Biotechnology Notes 4 (2023) 104–111

Contents lists available at ScienceDirect

Biotechnology Notes

journal homepage: www.keaipublishing.com/en/journals/biotechnology-notes/

Metabolic engineering of *Escherichia coli* to utilize methanol as a co-substrate for the production of (*R*)-1,3-butanediol

Qing Sun [a], Dehua Liu [a,b], Zhen Chen [a,b,*]

[a] Key Laboratory of Industrial Biocatalysis (Ministry of Education), Department of Chemical Engineering, Tsinghua University, Beijing, 100084, China
[b] Center for Synthetic and Systems Biology, Tsinghua University, Beijing, 100084, China

ARTICLE INFO

Keywords:
Methanol
(*R*)-1,3-Butanediol
Escherichia coli
Hybrid methanol assimilation pathway

ABSTRACT

Due to its abundance, cost-effectiveness, and high reducibility, methanol has gained considerable attention in the biomanufacturing industry as a nonfood feedstock for the production of value-added chemicals. The range of chemicals that can be derived from methanol, however, remains constrained and is currently in the concept validation phase. This study aimed to develop and evaluate a hybrid methanol assimilation pathway in *Escherichia coli* to improve the production of (*R*)-1,3-butanediol ((*R*)-1,3-BDO) by utilizing methanol and sugars as co-substrates. By combining the methanol dehydrogenase (MDH) from the prokaryotes with the dihydroxyacetone synthase (DAS) from the eukaryotes, the hybrid pathway facilitates methanol conversion into the central metabolism while generating NADH at the same time. Through pathway optimization and targeted gene deletions, we have successfully developed an *E. coli* strain capable of producing 5.79 g/L (*R*)-1,3-BDO in shake flask experiments and 13.71 g/L (*R*)-1,3-BDO with a yield of 0.35 C-mol/C-mol in batch fermentation using methanol and glucose as co-substrates. Our study also showed the incorporation of ^{13}C-methanol into cellular intermediates and an increase in NAD(P)H concentration, confirming the role of methanol as a co-substrate and supplier of NADH. In addition, our study also demonstrated the co-utilization of methanol with xylose for the production of (*R*)-1,3-BDO, expanding the substrate spectrum for sustainable 1,3-BDO production.

1. Introduction

In recent years, methanol has garnered substantial attention in the biomanufacturing industry due to its abundant supply, cost-effectiveness, and high degree of reduction. Since it can be produced from greenhouse gases like CO_2 and methane, methanol is a promising carbon source for producing value-added chemicals toward the goal of carbon neutrality. While the native methylotrophs such as *Methylobacterium extorquens*[1] and *Bacillus methanolicus*[2] can utilize methanol as sole carbon source, their real industrial application has been hindered by the lack of genetic engineering tools and our poor understanding of cellular metabolism and regulation.[3] Consequently, the modification of well-characterized model microbial chassis, like *Escherichia coli*, to serve as synthetic methylotrophs has gained increasing attention.

Native methylotrophs employ various pathways for C1 utilization. Methanol is initially oxidized to formaldehyde by different types of methanol dehydrogenases (MDHs). Formaldehyde is then assimilated through three different pathways, including the ribulose monophosphate pathway (RuMP), the serine cycle, and the xylulose monophosphate pathway (XuMP).[4] The RuMP pathway is regarded as the most energy-efficient pathway, prompting extensive efforts to implement it in *E. coli* or *Corynebacterium glutamicum* for methanol assimilation. Despite significant success in creating synthetic methylotrophs through the RuMP pathway, efficient conversion of methanol into value-added chemicals remains challenging due to several factors including the poor enzyme kinetics of MDHs, metabolic imbalances within the synthetic RuMP cycle, and formaldehyde's toxicity, etc.[5]

The XuMP pathway, which is highly efficient in methylotrophic yeast like *Pichia pastoris*, represents an alternative methanol assimilation pathway. However, its implementation in prokaryotic microorganisms has been limited due to the energy inefficiency of O_2-dependent alcohol dehydrogenase and the generation of toxic H_2O_2. Previous study has shown that expressing a hybrid XuMP pathway via the introduction of NAD-dependent MDH from *Acinetobacter garner* and dihydroxyacetone synthase (DAS) from *Pichia angusta* in *E. coli* increased its biosynthesis in a medium supplemented with methanol.[6] This hybrid XuMP pathway

* Corresponding author. Key Laboratory of Industrial Biocatalysis (Ministry of Education), Department of Chemical Engineering, Tsinghua University, Beijing, 100084, China.
E-mail address: zhenchen2013@mail.tsinghua.edu.cn (Z. Chen).

https://doi.org/10.1016/j.biotno.2023.11.005
Received 4 November 2023; Received in revised form 25 November 2023; Accepted 25 November 2023
Available online 6 December 2023

facilitates the production of the crucial glycolytic metabolite, glyceraldehyde 3-phosphate (GAP), through only two enzymatic steps. The energy efficiency of this process is on par with the well-established RuMP pathway when fructose-6-phosphate aldolase and transaldolase are co-utilized for the regeneration of xylulose 5-phosphate (Xu5P). However, the conversion of methanol into high valued chemicals via the hybrid XuMP pathway had not been fully explored yet.

This study aims to further explore the potential application of the hybrid XuMP pathway for production of a valuable chemical, (*R*)-1,3-butanediol ((*R*)-1,3-BDO). (*R*)-1,3-BDO is a platform chemical serving as an important solvent in the cosmetics industry and a key monomer in the polymer industry. Since (*R*)-1,3-BDO is a highly reduced compound, the production of 1 mol (*R*)-1,3-BDO from sugar consumes 3 mol NAD(P)H[7] (Fig. 1). Hence, the employment of methanol as a co-substrate through the hybrid XuMP pathway could potentially serve a dual purpose. Not only could it act as an economical carbon source, but it could also function as an effective supplier of NADH, thereby enhancing the production of (*R*)-1,3-BDO. In this study, we demonstrated that the production of (*R*)-1,3-BDO from glucose or xylose can be substantially improved by the concurrent utilization of methanol via the hybrid XuMP pathway. The incorporation of ^{13}C-labeled methanol into various cellular intermediates and an increase in intracellular NAD(P)H concentration were also experimentally verified. These findings indicate the potential viability of employing methanol as an economical supplementary feedstock for sustainable production of (*R*)-1,3-BDO through the hybrid XuMP pathway.

