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Engineering the Biological Conversion of Methanol to Specialty Chemicals in Escherichia coli


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Abstract

Methanol is an attractive substrate for biological production of chemicals and fuels. Engineering methylotrophic Escherichia coli as a platform organism for converting methanol to metabolites is desirable. Prior efforts to engineer methylotrophic E. coli were limited by methanol dehydrogenases (Mdhs) with unfavorable enzyme kinetics. We engineered E. coli to utilize methanol using a superior NAD-dependent Mdh from Bacillus stearothermophilus and ribulose monophosphate (RuMP) pathway enzymes from B. methanolicus. Using $^{13}$C-labeling, we demonstrate this E. coli strain converts methanol into biomass components. For example, the key TCA cycle intermediates, succinate and malate, exhibit labeling up to 39%, while the lower glycolytic intermediate, 3-phosphoglycerate, up to 53%. Multiple carbons are labeled for each compound, demonstrating a cycling RuMP pathway for methanol assimilation to support growth. By incorporating the pathway to synthesize the flavanone naringenin, we demonstrate the first example of in vivo conversion of methanol into a specialty chemical in E. coli.

Keywords: Methylotrophy; methanol; E. coli; pathway engineering for substrate utilization

1. Introduction

Methylotrophs are organisms capable of using C1 compounds such as methane and methanol as a carbon and energy source (Whitaker et al., 2015). They represent a polyphyletic microbial group consisting of both Gram negative and Gram positive bacteria as well as methylotrophic yeasts (Anthony, 1982; Lidstrom and Stirling, 1990). Bacteria can be further categorized by the type of enzyme used to oxidize methanol: pyrroloquinoline quinone (PQQ)-
dependent methanol dehydrogenase (Mdh) for Gram⁻ bacteria and NAD-dependent Mdh for Gram⁺ species (Whitaker et al., 2015). In both cases, oxidation of methanol yields the toxic product formaldehyde, which must then be assimilated into central metabolism. Native methylotrophs can be further categorized by the means in which they assimilate formaldehyde: either via the serine pathway, the ribulose monophosphate (RuMP) pathway or the Calvin-Benson-Bassham (CBB) cycle (Anthony, 1982; Chistoserdova et al., 2009; Lidstrom and Stirling, 1990; Whitaker et al., 2015). Both the serine and CBB pathways require an input of energy (ATP), and as such are inferior candidates for engineering synthetic methylotrophy to produce metabolites (Muller et al., 2015; Whitaker et al., 2015). The RuMP pathway consists of two core enzymes: 3-hexulose-6-phosphate synthase (Hps), which fixes formaldehyde to ribulose 5-phosphate (Ru5P) to yield hexulose 6-phosphate (Hu6P), and 6-phospho-3-hexulosisomerase (Phi), which isomerizes Hu6P to fructose 6-phosphate (F6P) (Kato et al., 2006; Yurimoto et al., 2009). There are several variations of this pathway based on how Ru5P is regenerated. Some variations generate 1 ATP and all variations generate 1 NADH per every 3 formaldehyde assimilated (Kato et al., 2006; Whitaker et al., 2015; Yurimoto et al., 2009), making this the most energetically favorable pathway for heterologous hosts such as E. coli.

The ability to produce commodity and specialty chemicals and biofuels from methanol is hindered by the fact that most natural methylotrophs lack well-developed genetic tools for the implementation of extensive synthetic production pathways. At the same time, platform organisms such as Escherichia coli have been extensively engineered for superior industrial growth and production of an enormous range of useful metabolites. Biosynthesis of most useful metabolites produced by industrial organisms requires electrons in the form of NADH, and culture conditions that are largely microaerobic or anaerobic (Papoutsakis, 2015; Whitaker et al.,
2015). As such, native methylotrophs are primarily confined to the production of amino acids and polyhydroxyalkanoates (Schrader et al., 2009). Recently, the serine pathway methylotroph, Methylobacterium extorquens, has been engineered to produce butanol from ethylamine, but not from methanol, though the butanol yields achieved by this strain are significantly lower than recombinant strains of E. coli or native clostridial butanol producers (Hu and Lidstrom, 2014). Thus, there is an impetus to engineer a non-native methanol utilization pathway into industrial microorganisms that would be capable of producing simple and complex metabolites using methanol as a substrate.

