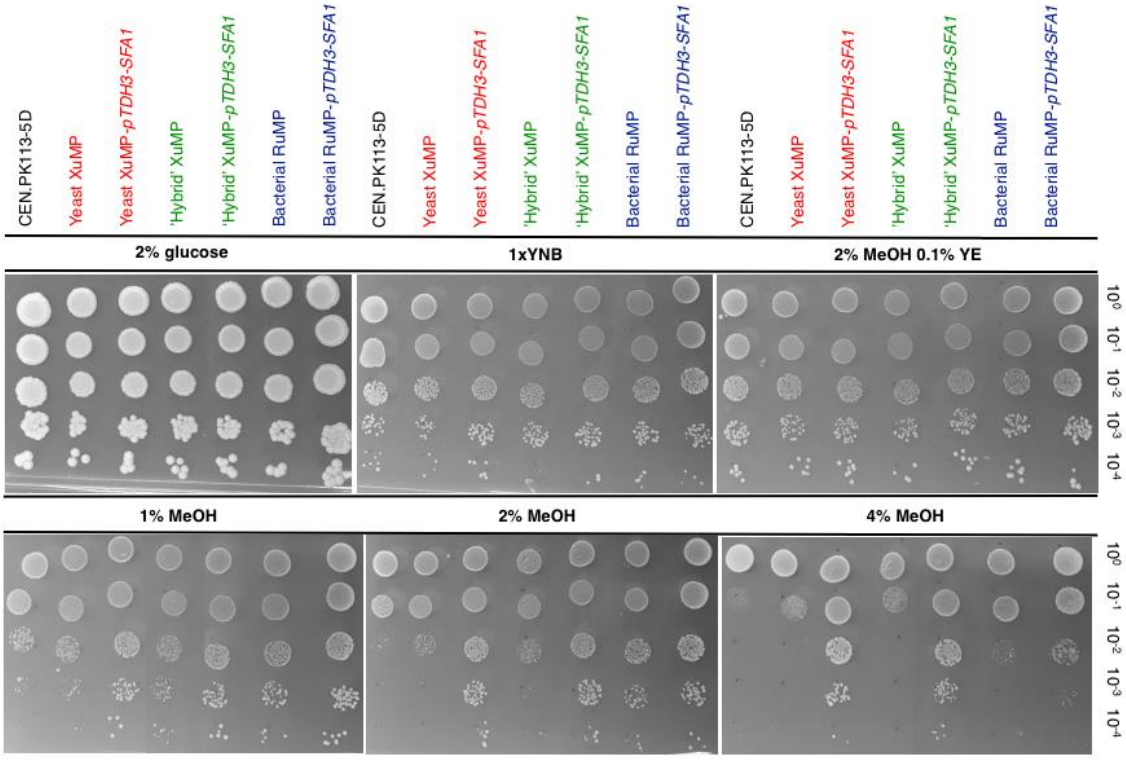
genome is an established method for gene over-expression [24,25]. *DAS1* was therefore targeted to the dispersed Ty1 delta sites in *S. cerevisiae*'s genome, but no improvement in methanol-dependent growth was observed. For the second approach of increasing flux through the pentose phosphate pathway, the *TKL1* and *TAL1* enzymes of the non-oxidative branch were over-expressed in both the 'hybrid' XuMP and bacterial RuMP strains. Over-expression of these enzymes has been shown to improve pentose phosphate pathway fluxes and availability of ribulose-5-phosphate [26-28]. Despite *TAL1* over-expression in both strains enabling a slight growth increase when spotted onto solid minimal medium with 1, 2 or 4 % methanol (Fig. 2), no growth increase was observed when tested in liquid yeast extract methanol medium.

Enhancing native formaldehyde detoxification improves methanol-specific growth

Methylotrophy is a delicate balance between formaldehyde assimilation and dissimilation. Formaldehyde dissimilation to CO_2 is the main source of energy for methylotrophs, as the TCA cycle is down-regulated when methanol is the sole carbon source [22]. While formaldehyde assimilation *in S. cerevisiae* requires heterologous enzyme expression, a native pathway for the dissimilation of formaldehyde to CO_2 and energy in the form of NADH already exists (Fig. 1) [29,30]. This pathway is initiated by an NAD^+ and glutathione-dependent formaldehyde dehydrogenase, Sfa1p, which has previously been shown to enhance formaldehyde tolerance when over-expressed in *S. cerevisiae* [31,32]. As formaldehyde accumulation in the cell can be toxic, and its dissimilation plays an important role in methylotrophs, we chose to over-express *SFA1* in *S. cerevisiae* alongside each of the three methanol assimilation pathways.