2. Materials and methods

2.1. Bacterial strains and plasmids

The bacterial strains and plasmids used in this study are listed in Table 1. *E. coli* DH5α was utilized for routine cloning and *E. coli* W3110 was used as the host for (*R*)-1,3-BDO production. Plasmids including pTrc99a, pCDFDuet, and pRSFDuet were employed for constructing the hybrid XuMP pathway and (*R*)-1,3-BDO biosynthesis pathway. Deletions of the *frmA* and *frmB* genes were accomplished through CRISPR-Cas9-mediated genome editing following the method described by Li et al.[8]

2.2. Plasmids and strains construction

The construction of plasmids pRSFDuet-mdh-das, pTrc99a-mdh-das, and pCDFDuet-mdh-das for the hybrid methanol assimilation pathway involved codon-optimized *mdh* gene encoding methanol dehydrogenase from *Acinetobacter garner* and *das* genes encoding dihydroxyacetone synthase from *Pichia angusta*. These genes were synthesized with a consensus RBS (AAGAAGGAGATATAC) under the control of the Trc promoter and inserted into the corresponding plasmids.

Plasmids pCDFDuet-bld^{L273T}-yqhD-phaA-phaB-pntAB and pRSFDuet-bld^{L273T}-yqhD-phaA-phaB-pntAB were constructed by amplifying the Trc promoter and bld^{L273T}-yqhD-phaA-phaB-pntAB fragment from plasmid pTrc99a-bld^{L273T}-yqhD-phaA-phaB-pntAB by Liu et al.[7] and inserting it into pCDFDuet and pRSFDuet by Gibson assembly method following the standard procedure.[9] A list of all primers used in this study can be found in Supplementary Table 1.

2.3. Medium and culture conditions

For 1,3-BDO production, strains were cultured in a modified MR medium with 4.5 g/L methanol.[7] The modified MR medium comprised $(NH_4)_2HPO_4$ (4 g/L), KH_2PO_4 (6.67 g/L), yeast extract (2.5 g/L), $MgSO_4{\cdot}7H_2O$ (0.8 g/L), citric acid (0.8 g/L), 3-(N-morpholino) propansulfonic acid (MOPS) (20.92 g/L), and 5 mL/L trace metal solution. The trace metal solution contained $FeSO_4{\cdot}7H_2O$ (10 g/L), $CaCl_2{\cdot}2H_2O$ (2 g/L), $ZnSO_4{\cdot}7H_2O$ (2.2 g/L), $MnSO_4{\cdot}4H_2O$ (0.5 g/L), $CuSO_4{\cdot}5H_2O$ (1.0 g/L), $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$ (0.1 g/L), and $Na_2B_4O_7{\cdot}10H_2O$ (0.02 g/L). The final antibiotics used were ampicillin (100 μg/mL), kanamycin (50 μg/mL), and spectinomycin (100 μg/mL). When necessary, 20 g/L glucose, 15 g/L xylose, and different concentration of methanol were added to the MR medium as mentioned in the main text.

Shake-flask fermentations were conducted in 500 mL baffled flasks with 50 mL medium. Inoculation was carried out with 5 % (v/v) seed cultures from overnight precultures in LB medium. Cells were grown at 37 °C and 100 rpm for 48 h. The expression of heterologous pathway enzymes was induced by adding 0.1 mM isopropyl β-D-1-thio-galactopyranoside (IPTG) when the OD_{600} reached approximately 0.6.

Batch fermentations were carried out in 1.3 L T&J MiniBox parallel bioreactors, each containing 500 mL of modified MR medium at 37 °C. Fermentations were controlled at pH 7.0 with the automatic addition of 5 M NaOH, with an aeration rate of 1.0 vvm and an agitation speed of 250 rpm.

2.4. Analytical methods

Cell concentration was determined at an optical density of 600 nm (OD_{600}). Glucose, methanol, (*R*)-1,3-BDO, ethanol, acetate, and other organic compounds were quantified by High-performance liquid chromatography (HPLC) with an Aminex HPX-87H Column (300 × 7.8 mm) by 5 mM sulfuric acid as the mobile phase with a flow rate of 0.8 mL/min at 65 °C.[6] The quantification of formaldehyde was conducted using the Nash reaction.[10] 0125 μL of cell supernatant and 125 μL Nash reagent (5 M ammonium acetate, 50 mM acetylacetone) were mixture thoroughly and incubated at 37 °C for 1 h. The mixtures were measured at 412 nm. A fresh formaldehyde standard solution was prepared daily, and the standard curve ranged from 0 to 100 μM.

2.5. ^{13}C labeling analysis

To carry out the ^{13}C-methanol labeling analysis for intracellular metabolites and free amino acids, the MR medium with 400 mM ^{13}C-methanol and 20 g/L^{12}C-glucose or 15 g/L^{12}C-xylose were used for cultivation. Strains were cultivated at 37 °C and 100 rpm for 48 h. Samples were prepared by standard protocols[11] and injected into the UHPLC-Q-Orbitrap LC-MS (Thermo Fisher Scientific, USA) equipped with the Waters BEH Amide Column (2.1 × 100 mm; 1.7 μm) at 35 °C with solvent A (acetonitrile with 0.1 % formic acid) and solvent B (deionized water with 0.1 % formic acid and 10 mM ammonium acetate) as the mobile phase with a flow rate of 0.3 mL/min[6]. Isotopologue fractions (IFs) and average carbon labeling were calculated according to equations (1) and (2).[12] m_i *and* m_j are the abundance of a mass isotopologue, n is the number of carbon atoms of the metabolite.