Efforts have recently been reported to engineer methanol utilization in Corynebacterium glutamicum (Leßmeier et al., 2015; Witthoff et al., 2015) and E. coli (Muller et al., 2015). For the C. glutamicum strains, methanol utilization was reported only in the presence of glucose. Using Mdh and RuMP genes from B. methanolicus, Muller et al. demonstrated labeling in glycolytic intermediates in E. coli. However, it should be noted that in their study, growth of the recombinant strain in methanol or quantifiable methanol consumption was not presented. Similarly, labeling in TCA cycle intermediates, amino acids or biomass was not presented.

Here we show that expressing a suitable NAD-dependent Mdh along with RuMP enzymes enables an engineered E. coli strain to convert methanol into biomass components and high-value specialty chemicals (Fig. 1). Specifically, we show that expressing the Mdh from Bacillus stearothermophilus in combination with the Hps and Phi from B. methanolicus enables the engineered E. coli strain to utilize methanol in the presence of low concentrations of yeast extract, resulting in superior growth with improved biomass yields. Importantly, we demonstrate, for the first time, that our methylotrophic E. coli utilizes methanol as a growth substrate, which leads to a 30% improvement in biomass when grown on a mixture of yeast
extract and methanol compared to yeast extract alone. We show that $^{13}$C from methanol is present in glycolytic and TCA cycle intermediates, free intracellular amino acids and biomass. To demonstrate the usefulness of this strain for metabolite production, we have also expressed the biosynthetic pathway for the flavonoid naringenin and show methanol-derived naringenin production by the engineered E. coli strain.
Fig 1. Methanol utilization in E. coli. Enzymes required for the assimilation of methanol into central metabolism are shown in red: MDH (methanol dehydrogenase), HPS (3-hexulose-6-phosphate synthase) and PHI (6-phospho-3-hexuloseisomerase). Methanol is oxidized to formaldehyde, which is fixed to the pentose phosphate pathway intermediate, ribulose-5-phosphate (Ru5P), yielding hexulose-6-phosphate (H6P). H6P is then isomerized to fructose-6-phosphate (F6P), which can enter the Embden–Meyerhof–Parnas (EMP) pathway to yield pyruvate and acetyl-CoA for metabolite production or the pentose phosphate pathway to regenerate Ru5P. The native formaldehyde detoxification pathway (formaldehyde to CO₂) has been disrupted by knocking out formaldehyde dehydrogenase (frmA).

2. Methods

2.1. Strains and plasmids

All strains and plasmids used in this study are listed in Supplementary Table 1.

Characterization of the genes required for methanol assimilation in E. coli was performed in a BW25113 ΔfrmA host strain. Methanol assimilation genes were expressed and characterized using the pETM6 vector that has been modified to employ the tac promoter upstream of the multiple cloning site. E. coli DH5α was used to propagate all plasmids, while the BL21star™ (DE3) was used as the host for flavonoid production. The ePathBrick vectors, pETM6, pCDM4 and pACM4 were used as the basis for all plasmid construction and pathway expression (Xu et al., 2012). Appropriate antibiotics were added at the following concentrations: Ampicillin, 80 μg/ml; Streptomycin, 50 μg/ml; Chloramphenicol, 25 μg/ml.

2.2. Genetic manipulations

To insert the tac promoter into the pETM6 and pACM4 vectors, inverse primers (Supplementary Table 2, primers 8 and 9) containing the tac promoter sequence were designed to amplify the vectors, omitting the existing T7 promoter. The resulting PCR products were
restriction endonuclease digested and re-circularized, yielding pM6tac. The methanol utilization pathway genes and their transcriptionally varied mutants were constructed using standard ePathBrick and ePathOptimize cloning procedures (Jones et al., 2015b; Xu et al., 2012). The flavonoid pathway was cloned from pETM6-At4CL-PhCHS to pCDM4 and pACM4 by digestion with ApaI and NheI (FastDigest, Thermo Scientific), gel purification (E.Z.N.A. MicroElute Gel Extraction Kit, Omega Bio-tek), and ligation (Rapid DNA Ligation, Thermo Scientific). Colonies were screened by restriction digest. Transcriptionally varied ePathOptimize mutants were sequenced (GENEWIZ, Inc.) to determine the specific promoters controlling expression of each gene in the pathway using primers 1-3, Supplementary Table 2. Sequencing results for ePathOptimize mutants are shown in Supplementary Table 1.