Growth of the yeast XuMP, 'hybrid' XuMP, or bacterial RuMP strains with or without over-expression of *SFA1* was tested on solid minimal YNB medium with increased concentrations of methanol, 2 % methanol supplemented with yeast extract, YNB medium with no added carbon source (1 x YNB), or 2 % glucose (Fig. 3). To confirm methanol assimilation, growth on methanol would have to be visible independently of *SFA1* over-expression. For example, from Fig. 2 and 3 a higher capacity for growth can be observed on 1 and 2 % methanol for the

198 'hybrid' XuMP and bacterial RuMP strains. Enhanced formaldehyde dissimilation via *SFA1*
199 over-expression had a further positive effect on the growth of these strains at 2 % and 4 %
200 methanol (Fig. 3). This distinct growth increase was most pronounced in the bacterial RuMP
201 strain relative to both the empty vector control strain on the same media, and relative to the
202 same engineered strains on 1 x YNB medium. This experiment indicated four critical things: (i)
203 all engineered strains were oxidising methanol to formaldehyde; (ii) the RuMP and 'hybrid'
204 pathways enabled methanol and formaldehyde assimilation in *S. cerevisiae*; (iii) the RuMP
205 pathway is superior to the 'hybrid' for formaldehyde assimilation; and (iv) *SFA1* over-
206 expression is an effective method for formaldehyde dissimilation, which is beneficial for growth
207 on methanol. These experiments show that although the 'hybrid' XuMP and RuMP pathways
208 enable methanol and formaldehyde assimilation and improved growth relative to the control
209 strain, they do not facilitate methanol-dependent growth increases relative to 1 x YNB medium
210 unless *SFA1* is also expressed.



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212 **Fig. 3. Over-expression of the native formaldehyde detoxification pathway improves growth on**
213 **methanol.** Growth on increasing concentrations of methanol was tested in serial 10-fold dilutions for an
214 empty vector control, the yeast XuMP (red), 'hybrid' XuMP (green), or bacterial RuMP (blue) strain with

or without over-expression of *SFA1*. Yeast Nitrogen Base (YNB), Yeast Extract (YE), Methanol (MeOH). Images were taken after incubating at 30 ºC for 5 days.

Liquid growth and ^{13}C-methanol tracer analysis identifies methanol-specific growth and methanol assimilation to formaldehyde

After observing methanol- and strain-specific growth differences on solid medium (Fig. 2 and 3), methanol-dependent growth of the 'hybrid' XuMP-*pTDH3-SFA1*, bacterial RuMP-*pTDH3-SFA1*, and empty vector control strains was tested in liquid medium. No growth was observed with 2 % methanol as the sole carbon source. Previous studies have highlighted the importance of yeast extract for growth on liquid methanol media [33]. We therefore tested growth with and without 2 % methanol in liquid YNB medium with 0.1 % yeast extract (Fig. 4A). The presence of methanol in the medium resulted in significant ($p < 0.05$) final OD_{600} increases relative to yeast-extract-only medium of 58 %, 47 %, and 39 % for the 'hybrid' XuMP-*pTDH3-SFA1*, bacterial RuMP-*pTDH3-SFA1*, and control strains, respectively. This result clearly identified a methanol-specific growth increase in liquid medium containing yeast-extract. In contrast to the tests on solid methanol YNB medium (Fig. 2 and 3), comparing growth of the different strains on liquid yeast extract methanol medium revealed only marginal and non-significant differences. The presence of yeast extract appeared to largely eliminate the strain-specific differences that were observed on solid YNB-methanol plates, which is consistent with the trend observed on solid YNB-methanol plates with 0.1% yeast extract (Fig. 2 and 3).

To accurately assess the metabolic performance of the 'hybrid' XuMP-*pTDH3-SFA1* and bacterial RuMP-*pTDH3-SFA1* strains and determine if methanol was being metabolised in the presence of yeast extract, fermentations with 1 % ^{13}C-methanol were conducted. For ^{13}C-methanol tracer analysis, bioreactor off-gases such as ^{13}C-methanol, ^{13}C-CO_2, CO_2, ^{13}C-ethanol, and ethanol were measured in real-time using a mass spectrometer connected to the bioreactors. The ratio of labelled to unlabelled CO_2 was compared at the maximum ^{13}C-CO_2 production point, showing that the bacterial RuMP-*pTDH3-SFA1* strain produced 50 % of total CO_2 as ^{13}C-

CO_2, while the 'hybrid' XuMP-*pTDH3-SFA1* strain produced 30 % ^{13}C-CO_2 (Fig. 4B). The bacterial RuMP-*pTDH3-SFA1* strain had the highest labelled CO_2 production despite the only difference being the formaldehyde assimilation enzymes (bacterial Hps-Phi instead of *P. pastoris* Das1p), suggesting the different ^{13}C-CO_2 production levels can be partly attributed to the fluxes in the different formaldehyde assimilation pathways. No ^{13}C- ethanol was detected in the off-gas measurements of both strains, making it unclear if methanol was assimilated beyond formaldehyde. Nonetheless, ^{13}C-methanol tracer analysis confirmed methanol assimilation to formaldehyde in the engineered strains, and further established the bacterial RuMP-*pTDH3-SFA1* pathway as the superior option for development of synthetic methylotrophy in *S. cerevisiae*.