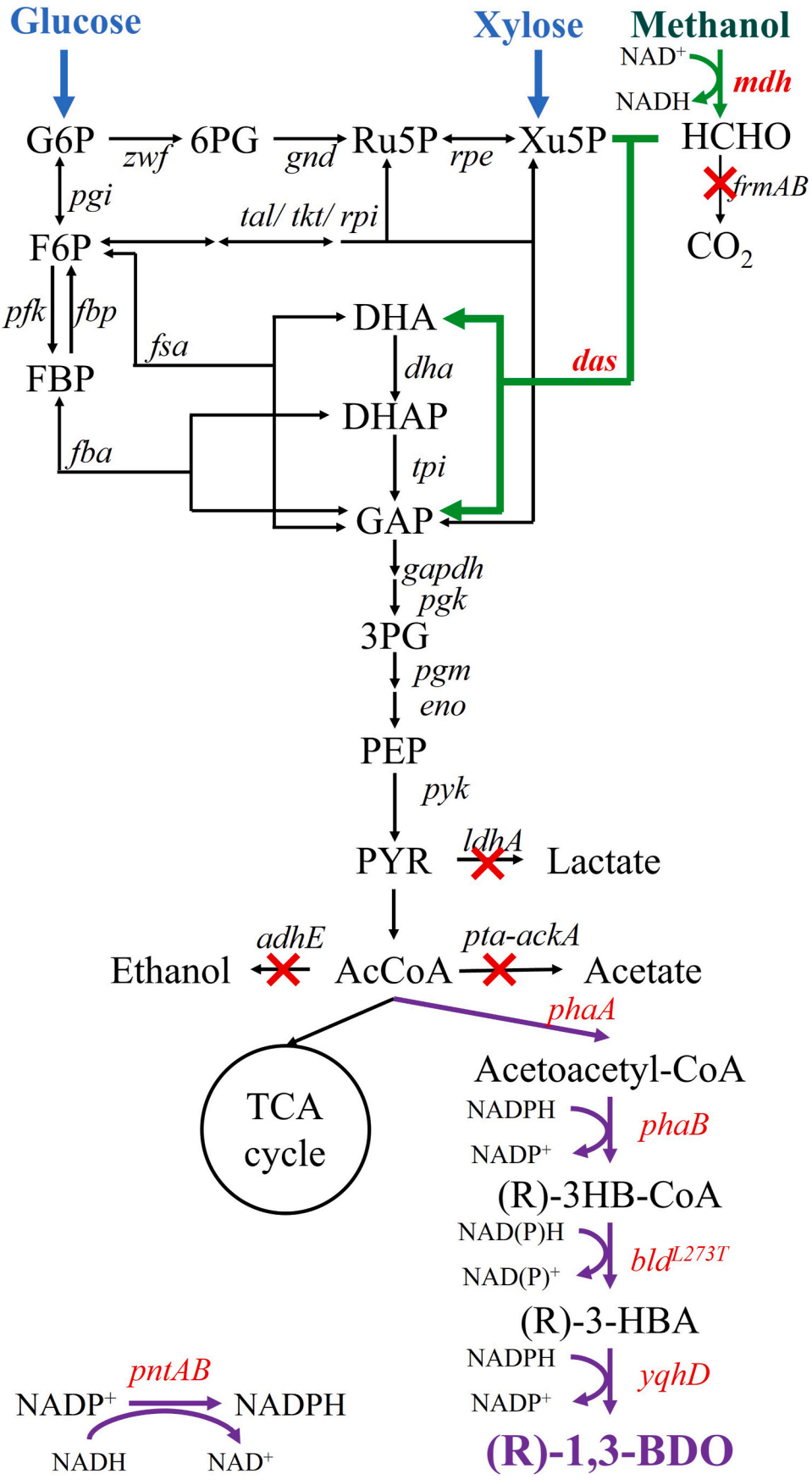
$$\text{Isotopologue fractions} = \frac{m_i}{\sum_{j=0}^{n} m_j} \tag{1}$$

$$\text{Average carbon labeling} = \frac{\sum_{j=0}^{n} m_j}{n * \sum_{j=0}^{n} m_j} \tag{2}$$

3. Results and discussion

3.1. Construction of the hybrid XuMP pathway in E. coli

The hybrid XuMP pathway combines the prokaryotic NAD-dependent MDH with the eukaryotic DAS, resulting in the conversion of methanol and Xu5P into GAP and dihydroxyacetone (DHA), accompanied by the generation of NADH (Fig. 1). Subsequently, DHA can be further transformed into dihydroxyacetone phosphate (DHAP) by the endogenous DHA kinase in *E. coli*. In previous study, we have demonstrated the potential of the hybrid methanol assimilation pathway comprised of the MDH from *A. garner* and the DAS from *P. angusta* to enhance both biomass concentration and growth rate in engineered *E. coli*.[6]



(caption on next page)

Fig. 1. Metabolic engineering strategies used for the construction of an engineered *E. coli* for 1,3-butanediol production from methanol and sugars. The symbol "X'' indicates gene deletion. The amplified targets are shown with thick lines and red characters. Abbreviations: G6P: glucose-6-phosphate; 6 PG: 6-phosphogluconate; Ru5P: ribulose-5-phosphate; Xu5P: xylulose 5-phosphate; F6P: fructose-6-phosphate; FBP: fructose-1,6-diphosphate; DHA: dihydroxyacetone; DHAP: dihydroxyacetone phosphate; GAP: glyceraldehyde-3-phosphate; 3 PG: glycerate-3-phosphate; PEP: phosphoenolpyruvate; PYR: pyruvate; AcCoA: acetyl-coenzyme A; (R)-3HB-CoA: (R)-3-Hydroxybutyryl-CoA; (R)-3-HBA: (R)-3-hydroxybutyraldehyde; (R)-1,3-BDO: (R)-1,3-Butanediol.

Table 1
Strains and plasmids used in this study.