2.3. Media and growth conditions

E. coli strains were cultured in Luria Bertani (LB) medium or M9 minimal medium for methanol consumption and/or metabolite analysis. Methylomonas L3 was cultured as described previously (Chu and Papoutsakis, 1987). Pre-culture conditions for the methanol-assimilating strains were as follows: a single colony was picked from a plate and grown in M9 minimal medium supplemented with 5 g/L of growth substrate (glucose, xylose, tryptone or yeast extract). After overnight growth, these cultures were pelleted, washed and re-suspended in 100 mL M9 minimal medium supplemented with 60 mM methanol plus 1 g/L of an additional growth substrate and incubated in a 500 mL baffled flask at 37°C with shaking (225 rpm). The optimal induction point was determined to be 4.5 hours post inoculation for all growth experiments in BL21star™ (DE3). Scale-up batch cultures were performed as above, except that cultures were grown in 1.5 L in a 4 L bioreactor (Bioflow II and 110, New Brunswick Scientific, Edison, NJ,
USA). The pH was monitored and adjusted to 7 using 2 N NaOH, temperature was maintained at 37°C, agitation was maintained at 200 rpm and airflow was maintained at ca. 0.67 vvm. Methanol was maintained at ca. 80 mM by feeding methanol every 24 hours. The methanol evaporation rate, along with any native methanol oxidation, was determined from cultures of the empty vector control strain (ΔfrmA pM6tac), and the data were used to adjust the biomass yield and uptake rate of methanol in the methanol-assimilating strain in both flask cultures and bioreactors.

To quantify the effect of methanol on the recombinant E. coli strain, the following growth parameters were calculated. To calculate the biomass yield on a substrate, S, (e.g., YE) \((Y_{SX}; \text{gCDW/gS})\), Eq. 1 was employed, where X and S represent the concentrations of biomass and substrate, respectively. Cell dry weight (CDW; in g/L) was determined using the conversion factor (Soini et al., 2008): 1 OD_{600} unit = 0.33 CDW. For biomass yield on methanol \((Y_{MX}; \text{gCDW/gMeOH})\), Eq. 2 was employed, where M represents the concentration of methanol. \(Y_{MX}\) was based on the assumption that the total methanol consumption accounted for all of the additional biomass in co-substrate cultures. For the specific methanol uptake rate \((q_M; \text{gMeOH/gCDW·h})\), Eq. 3 was employed, which determined methanol uptake of resting cells.

\[
Y_{SX} = \frac{\frac{dX}{dt}}{\frac{dS}{dt}} = \frac{X}{S} \text{ (gCDW/gS)} \tag{Eq. 1}
\]

\[
Y_{MX} = \frac{(\frac{dX}{dt})^{+\text{MeOH}} - (\frac{dX}{dt})^{-\text{MeOH}}}{(\frac{dM}{dt})^{+\text{MeOH}} - (\frac{dM}{dt})^{-\text{MeOH}}} = \frac{X^{+\text{MeOH}} - X^{-\text{MeOH}}}{M} \text{ (gCDW/gMeOH)} \tag{Eq. 2}
\]

\[
q_M = \frac{\frac{dM}{dt}}{X} = \frac{M}{Xt} \text{ (gMeOH/gCDW·h)} \tag{Eq. 3}
\]
2.4. Culture conditions for naringenin production

Preparation of overnight cultures was carried out as reported above. After 14 hours of growth, the overnight culture was used to inoculate 20 mL of media at 4% (v/v) and grown at 37°C until induction with 1 mM IPTG. At the induction point, 25 mg/L of p-coumaric acid precursor, dissolved in DMSO, was also added and the cultures were transferred to either 30 or 37°C for 48 hours. The optimal induction point was determined to be 0.5 hours post inoculation for all naringenin production experiments.

2.5. Analytical methods

Cell growth was determined by measuring the OD600 using a Beckman-Coulter DU370 spectrophotometer. Methanol and secreted metabolite concentrations were measured via high performance liquid chromatography (HPLC; Agilent 1200 series) with an Aminex HPX-87H column (Bio Rad) as described (Yuan et al., 2014).