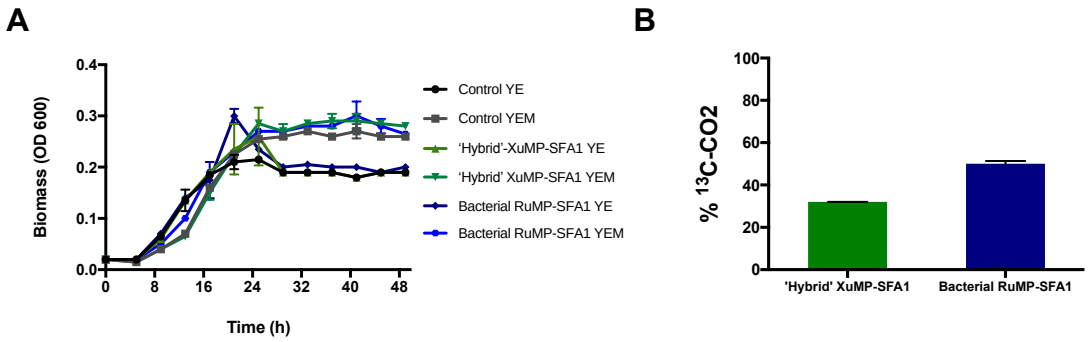

Fig. 4. Liquid growth in yeast extract (YE) medium or yeast extract methanol medium (YEM) and ^{13}C-CO_2 production. A. Growth profiles of the empty vector control, the 'hybrid' XuMP-*pTDH3-SFA1* and the bacterial RuMP-*pTDH3-SFA1* strain grown in liquid YNB medium with 0.1 % yeast extract (YE) or with 0.1 % yeast extract and 2 % methanol (YEM). Data points represent the average of three biological replicates and error bars are the standard deviation. **B.** Percentage of ^{13}C-CO_2/CO_2 production of the yeast synthetic strains on yeast extract ^{13}C-methanol (1 %) medium. Data points represent the average of two biological replicates and error bars are the standard deviation.

^{13}C-methanol to ^{13}C-ethanol production confirms methanol assimilation through central carbon metabolism

To determine whether formaldehyde was being assimilated through central carbon metabolism or only dissimilated to CO_2, fermentations with a higher percentage of ^{13}C-methanol (2 %) were performed (Fig. 5A), the assumption being more methanol available for consumption would result in a greater driving force for methanol assimilation through central carbon metabolism and therefore detectable production of ^{13}C-ethanol. The bacterial RuMP-*pTDH3-SFA1* strain was selected as the superior candidate due to its better growth on solid methanol media (Fig. 2 and 3) and higher ^{13}C-CO_2 production when grown in 1 % ^{13}C-methanol compared to the 'hybrid' XuMP-*pTDH3-SFA1* strain (Fig. 4B). An empty vector control strain was included to allow for a wild-type comparison of ^{13}C-CO_2 and ^{13}C-ethanol production. As previously observed, the bacterial RuMP-*pTDH3-SFA1* and control strains had similar growth profiles (Fig. 4A and 5A). The bacterial RuMP-*pTDH3-SFA1* strain had higher ^{13}C-CO_2 production than in the 1 % methanol medium, producing 80 % ^{13}C-CO_2 instead of 50 % (Fig. 4B and 5B), supporting the hypothesis that the higher 2 % methanol concentration in the medium had enabled a greater driving force for methanol metabolism. Surprisingly, both strains had the same ^{13}C-CO_2 and ^{13}C-ethanol production levels (Fig. 5B, C). A high sensitivity detector was used to scan for the unique masses corresponding to ^{13}C-ethanol (Supplementary Fig. 1) at two different time points during the fermentation, and ^{13}C-ethanol was detected at both time points (Fig. 5C). Methanol assimilation through central carbon metabolism was therefore confirmed for both the wild-type and the bacterial RuMP-*pTDH3-SFA1* strain with the detection of ^{13}C-ethanol, which could only arise through the conversion of ^{13}C-methanol through central carbon metabolism (Fig. 5C). This result showed that *S. cerevisiae* has a previously undiscovered native capacity for methanol assimilation. HPLC analysis of extracellular metabolites also verified the presence of glycerol in the wild-type strain, while fumarate was detected in the bacterial RuMP-*pTDH3-SFA1* strain (Supplementary Table 1). These distinct extracellular metabolite accumulation levels support the spot assay tests (Fig. 2 and 3) in showing that these strains have different metabolic characteristics, despite their similar growth profiles in liquid yeast extract methanol medium (Fig. 4A).