Strain or plasmid	Description	Sources
Plasmids		
pTrc99a	High-copy plasmid, ColE1 ori, Amp^r	Lab stock
pCDFDuet	High-copy plasmid, ColDF13 ori, Sm^r	Lab stock
pRSFDuet	High-copy plasmid, RSF1030 ori, Kan^r	Lab stock
pRSFDuet-mdh-das	pRSFDuet with *mdh* gene from *Acinetobacter garner* and *das* gene from *Pichia angusta* under the control of Tac promoter	This study
pTrc99a-mdh-das	pTrc99a with *mdh* gene from *Acinetobacter garner* and *das* gene from *Pichia angusta* under the control of Tac promoter	This study
pCDFDuet-mdh-das	pCDFDuet with *mdh* gene from *Acinetobacter garner* and *das* gene from *Pichia angusta* under the control of Tac promoter	This study
pTrc99a-bld^{L273T}-yqhD-phaA-phaB-pntAB	pTrc99a containing *bld* gene from *C. saccharoperbutylacetonicum* containing a mutation of L273T, *yqhD* gene from *E. coli*, *phaAB* genes from *C. necator* and *pntAB* genes from *E. coli* under the control of Tac promoter	Liu et al. (2021)
Strains		
W3110	Wild type *E. coli*, ATCC27325	Lab stock
W43	W3110, Δ*adhE* Δ*ldh* Δ*pta-ackA* Δ*yciA* Δ*tesB*	Liu et al. (2021)
S01	W43, harboring pRSFDuet-mdh-das	This study
S02	W43, harboring pTrc99a-mdh-das	This study
S03	W43, harboring pCDFDuet-mdh-das	This study
S04	W43, harboring pTrc99a-bld^{L273T}-yqhD-phaA-phaB-pntAB and pCDFDuet-mdh-das	This study
S05	S04 Δ*frmAB*	This study

To optimize the expression of the hybrid methanol assimilation module, we overexpressed the two pathway genes in three different plasmids: pRSFDuet (copy number >100), pTrc99a (copy number ~40), and pCDFDuet (copy number 20–40). The resulting plasmids were introduced into strain W43 (Δ*adhE*Δ*ldh*Δ*pta-ackA*Δ*yciA* Δ*tesB*), which blocked the synthesis pathways of ethanol, lactate, acetate, and 3-hydroxybutyric acid (3-HB), generating strains S01, S02, and S03. The recombinant cells were then cultured in a modified MR medium containing 20 g/L glucose and 4.5 g/L methanol. As shown in Fig. 2, compared with strain W43, all recombinant strains showed significant methanol consumption and formaldehyde accumulation, confirming the successful expression of the hybrid methanol assimilation module (Fig. 2). Strain S01, which has the highest plasmid copy number (>100), demonstrated the least formaldehyde accumulation (41.9 ± 8.6 μmol/L), the most significant pyruvate accumulation (7.2 ± 0.0 g/L), and retained 6.0 ± 0.0 g/L of unused glucose at 48 h, indicating that the introduction of high copy plasmid disturbed glucose metabolism and reduced methanol consumption. In contrast, strain S02 exhibited a greater formaldehyde accumulation (75.3 ± 6.1 μmol/L) and a higher methanol consumption (1.20 ± 0.02 g/L). However, strain S02 also accumulated 3.84 ± 0.04 g/L of acetate and 1.97 ± 0.17 g/L of pyruvate as byproducts, implying the disturbance of the glucose metabolism. Strain S03 showed moderate methanol consumption (0.77 ± 0.07 g/L) and formaldehyde accumulation (56.4 ± 22.7 μmol/L) without significant accumulations of pyruvate and acetate. These characteristics rendered it more suitable for (*R*)-1,3-BDO production, and strain S03 was thus selected for further investigation.

3.2. Enhanced production of (R)-1,3-BDO driven by methanol

In our previous study, we have successfully established a synthetic pathway for (*R*)-1,3-BDO, which contains four enzymatic steps.[7] In this pathway, two acetyl-CoA molecules are transformed into one (*R*)-1,3-BDO molecule by the sequential action of acetoacetyl-CoA thiolase (encoded by the *phaA* gene), (R)-3-hydroxybutyryl-CoA dehydrogenase (encoded by the *phaB* gene), a mutated butyraldehyde dehydrogenase (encoded by the *bld*^{273T} gene), and alcohol dehydrogenase (encoded by the *yqhD* gene). Given that this pathway necessitates the consumption of three molecules of NAD(P)H, the incorporation of the hybrid methanol assimilation pathway is anticipated to supply both an auxiliary carbon source and NADH, thereby promoting the production of (*R*)-1,3-BDO. Thus, the 1,3-BDO synthesis module plasmid pTrc99a-bld^{L273T}-yqhD--phaA-phaB-pntAB was transformed into strain S03, resulting in the generation of strain S04 (Fig. 3). Upon cultivation in MR medium with 20 g/L of glucose, strain S04 demonstrated a relatively low (*R*)-1,3-BDO production of 1.15 ± 0.02 g/L, accompanied by an accumulation of 7.24 ± 0.11 g/L of acetate and a residual 6.50 ± 0.22 g/L of glucose at 48 h. These observations suggest that the hybrid methanol assimilation pathway interfered with the synthesis of (*R*)-1,3-BDO and the consumption of glucose. When the medium was further fortified with 4.5 g/L methanol, strain S04 exhibited a marginally enhanced (*R*)-1,3-BDO production of 1.30 ± 0.04 g/L (Fig. 3).

To further increase the production of (*R*)-1,3-BDO, we knocked out the glutathione-dependent formaldehyde dehydrogenase gene (*frmA*) and the formylglutathione hydrolase gene (*frmB*) to reduce the dissimilation of formaldehyde into CO_2, generating strain S05. When cultured in MR medium with 20 g/L of glucose, strain S05 was able to consume all glucose and produced 5.41 ± 0.32 g/L of (*R*)-1,3-BDO with significant reduced acetate accumulation. Notably, when the medium was further supplemented with methanol, strain S05 was able to consume 1.41 ± 0.04 g/L of methanol, leading to an enhanced production of (*R*)-1,3-BDO (5.79 ± 0.17 g/L) and 3-HB (1.50 ± 0.12 g/L), compared to the production levels observed in the absence of methanol (5.41 ± 0.32 g/L (*R*)-1,3-BDO and 1.28 ± 0.00 g/L 3-HB). These observations imply that the assimilation of methanol augments the metabolic flux towards the biosynthesis of (*R*)-1,3-BDO or 3-HB.