Intracellular metabolites were extracted and analyzed as follows. Samples were taken at several time points throughout the culture and frozen at -20°C. For extraction, 1 mL of 70% (v/v) aqueous ethanol was heated to 70°C, added to each frozen cell pellet and vortexed for 30 seconds. This mixture was placed at 95°C for 5 minutes and then cooled on ice for 5 minutes. The sample was centrifuged for 5 minutes at 14,000 rpm. The supernatant was removed and dried under nitrogen at 45°C. To derivatize the samples, 50 µL of MTBSTFA + 1% tBDMS and 35 µL of pyridine were added to the sample and incubated for 30 minutes at 60°C. The derivatized samples were centrifuged for 5 minutes at 14,000 rpm and 50 µL was transferred to a GC injection vial. Biomass hydrolysis and derivatization were performed as follows. 500 µL of
6 N HCl was added to the cell pellet and heated at 110°C for 24 hours. Samples were then dried under air at 65°C. After drying, samples were dissolved in 35 µL of pyridine and 50 µL of MTBSTFA + 1% tBDMS and incubated for 30 minutes at 60°C. Samples were centrifuged for 5 minutes at 14,000 rpm and 50 µL was transferred to a GC injection vial. GC-MS analysis was performed as described previously (Long and Antoniewicz, 2014).

Naringenin analysis was carried out with slight modification from published methods (Jones et al., 2015b). Briefly, fermentation broth was mixed with an equal volume of absolute ethanol and vortexed for 10 seconds prior to centrifugation (10 minutes, 20,000 x g). The supernatant (25 µL) was used for HPLC analysis, which was carried out using an Agilent 1200 series HPLC equipped with a ZORBAX SB-18 column (5 µm, 4.6×150 mm) and a diode array detector. Absorbance at 280 nm was monitored for all cases. The mobile phase was acetonitrile (solvent A) and water (solvent B) (both contain 0.1% formic acid) at a flow rate of 1 mL/min. HPLC program was as follows: 10 to 40% A (0–10 minutes) and 40 to 60% A (10–15 minutes). Product titers were determined using authentic standards with retention times: naringenin (12.8 minutes) and p-coumaric acid (7.5 minutes).

Flavonoids were extracted for LCMS analysis from 20 mL of cell culture with 3 mL of ethyl acetate, and 1 mL of the organic layer was dried using a centrifugal concentrator (Thermo Scientific SpeedVac Savant) maintained at 60°C for 1 hour. The resulting pellet was re-suspended in 200 µL of pure methanol and 5 µL was injected for analysis. LCMS analysis was carried out using an Agilent 1200 series HPLC equipped with a ZORBAX SB-18 column (5 µm, 4.6×150 mm) and a LTQ-ORBITRAP XL mass spectrometer detector. The mobile phase was acetonitrile (solvent A) and water (solvent B) (both contain 0.1% formic acid) at a flow rate of 250 µL/min. HPLC program was as follows: 40 to 90% A (0–15 minutes), 90-40% A (15-15.1
minutes) and 40% A (15.1–20 minutes). The instrument was operated in negative ion mode with 2-ppm mass accuracy. Significance of data was determined using a two-tailed unpaired t-test with 95% confidence interval.

$^{13}$C labeling was determined from the measured mass isotopomer data. First, the mass isotopomer distributions were corrected for natural isotope abundance using the method by Fernandez and colleagues (Fernandez et al., 1996). Next, average $^{13}$C labeling was determined as follows: $^{13}$C-labeling (%) = sum(Mi*i)/n, where n is the number of carbon atoms for the measured fragment and Mi is the corrected mass isotopomer abundance. For naringenin analysis, n is the maximum number of carbon atoms that can be labeled (6 carbons). For GC-MS fragments, n is the number of carbon atoms originating from the measured metabolite, i.e. carbon atoms due to the derivatization reagent are not counted.

2.6. in vitro enzyme assays

Cell extracts from cultures harvested during the indicated time points were used for enzyme assays. Briefly, cell pellets were washed and suspended in potassium phosphate buffer (0.1 M, pH 7) containing 2-mercaptoethanol (2 mM), then lysed via sonication (Fisher Scientific Model 505 Sonic Dismembrator, Qsonica Cup Horn) at 40% amplitude for 10 minutes (30 seconds on/off cycles). Resulting cell debris was removed via centrifugation at 13,000 x g for 10 minutes at 4°C, and the remaining supernatant was used as cell extract.