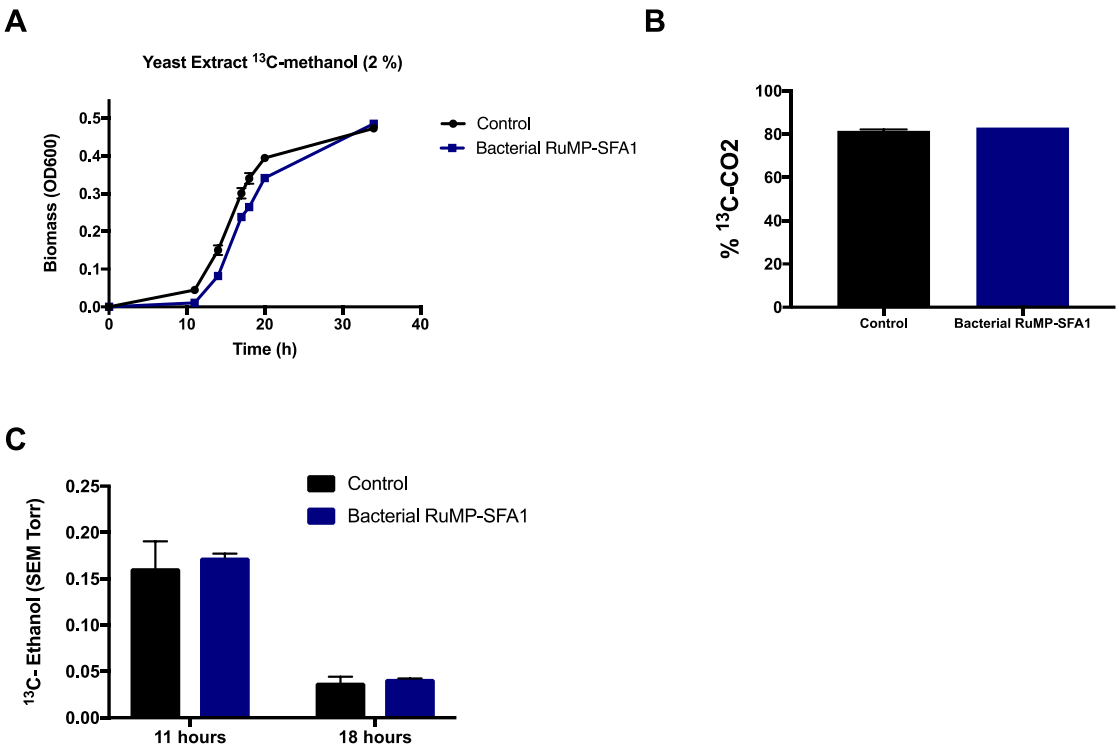

Fig 5. ^{13}C-methanol fermentations confirm methanol assimilation in *S. cerevisiae*. A. Growth profiles of the empty vector control (black) and the bacterial RuMP-*pTDH3-SFA1* strain (blue) grown in liquid YNB medium with 2 % ^{13}C-methanol supplemented with 0.1 % yeast extract. **B.** Percentage of ^{13}C-CO_2/CO_2 production of the two yeast strains on yeast extract ^{13}C-methanol (2 %) medium. **C.** ^{13}C-ethanol was produced by the empty vector control (black) and the bacterial RuMP-*pTDH3-SFA1* strain (blue). The signal intensity was normalised to the inert gas nitrogen, and then to biomass for each strain. Data shows the average ^{13}C-ethanol intensity at 47 amu for two biological replicates during independent scanning cycles using a Hiden HPR-20-QIC mass spectrometer. Error bars are the standard deviation of the ^{13}C-ethanol intensity. Secondary Electron Multiplier (SEM). Data points represent the average of two biological replicates and error bars are the standard deviation.

Discussion

We engineered the XuMP and RuMP methanol assimilation pathways in *S. cerevisiae* (Fig. 1) and analysed growth on methanol as well as ^{13}C-metabolite production from ^{13}C-methanol. The XuMP pathway was targeted either to the peroxisome or to the cytosol, where a 'hybrid' version of the pathway was designed. A subtle growth improvement only on solid 1 % methanol medium was observed when the enzymes were targeted to the peroxisome (Fig. 2). This

pathway is based on the methylotrophic yeast *P. pastoris*, in which peroxisome proliferation plays a major role in its ability to grow on methanol, a common characteristic of other methylotrophic yeasts [32,34]. However, peroxisome proliferation in *S. cerevisiae* is limited to specific stress responses such as fatty acid oxidation and requires the regulation of multiple genes [35]. It is possible that peroxisome proliferation was a limiting step for function of the XuMP pathway as peroxisome proliferation in response to methanol has not evolved in *S. cerevisiae*. In conclusion, a XuMP methanol assimilation pathway targeted to the peroxisome did not appear to be a viable option for synthetic methylotrophy in *S. cerevisiae*. This contradicts a previous report by Dai et al. where a 9.23 or 11.70 % improvement in growth was recorded in yeast extract methanol (1 %) medium after introduction of two yeast XuMP pathway variants (Aox1p-Das1p-Cat1p-Dak1p or Aox1p-Das2p-Cat1p-Dak1p, respectively) [36].