3.3. ^{13}C-labeling analysis and determination of intracellular NAD(P)H

To ascertain the integration of methanol into central metabolism as a co-substrate, we conducted ^{13}C-labeling analysis using strain S05. Cells were cultivated in MR medium containing 4.5 g/L ^{13}C-methanol and 20 g/L ^{12}C-glucose, and the carbon labeling percentages of intracellular metabolites and amino acids were quantified by LC-MS. The introduction of the hybrid methanol assimilation module yielded relatively low but significant average ^{13}C-labeled Xu5P/Ru5P (0.68 % ± 0.16 %), G6P (0.65 % ± 0.14 %), 3-HB (0.19 % ± 0.03 %), and citric acid (0.31 % ± 0.35 %) in strain S05. This finding strongly suggests that methanol was utilized as a co-substrate and converted into glycolysis, the TCA cycle, and the (*R*)-1,3-BDO synthesis module (Fig. 4C). However, most of these labeled metabolites featured only a single ^{13}C atom (M+1). Notably, M+1 labeled 3-HB accounted for 0.66 % ± 0.15 %, and no detectable ^{13}C-labeled (*R*)-1,3-BDO was observed (Fig. 4A). This observation implies that the C1 regeneration cycle could run only once, and ^{13}C-methanol contributed primarily to 3-HB rather than the final (*R*)-1,3-BDO. The formation of 3-HB may be caused by the hydrolysis of 3-HB-CoA by the endogenous acyl-CoA hydrolases/thioesterases in *E. coli*.[7] Despite the deletion of the *tesB* and *yciA* genes, 3-HB continues to accumulate in strain S05, indicating the existence of other acyl-CoA hydrolases/thioesterases capable of catalyzing the hydrolysis of

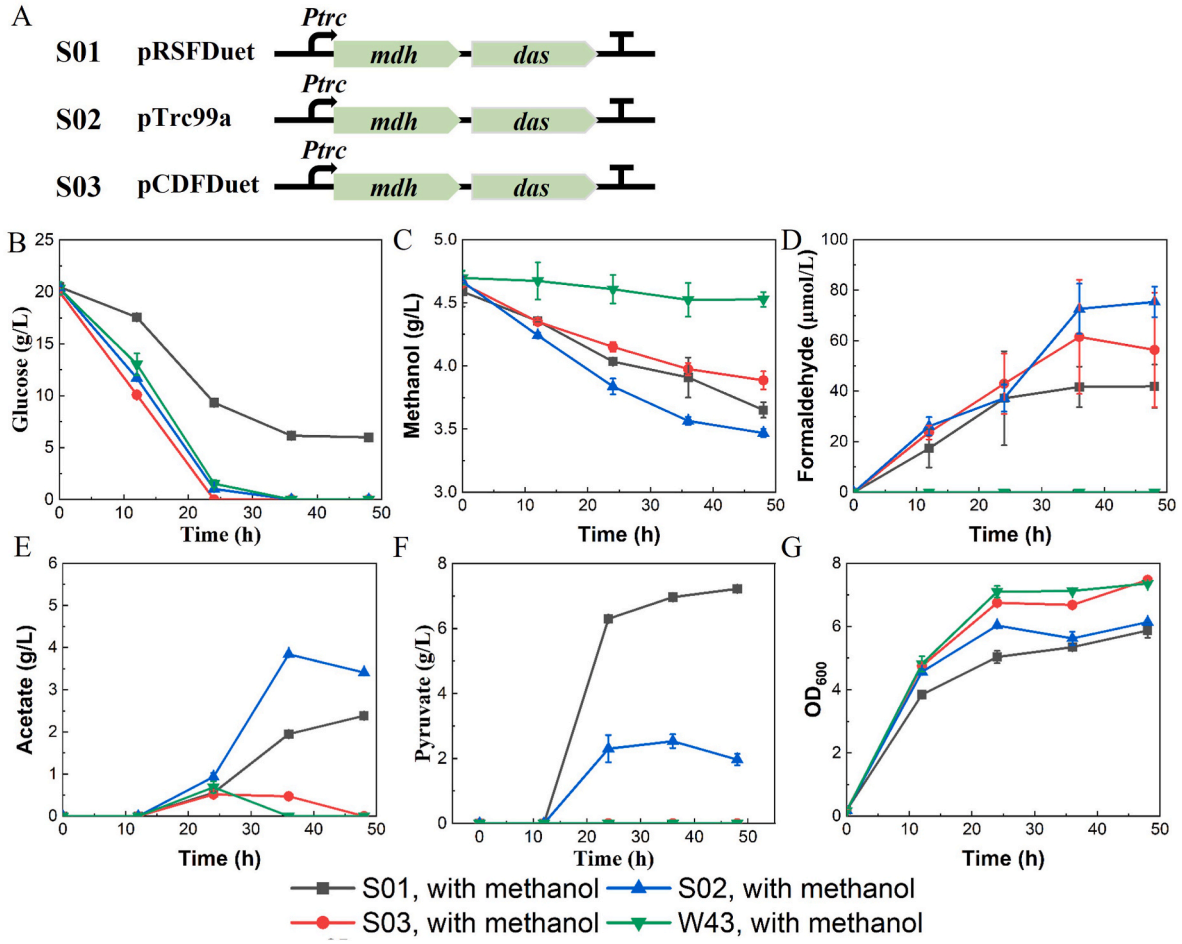


Fig. 2. Construction and optimization of the hybrid methanol assimilation pathway. (A) Plasmid composition; (B) glucose consumption; (C) methanol consumption; (D) formaldehyde accumulation; (E) acetate accumulation; (F) pyruvate accumulation; (G) cell growth (OD_{600}).