Mdh from exponential Methylomonas L3 cultures was assayed as reported (Day and Anthony, 1990). Briefly, 0.1 M, pH 9 tris-HCL buffer, 15 mM ammonium chloride, 87 µM 2,6-dichlorophenolindophenol (DCPIP), 10 mM methanol and cell extract were combined in a 1 mL
cuvette. The reaction was initiated by addition of 1.1 mM phenazine ethosulfate and the decrease in absorbance at 600 nm was measured at 30°C in a spectrophotometer. Enzyme units were calculated as μmol DCPIP reduced per minute and normalized to mg of cell lysate.

Mdh from B. stearothermophilus and Mdh2 from B. methanolicus were assayed by the method of Muller et al. Recombinant enzymes were obtained from exponential cultures of E. coli ΔfrmA housing the respective genes in pM6tac and grown in M9 minimal media supplemented with 1 g/L yeast extract. The reaction mixture consisted of 50 mM, pH 7.4 K₂HPO₄, 5 mM MgSO₄, 1 mM NAD⁺ and 250-400 μg cell extract in a 1 mL quartz cuvette. The reaction was initiated by addition of 1 M methanol and the increase in absorbance at 340 nm was measured at 37°C in a spectrophotometer. Enzyme units were calculated as μmol NADH produced per minute and normalized to mg of cell lysate.

Hps activity from exponential Methylomonas L3 cultures was determined based on a previously described assay (Kato, 1990). Briefly, the assay contained the following: 50 mM potassium phosphate buffer (pH 7.5), 5 mM magnesium chloride, 5 mM D-ribose-5-phosphate (sodium salt, Sigma Aldrich), cell lysate and 2 U phosphoriboisomerase (Sigma Aldrich). This mixture was incubated at 30°C for 10 minutes to generate ribulose-5-phosphate. Hps activity was started by addition of 2 mM formaldehyde and incubated at 37°C. The reaction was stopped by the addition of 0.1 mL of 0.1 M HCl and formaldehyde concentrations were determined (Nash, 1953). Enzyme units were calculated as μmol formaldehyde consumed per minute and normalized to mg of cell lysate.

Hps from B. methanolicus was assayed by the method of Muller et al. Recombinant enzymes were obtained from exponential cultures of E. coli ΔfrmA housing the respective genes
in pM6tac and grown in M9 minimal media supplemented with 1 g/L yeast extract. Briefly, the assay contained the following: 50 mM potassium phosphate buffer (pH 7.4), 5 mM magnesium chloride, 5 mM formaldehyde, 5 mM D-ribose-5-phosphate (sodium salt, Sigma Aldrich) and 2 U phosphoriboisomerase (Sigma Aldrich). This mixture was incubated at 37°C for 15 minutes to generate ribulose-5-phosphate. Hps activity was started by addition of cell lysate and incubated at 37°C. The reaction was stopped by the addition of 0.1 ml of 0.1 M HCl and formaldehyde concentrations were determined (Nash, 1953). Enzyme units were calculated as µmol formaldehyde consumed per minute and normalized to mg of cell lysate.

2.7. in vivo enzyme assays

ΔfrmA strains expressing either B. stearothermophilus Mdh or B. methanolicus Mdh2 on pM6tac were grown from a colony in LB for 8 hours at 37°C with shaking (225 rpm). Cells were then washed twice in M9 minimal medium and adjusted to an OD$_{600nm}$ of 4 in M9 minimal medium and methanol was added at a final concentration of 60 mM. At the indicated time points, samples were collected and 400 µL of culture supernatant was mixed with 800 µL of Nash reagent to assay for the production of formaldehyde (Nash, 1953).

Activity of the RuMP genes was assessed by growing the ΔfrmA strain expressing RuMP genes on the pM6tac vector in LB as above for 8 hours at 37°C with shaking (225 rpm). The culture was then washed twice with M9 minimal medium and adjusted to an OD$_{600nm}$ of 1 in M9 minimal medium supplemented with 0.5 mM formaldehyde. At the indicated time points, samples were collected and 400 µL of culture supernatant was mixed with 800 µL of Nash reagent to assay for the consumption of formaldehyde (Nash, 1953).
3. Results

3.1. Identification of a thermophilic NAD-dependent Mdh with a low $K_m$ toward methanol for engineering a methylotrophic E. coli