The second, cytosol localised, 'hybrid' XuMP pathway utilised a bacterial NAD^+-dependent Mdh alongside the *P. pastoris DAS1*. The third 'bacterial RuMP' pathway used enzymes from methylotrophic bacteria (Mdh and Hps-Phi). An NAD^+-dependent methanol dehydrogenase was chosen to oxidise methanol to formaldehyde (first step on the pathway), as it has been identified as having higher affinity for methanol and the most efficient conversion compared to other methanol dehydrogenases, especially the variant from *B. stearothermophilus* [11,13]. To assimilate formaldehyde to biomass, enzymes utilising two different cyclic pathways were chosen, Das1p (XuMP pathway) or Hps and Phi (RuMP pathway). These two alternatives were tested as they use different co-substrates for formaldehyde assimilation (xylulose-5- phosphate or ribulose-5-phosphate) and produce either glyceraldehyde-3-phosphate or fructose-6-phosphate as the precursors for biomass formation (Fig. 1). Both pathways enabled a greater capacity for growth on solid minimal methanol medium relative to an empty vector control strain, with the bacterial RuMP pathway being superior to the hybrid-XuMP (Fig. 2). This result is supported by an *in silico* flux balance analysis performed by Comer et al., which analysed different C1 carbon sources and also found the RuMP pathway to be the more promising option in *S. cerevisiae* [37]. Further modifications aimed at improving growth on methanol included targeting of *DAS1* to

Ty1 sites for multiple genome insertion or including the second variant of the enzyme Das2p, as well as attempting to modulate flux through the pentose phosphate pathway by over-expressing *TKL1* or *TAL1* in either strain. Although *TKL1* and *TAL1* expression resulted in a small improvement in growth of the RuMP pathway strain on solid medium (Fig. 2), no modifications enabled growth in liquid medium with methanol as the sole carbon source.

An interesting development while testing the synthetic methylotrophic pathways in yeast was the identification of formaldehyde detoxification as a critical component for methanol-dependent growth improvement. Studies on methylotrophic species have previously highlighted the importance of the balance between assimilation and dissimilation of formaldehyde. Over-expression of *SFA1*, the first gene in the native formaldehyde detoxification pathway, was therefore tested alongside the three synthetic pathways. Growth on methanol was significantly improved in all strains upon introduction of an over-expressed *SFA1* (Fig. 3). Likewise, methanol assimilation in solid minimal YNB-methanol media was confirmed in both the 'hybrid' XuMP and bacterial RuMP strains by comparing growth against an empty vector control strain on increasing and toxic concentrations of methanol. These results enabled identification of the bacterial RuMP-*pTDH3*-*SFA1* pathway as a the most promising for engineering synthetic methanol assimilation in *S. cerevisiae*.

Recent work has also highlighted the importance of formaldehyde dissimilation through the engineering of synthetic methylotrophy in *E. coli*. For example, Müller et al. detected an overall higher percentage of total ^{13}C-labelling when they assessed methanol assimilation in a wild-type background compared to a genetic background unable to dissimilate formaldehyde (Δ*frmA*; 39.4 % versus 20.2 %), results that also contradicted their *in silico* simulations where formaldehyde detoxification was not found to be essential for the conversion of methanol [11]. Woolston et al. also investigated the improvement of formaldehyde assimilation in a synthetic methylotrophic *E. coli* strain. They found that the unfavourable thermodynamics of the Mdh enzyme need to be overcome to maintain the forward reaction direction of methanol oxidation [16]. One way to

achieve this is to maintain low intracellular formaldehyde levels through formaldehyde dissimilation pathway over-expression. This observation could explain why Müller et al. saw higher ^{13}C-labelling levels in a wild-type background that was able to dissimilate formaldehyde, and together these observations support the importance of formaldehyde dissimilation for methanol assimilation in *S. cerevisiae*.