3-HB-CoA. The accumulation of ^{13}C-labeled 3-HB may be attributed to the reduced activity of butyraldehyde dehydrogenase possibly due to its sensitivity to oxygen, coupled with the functional activity of endogenous acyl-CoA hydrolases/thioesterases. Furthermore, the average labeling of intracellular free amino acids was also evident in strain S05, including L-methionine (2.49 % ± 0.33 %), L-histidine (1.08 % ± 0.45 %), and L-glutamic acid (0.94 % ± 0.02 %), suggesting that methanol was mainly used for cellular biosynthesis.

Considering that methanol is not directly transformed into (*R*)-1,3-BDO, it was postulated that the augmented availability of NAD(P)H via methanol assimilation may contribute to improved (*R*)-1,3-BDO production given that synthesis of 1 mol 1,3-BDO from glucose consumes 3 mol of NAD(P)H. As illustrated in Fig. 4D, the intracellular pool of NADP(H) and NAD(H) increased by 39 % and 43 %, respectively. Therefore, the enhanced production of 1,3-BDO is primarily attributed to the supplementary NAD(P)H provided by the methanol assimilation process.

3.4. Batch fermentation

To evaluate the practical applicability of strain S05, we conducted batch fermentations in bioreactors. The fermentations were performed in MR medium with 50 g/L glucose and methanol at four different concentrations: 0 g/L, 2 g/L, 4 g/L, and 10 g/L. It was observed that the presence of 2 g/L and 4 g/L methanol markedly amplified both the production and yield of (*R*)-1,3-BDO, affirming the positive impact of methanol assimilation on 1,3-BDO production (Fig. 5). The introduction of 4 g/L methanol yielded the best results with the production of 13.71 g/L of (*R*)-1,3-BDO with a yield of 0.35 C-mol/C-mol. The production and yield of (*R*)-1,3-BDO in the presence of 2 g/L methanol reached 12.39 g/L and 0.34 C-mol/C-mol, respectively. However, when 10 g/L of methanol was added, the production and yield of 1,3-BDO production was reduced with the high accumulation of pyruvate, suggesting that excessive levels of methanol and formaldehyde may exert toxic effects for cellular metabolism.

3.5. Production of (R)-1,3-BDO using methanol and xylose as Co-substrates

Considering that xylose constitutes a significant component of lignocellulosic sugars and its metabolism can furnish a sufficient C1 receptor Xu5P for the XuMP cycle,[13] we extended our investigation to test strain S05 for the co-utilization of methanol and xylose for (*R*)-1,3-BDO production. In shake flask fermentation, strain S05 was able to metabolize 15.0 ± 0.0 g/L of xylose and 1.21 ± 0.06 g/L of methanol, resulting in the generation of 2.35 ± 0.15 g/L of (*R*)-1,3-BDO with an yield of 0.30 C-mol/C-mol (Fig. 6). When compared to cultures that utilized xylose as the exclusive carbon source (yielding 1.99 ± 0.07 g/L of (*R*)-1,3-BDO with a yield of 0.24 C-mol/C-mol), the incorporation of methanol significantly augmented the production and yield of (*R*)-1,3-BDO.^{13}C-labeling analysis showed high incorporation of ^{13}C-methanol into the key metabolites, such as 5.82 % ± 1.84 % of Xu5P/Ru5P with one ^{13}C-atom labeling (M+1) and 7.01 % ± 2.24 % of G6P with one ^{13}C-atom. Additionally, the intracellular pool of NADP(H) and NAD(H) were increased by 80 % and 17 %, respectively, highlighting the key role of augmented reducing power for (*R*)-1,3-BDO synthesis.

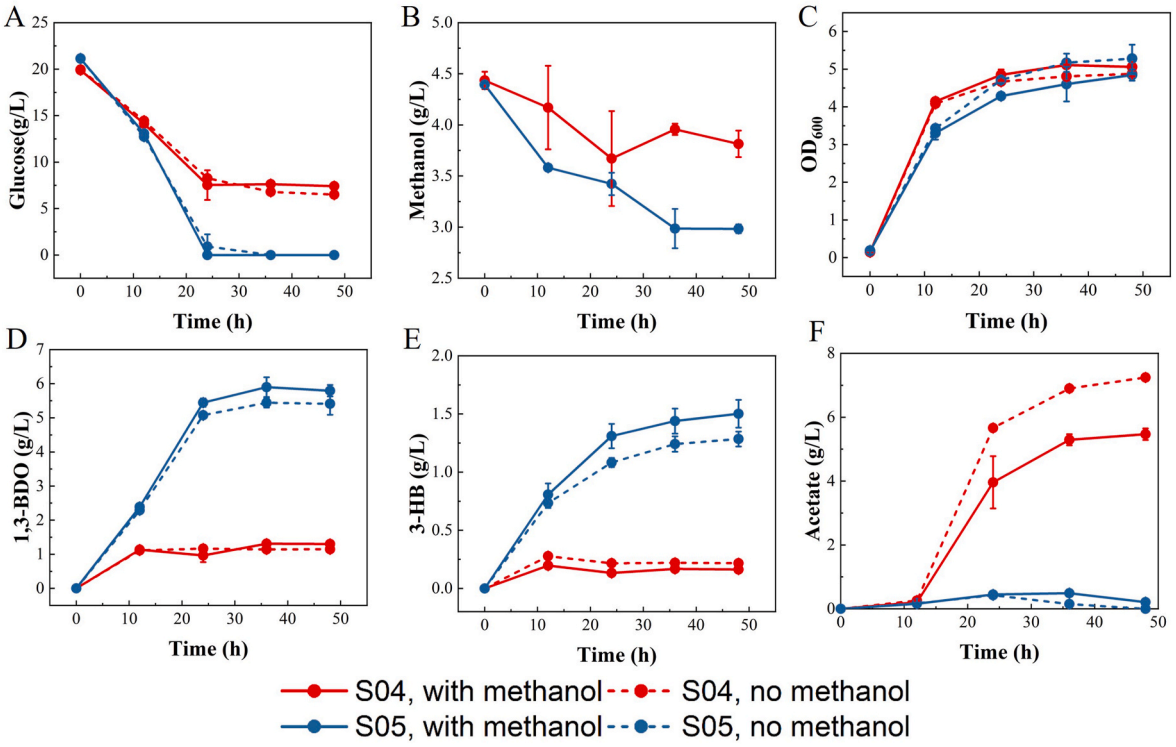


Fig. 3. Production of 1,3-BDO from glucose and methanol by recombinant strains with the integrated 1,3-BDO synthesis module and hybrid methanol assimilation module. (A) Glucose consumption; (B) methanol consumption; (C) cell growth (OD_{600}); (D) 1,3-BDO accumulation; (E) 3-HB accumulation; (F) acetate accumulation.