Methanol-specific growth of the control, 'hybrid' XuMP-*pTDH3-SFA1* and bacterial RuMP-*pTDH3-SFA1* strains, was observed in liquid yeast extract media with and without methanol, with final biomass increasing by between 39 % and 58 % for the control and engineered strains, respectively (Fig. 4A). However, strain-specific differences in liquid yeast extract methanol medium were dramatically diminished relative to those observed on minimal solid methanol medium (Fig. 2 and 3). ^{13}C-labelled methanol was therefore used to elucidate metabolic differences between the engineered strains in liquid medium. The proportion of CO_2 identified as ^{13}C-CO_2 was 30 % and 50 % in the 'hybrid' XuMP-*pTDH3-SFA1* and bacterial RuMP-*pTDH3-SFA1* strains, respectively, which only differed in their formaldehyde assimilation enzymes (Fig. 4B). Although these initial ^{13}C methanol fermentations were useful for differentiating between the methylotrophic capacities of the 'hybrid' XuMP-*pTDH3-SFA1* and RuMP-*pTDH3-SFA1* strains in liquid medium, no ^{13}C labelled metabolites other than CO_2 were detected. Fermentations with a higher ^{13}C-methanol concentration (2 %, Fig. 5, compared to 1 %, Fig. 4) were performed with the wild-type and bacterial RuMP-*pTDH3-SFA1* strains to facilitate ^{13}C ethanol detection. Quite surprisingly, both strains produced the same amount of ^{13}C-CO_2 and ^{13}C-ethanol (Fig. 5), confirming methanol assimilation through central carbon metabolism (Fig. 5C and Supplementary Table 1). The increased concentration of methanol appeared to have a positive effect on the methanol-metabolism of *S. cerevisiae*, with higher $^{13}CO_2$ levels detected in the bacterial RuMP-*pTDH3-SFA1* strain (Fig. 4 and 5). Importantly, *S. cerevisiae* has always been recognised as a non-native methylotroph and no previous growth on- or metabolism of- methanol has ever been previously recorded in the literature. The

identification of ^{13}C-ethanol from ^{13}C-methanol here clearly and convincingly shows for the first time that methanol is assimilated through central carbon metabolism in *S. cerevisiae*.

In a separate study, we have recently identified *S. cerevisiae* genes that are significantly up-regulated in response to methanol in the growth medium [38]. Such genes include the alcohol dehydrogenase 2 (*ADH2*), *PEX11* (involved in peroxisome biogenesis and metabolism), as well as *GTT2*, *GSH2*, *ECM4* involved in glutathione metabolism, which supports formaldehyde detoxification. It is not inconceivable that *S. cerevisiae* shares some of the characteristics of methylotrophic yeasts like *P. pastoris* and is assimilating methanol through an incomplete version of this methylotrophic pathway (Fig. 1). Another possibility that has been recently explored is the formation of biomass through formaldehyde detoxification to formate and then assimilation through flux rearrangements in the glycine cleavage complex [39]. Both avenues open the possibility to identify and characterise the native methanol assimilation pathway of *S. cerevisiae*.

This multi-pathway comparison identified the bacterial RuMP-*pTDH3-SFA1* strain as the best candidate for synthetic methanol assimilation in *S. cerevisiae.* Additionally, native methanol metabolism was identified in *S. cerevisiae* for the first time, providing the possibility of exploring a newly discovered native methanol metabolism. Higher biomass formation and the elimination of the requirement for yeast extract in liquid methanol medium are the most immediate challenges remaining for the implementation of industrially-relevant methylotrophy in *S. cerevisiae*. Higher biomass formation is likely limited in the bacterial RuMP-*pTDH3-SFA1* strain by the inefficient regeneration of ribulose-5-phosphate and the cell's adaptation to this stressful condition. The regeneration of ribulose-5-phosphate could not be optimized through our rational engineering attempts, thus, other avenues like laboratory evolution remain to be explored. Evolutionary experiments have shed some light on engineering methanol assimilation in *E. coli* [10], where increased formaldehyde dissimilation via the native formaldehyde dehydrogenase was selected for via serial passaging on methanol medium. After robust

methylotrophy is established in *S. cerevisiae*, a plethora of existing metabolite production pathways could potentially be coupled to a methanol converting 'platform strain' using the state-of-the art genetic tools and physiological characterizations that are available. Together, the results from this work represent an exciting step towards using sustainable feed-stocks during microbial fermentations for the conversion to chemicals, fuels, materials, pharmaceuticals, and foods.

Methods

Strains and Plasmids

S. cerevisiae plasmids and strains used in this study are shown in Tables 2 and 3, respectively. The genes were codon optimised for *S. cerevisiae* and synthesised by GenScript USA Inc. DNA manipulation and propagation were performed using standard techniques [40]. All *S. cerevisiae* transformations were carried out using the lithium acetate method [41]. *AOX1*, *DAS1*, *mdh*, *hps* and *phi* genes were designed with flanking loxPsym sites to enable compatibility with the synthetic yeast genome SCRaMbLE system, however, this system was not tested during this study [42]. The synthetic genes were amplified using PCR and cloned into the pRS416 plasmid digested with SmaI using the NEBuilder® HiFi DNA Assembly Master Mix. The *SFA1*, *TAL1* and *TLK1* genes from *S. cerevisiae* were overexpressed by amplifying the gene and its native terminator from genomic DNA, and cloned under the control of the *TDH3* promoter. These over-expression constructs were cloned into the relevant pRS416 plasmids containing methanol assimilation enzymes, the NEBuilder® HiFi DNA Assembly Master Mix was used for Gibson Assembly [43]. For multiple site integration of *DAS1*, the gene was cloned into the pBDK1 plasmid between the *PGK1* promoter and terminator flanked by Ty1 delta sites, and transformed into yeast as described previously [24], cells were plated on minimal media with 0.5 % glucose and toxic levels of formaldehyde (4 mM) and incubated at 30 °C.

Media and growth conditions

Escherichia coli DH5α cells were used for plasmid propagation/storage and grown in lysogeny broth media (1 % tryptone, 0.5 % yeast extract, 1 % NaCl) with ampicillin.

The strains were pre-cultured on Yeast Nitrogen Base (YNB) medium without amino acids and with 5 g/L ammonium sulfate (Sigma-Aldrich Y0626) and 10 g/L glucose. Growth experiments were performed on YNB medium with 1 or 2 % methanol supplemented with 1 g/L yeast extract (Merck 103753). Optical density readings at 600 nm (OD_{600}) were used to track growth. For spot assays, swabs from streaked agar plates were pre-cultured twice in 10 mL of 1x YNB,

1% glucose in sterile 50 mL Falcon tubes. During the log phase of the final pre-culture, cells were washed twice in 10 mL of sterile MilliQ water and serially diluted 10-fold up to 10^{-4}, prior to spotting 5 µl from each dilution onto the indicated agar plates. Plates were incubated at 30°C for 5 days. Spot assay photos are representative of many repeated experiments.

ambr® 250 Bioreactor Fermentations

Strains were pre-cultured as above in sterile 50 mL Falcon tubes prior to inoculation at an OD_{600} of 0.02 in ambr® 250 (Sartorius Stedim) microbial bioreactors with 140 mL of medium. Dissolved oxygen was maintained at a minimum level of 20 % via automatic control of stirring speed, air sparging, and O_2 sparging. pH was maintained at 5 via the automatic addition of 5 M potassium hydroxide or 1 M phosphoric acid, and temperature was kept at 30 °C. Bioreactors were sampled robotically at indicated time-points, with 1 mL samples stored at -20 °C prior to measurement of growth using OD_{600}.

^{13}C- Methanol Fermentations

Growth experiments in YNB medium supplemented with yeast extract (1 g/L) and ^{13}C-methanol (1 % or 2 %; Sigma-Aldrich 277177) were carried out aerobically (sparging air) at 30°C, agitation of 300 RPM, and with a starting volume of 250 mL of medium using the Multifors 2 bioreactor system (Infors AG). pH was maintained between 4.8 and 5.2 using 3M H_3PO_4. For pre-culturing, biological replicates of each strain were grown in liquid YNB medium for approximately 15 h. Cultures were then passaged into a second pre-culture and grown to mid-exponential phase (OD_{600nm} between 1 and 3). A third pre-culture was also grown to mid-exponential phase and washed twice with sterile water before inoculating the experimental cultures at an OD_{600nm} of 0.02.

Analytical methods

Cell growth was determined by measuring the OD_{600nm} using a UV-spectrophotometer. Extracellular metabolites were obtained from cultures at indicated time points by centrifuging 0.5 – 1 mL of culture at 12,000 x g for 1 min prior to transfer of the supernatant to a fresh microtube. Samples were stored at - 20 °C until analysis. Methanol and extracellular metabolite concentrations were measured using High-Performance Liquid Chromatography (HPLC, Agilent Technologies) with a Hiplex-H column heated at 65 °C, samples ran for 26 min at a flow rate of 0.6 mL/min using 4 mM H_2SO_4 as mobile phase. The detection wavelength was set at 210 nm and Refractive Index was at positive polarity and at 40 °C optical unit temperature. 30 µLs of sample was injected. Standard curves of each analyte were constructed using purified chemicals in water.

Real-time analysis of bioreactor culture off-gas was achieved using a Hiden HPR-20-QIC mass spectrometer (Hiden Analytical) that was connected to the bioreactors. The Faraday Cup detector was used to monitor the intensities of N, Ar, CO_2, ^{13}C-CO_2, ethanol, ^{13}C-ethanol, and ^{13}C-methanol at 28, 40, 44, 45, 27, 47, and 30 amu, respectively. To increase sensitivity and detect the presence of ^{13}C-ethanol from the off-gas data, the Secondary Electron Multiplier (SEM) detector was used to scan any intensities from 15 to 50 amu, with 47 and 48 amu corresponding to ^{13}C-ethanol. N intensity (constant during fermentation as nitrogen was an inert gas in our experiments) at 28 amu was used to normalise the intensity from ^{13}C-ethanol. The SEM detector scanned the intensities during two to six independent cycles for each bioreactor and at two different time points.

Table 2. Plasmids used in this study.