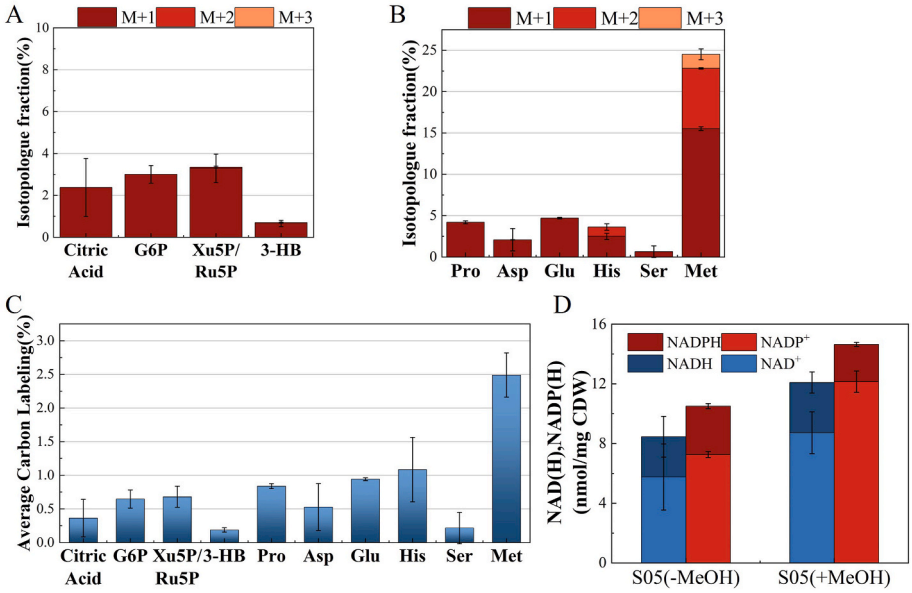


Fig. 4. Accumulation of key metabolites in strain S05. Isotopologue fraction (% ^{13}C) of intracellular metabolites (A), amino acids (B) and corresponding average carbon labeling (C) from ^{12}C-glucose and ^{13}C-methanol. (D) Intracellular concentrations of NAD(H) and NADP(H).

4. Conclusion

The engineering of synthetic methylotrophy for C1 utilization and the production of high-value chemicals presents a significant challenge. In this study, we successfully engineered a synthetic *E. coli* strain by incorporating a XuMP-based hybrid methanol assimilation pathway and a (*R*)-1,3-BDO synthesis pathway. Our findings revealed that the production of (*R*)-1,3-BDO was substantially enhanced through methanol assimilation, thereby establishing methanol as an effective co-substrate and supplier of NADH. By balance the heterologous modules and deletion of targeted gene, an engineered strain can produce 5.79 g/L (*R*)-1,3-BDO in shake-flask and 13.71 g/L (*R*)-1,3-BDO with a yield of 0.35 C-mol/C-mol in batch fermentation by methanol and glucose. The integration of methanol into central metabolites and cellular free amino acids was validated through ^{13}C-labelling analysis. Moreover, the supplementation of methanol led to an increase in the intracellular pools of NADP(H) and NAD(H), thereby affirming the role of methanol in providing additional reducing power. The same engineered strain was

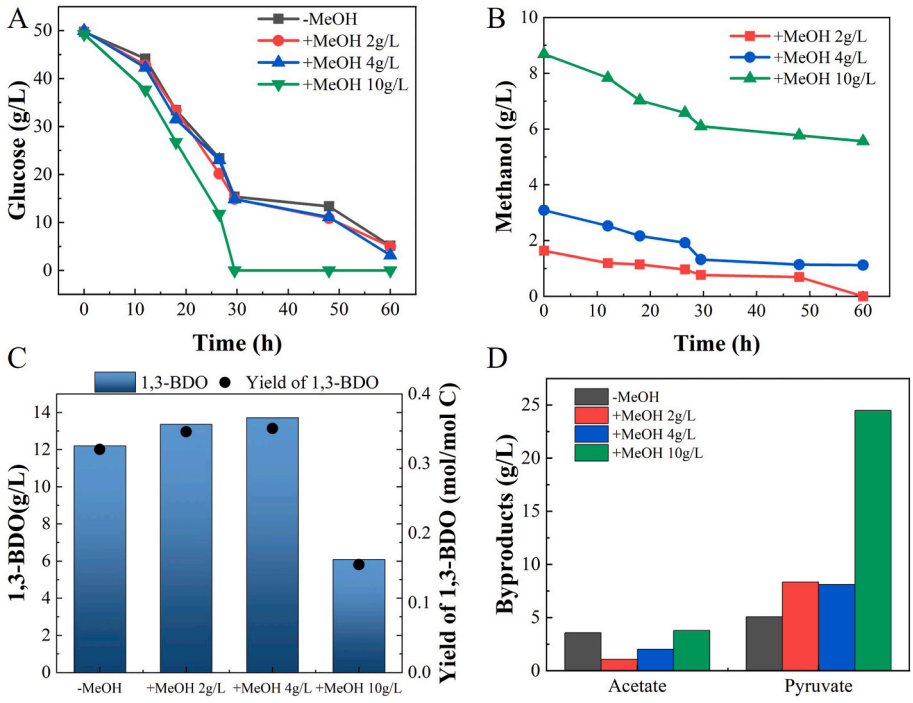


Fig. 5. Batch fermentation of strain S05 in bioreactor. (A) Glucose consumption; (B) methanol consumption; (C) 1,3-BDO production; (D) byproducts accumulation.