Name	Details	Origin
pRS416	Yeast centromeric plasmid, URA3 marker	Euroscarf [44]
ScMOX3a-416	*pTDH3-AOX1-CYC1t-pTEF1-DAS1-PHO5t-pSSA1-PYC1-ADH1t*-pRS416	This study
ScMOX3a-SFA1-416	*pTDH3-AOX1-CYC1t-pTEF1-DAS1-PHO5t-pSSA1-PYC1-ADH1t- pTDH3-SFA1-SFA1t* -pRS416	This study

ScMOX3e-416	*pTEF1-DAS1-PHO5t-pTDH3-mdh-CYC1t* -pRS416	This study
ScMOX3e2-416	*pTEF1-DAS1-PHO5t-pTDH3-mdh-CYC1t-pTEF2-DAS2-ADH1t*-pRS416	This study
ScMOX3e-SFA1-416	*pTEF1-DAS1-PHO5t-pTDH3-mdh-CYC1t-pTDH3-SFA1-SFA1t* -pRS416	This study
ScMOX3e-TLK1-416	*pTEF1-TKL1-TKL1t-pTEF1-DAS1-PHO5t-pTDH3-mdh-CYC1t*-pRS416	This study
ScMOX3e-TAL1-416	*pSSA1-TAL1-TAL1t-pTEF1-DAS1-PHO5t-pTDH3-mdh-CYC1t*-pRS416	This study
ScMOX3g-416	*pTEF1-hps-ADH1t-pTEF2-phi-PHO5t-pTDH3-mdh-CYC1t*-pRS416	This study
ScMOX3g-SFA1-416	*pTEF1-hps-ADH1t-pTEF2-phi-PHO5t-pTDH3-mdh-CYC1t-pTDH3-SFA1-SFA1t*- pRS416	This study
ScMOX3g-TKL1-416	*pTEF1-TKL1-TKL1t-pTEF1-hps-ADH1t-pTEF2-phi-PHO5t-pTDH3-mdh-CYC1t* -pRS416	This study
ScMOX3g-TAL1-416	*pSSA1-TAL1-TAL1t-pTEF1-hps-ADH1t-pTEF2-phi-PHO5t-pTDH3-mdh-CYC1t* -pRS416	This study

Table 3. *Saccharomyces cerevisiae* strains used in this study.

Name	Genotype, plasmids	Notes	Origin
CEN.PK 113-5D	MATa; ura3-52	Haploid laboratory strain with uracil auxotrophy, mating type 'a'	Euroscarf [44]
CEN01	CEN.PK 113-5D, pRS416	Prototrophic strain with empty *URA3* vector	This study
CEN02	CEN.PK 113-5D, ScMOX3a-416	Strain expressing yeast (XuMP) methanol assimilation pathway	This study
CEN03	CEN.PK 113-5D, ScMOX3a-SFA1-416	Strain expressing yeast (XuMP) methanol assimilation pathway and over-expression of *SFA1*	This study
CEN04	CEN.PK 113-5D, ScMOX3e-416	Strain expressing 'hybrid' (XuMP) methanol assimilation pathway	This study
CEN05	CEN.PK 113-5D, ScMOX3g-416	Strain expressing bacterial (RuMP) methanol assimilation pathway	This study
CEN06	CEN.PK 113-5D, ScMOX3e-TKL1-416	Strain expressing 'hybrid' methanol assimilation pathway and over-expression of *TKL1*	This study
CEN07	CEN.PK 113-5D, ScMOX3e-TAL1-416	Strain expressing 'hybrid' methanol assimilation pathway and over-expression of *TAL1*	This study
CEN08	CEN.PK 113-5D, ScMOX3e2-416	Strain expressing second version of the 'hybrid' methanol assimilation pathway (*mdh-DAS1/DAS2*)	This study
CEN09	CEN.PK 113-5D, ScMOX3g-TKL1-416	Strain expressing bacterial (RuMP) methanol assimilation pathway and over-expression of *TKL1*	This study
CEN10	CEN.PK 113-5D, ScMOX3g-TAL1-416	Strain expressing bacterial (RuMP) methanol assimilation pathway and over-expression of *TAL1*	This study
MOX01	CEN.PK 113-5D, ScMOX3e-SFA1-416	Strain expressing 'hybrid' (XuMP) methanol assimilation pathway and over-expression of *SFA1*	This study
MOX02	CEN.PK 113-5D, ScMOX3g-SFA1-416	Strain expressing bacterial (RuMP) methanol assimilation pathway and over-expression of *SFA1*	This study

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Author contributions

M.I.E and T.C.W conceived the study and designed the experiments. M.I.E performed the experiments. M.I.E, E.M, K.V and R.A.G.G designed and performed the 13C-labelling and bioreactor experiments. C.S, T.C.W, I.S.P and I.T.P participated in the design, support, and coordination of the project. All authors read and approved the final manuscript.

Competing interests

None

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