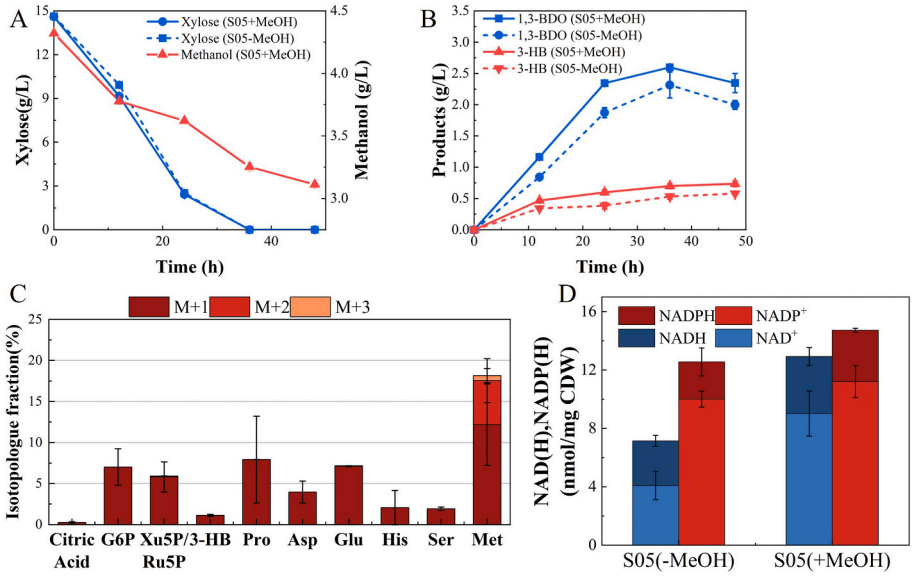


Fig. 6. Production of 1,3-BDO from xylose and methanol by strain S05. (A) Xylose and methanol consumption; (B) 1,3-BDO and 3-HB accumulation; (C) isotopologue fraction (% ^{13}C) of metabolites from ^{12}C-xylose and ^{13}C-methanol; (D) intracellular concentrations of NAD(H) and NADP(H).

also adopted to produce (*R*)-1,3-BDO from xylose and methanol, expanding the substrate spectrum of this system. In summary, this study contributes novel insights into the engineering of synthetic *E. coli* for the production of high-value chemicals via the integration of a hybrid methanol assimilation pathway.

CRediT authorship contribution statement

Qing Sun: Methodology, Investigation, Formal analysis, Data curation, Validation, Writing - original draft, preparation. Dehua Liu: Supervision, Project administration. Zhen Chen: Conceptualization, Investigation, Formal analysis, Writing - review & editing, Funding acquisition, Resources, Supervision, Project administration.

Declaration of competing interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Acknowledgement

This work was supported by the National Key R&D Program of China (No. 2018YFA0901500), the National Natural Science Foundation of

China (Grant Nos. 22078172 and 21938004).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biotno.2023.11.005.

References

1. Yuan XJ, Chen WJ, Ma ZX, et al. Rewiring the native methanol assimilation metabolism by incorporating the heterologous ribulose monophosphate cycle into *Methylorubrum extorquens*. *Metab Eng*. 2021;64:95–110.
2. Brito LF, Irla M, Nærdal I, et al. Evaluation of heterologous biosynthetic pathways for methanol-based 5-aminovalerate production by thermophilic *Bacillus methanolicus*. *Front Bioeng Biotechnol*. 2021;9, 686319.
3. Zhu T, Zhao T, Bankefa OE, Li Y. Engineering unnatural methylotrophic cell factories for methanol-based biomanufacturing: challenges and opportunities. *Biotechnol Adv*. 2020;39, 107467.
4. Zhang W, Zhang T, Wu S, et al. Guidance for engineering of synthetic methylotrophy based on methanol metabolism in methylotrophy. *RSC Adv*. 2017;7(7):4083–4091.
5. Chen FY, Jung HW, Tsuei CY, Liao JC. Converting *Escherichia coli* to a synthetic methylotroph growing solely on methanol. *Cell*. 2020;182(4):933–946.e14.
6. Sun Q, Liu D, Chen Z. Engineering and adaptive laboratory evolution of *Escherichia coli* for improving methanol utilization based on a hybrid methanol assimilation pathway. *Front Bioeng Biotechnol*. 2022;10, 1089639.
7. Liu Y, Cen X, Liu D, Chen Z. Metabolic engineering of *Escherichia coli* for high-yield production of (R)-1,3-Butanediol. *ACS Synth Biol*. 2021;10(8):1946–1955.
8. Li Y, Lin Z, Huang C, et al. Metabolic engineering of *Escherichia coli* using CRISPR-Cas9 meditated genome editing. *Metab Eng*. 2015;31:13–21.
9. Gibson DG, Young L, Chuang RY, Venter JC, Hutchison 3rd CA, Smith HO. Enzymatic assembly of DNA molecules up to several hundred kilobases. *Nat Methods*. 2009;6(5):343–345.
10. Woolston BM, King JR, Reiter M, Van Hove B, Stephanopoulos G. Improving formaldehyde consumption drives methanol assimilation in engineered *E. coli*. *Nat Commun*. 2018;9(1):2387.
11. Long CP, Antoniewicz MR. High-resolution ^{13}C metabolic flux analysis. *Nat Protoc*. 2019;14(10):2856–2877.
12. Keller P, Noor E, Meyer F, et al. Methanol-dependent *Escherichia coli* strains with a complete ribulose monophosphate cycle. *Nat Commun*. 2020;11(1):5403.
13. Clomburg J, Crumbley A, Gonzalez R. Industrial biomanufacturing: the future of chemical production. *Science*. 2017;355, aag0804.