As already discussed, to engineer a methylotrophic E. coli strain for the production of metabolites, the most logical choice is an NAD-dependent Mdh for three reasons: biosynthesis of the electron carrier PQQ requires oxygen (Puehringer et al., 2008), E. coli cannot natively synthesize PQQ and reduced PQQ cannot carry and donate high-energy electrons as universally as NADH. NAD-dependent Mdhs have thus far been found in thermophilic Gram$^+$ Bacillus sp. that have optimal growth temperatures outside the normal growth range of E. coli. The thermodynamics for NAD-dependent methanol oxidation are unfavorable at low temperatures but become more favorable at higher temperatures (Whitaker et al., 2015). Still, it has been shown that the NAD-dependent Mdhs from B. methanolicus can be expressed in E. coli at 37°C and are functional. We confirmed that Mdhs from B. methanolicus expressed in E. coli can function in vivo under normal physiological conditions for E. coli (data not shown). However, these enzymes have a $K_m$ for methanol of ca. 200 mM (Krog et al., 2013), which is near the concentration where methanol exerts a bactericidal effect on E. coli (Supplementary Fig. 1). In any case, under practical operating conditions, substrate concentrations in fermenters are maintained at relatively low levels, typically below 80-100 mM and frequently lower in order to achieve complete substrate utilization and minimize the evaporation of volatile compounds such as methanol (Papoutsakis, 2015). Therefore, we aimed to identify a more suitable Mdh to engineer methylotrophic E. coli strains. The Gram$^+$ thermophile B. stearothermophilus encodes a
methanol dehydrogenase that shares 21-23% amino acid identity with Mdhs from B. methanolicus. We expressed the codon optimized B. stearothermophilus Mdh (Supplementary Table 3) in E. coli to confirm enzyme activity. Purification of this Mdh yields a $K_m$ of 20 mM and $V_{\text{max}}$ of 2.1 U/mg for methanol based on previous literature (Sheehan et al., 1988). Taken together, these characteristics are improvements upon the kinetic properties of the B. methanolicus Mdhs (Krog et al., 2013). Furthermore, this Mdh functions well in E. coli at 37°C, as we observe high rates of formaldehyde production in vivo when cells are exposed to methanol (Fig. 2a).

3.2. in vivo assays in an E. coli ∆frmA strain to identify hps and phi genes enabling fast formaldehyde assimilation

For reasons already discussed, we have chosen the RuMP pathway downstream of methanol oxidation to engineer synthetic methylotrophy in E. coli. Enzymes comprising the RuMP pathway have previously been expressed in E. coli and other species aiming to either utilize formaldehyde as a co-substrate or enhance resistance to formaldehyde (Koopman et al., 2009; Orita et al., 2007). In particular, a Mycobacterium gastri Hps and Phi fusion and B. methanolicus Hps and Phi were previously shown to be functional in E. coli (Muller et al., 2015; Orita et al., 2007). Using an E. coli ∆frmA background strain, we measured 0.058 ± 0.013 mM, or 0.174 ± 0.039 mmol/gCDW, formaldehyde consumption in 60 minutes, which likely results from non-specific dehydrogenase activity (Fig. 2b). When we expressed the M. gasti RuMP fusion in this strain, we observed that formaldehyde consumption increased to 0.074 ± 0.001 mM, or 0.222 ± 0.003 mmol/gCDW, in 60 minutes, demonstrating 0.016 mM, or 0.048
mmol/gCDW, formaldehyde assimilation by RuMP enzymes (Fig. 2b). We then expressed codon optimized hps and phi genes from B. methanolicus (Supplementary Table 3) in the E. coli ∆frmA strain and compared this strain’s ability to consume formaldehyde with the strain expressing the M. gastri fusion gene. The strain expressing the B. methanolicus RuMP genes consumed 0.113 ± 0.002 mM, or 0.339 ± 0.006 mmol/gCDW, formaldehyde in 60 minutes, demonstrating 0.055 mM, or 0.165 mmol/gCDW, formaldehyde assimilation by RuMP enzymes, which represents a nearly 2.5-fold improvement over the M. gastri RuMP fusion (Fig. 2b). These results are similar to the data reported by Muller et al. for the B. methanolicus RuMP enzymes, which showed that an E. coli ∆frmA strain expressing the B. methanolicus RuMP genes was able to consume about 0.1 mM, or 0.3 mmol/gCDW, formaldehyde in 60 minutes in the absence of ribose. Higher in vivo activity in our strain may be attributed to the use of codon optimized genes compared to the native sequences of the hps and phi genes.
Fig 2. Activities of methanol utilization enzymes. (A) Activity of B. stearothermophilus Mdh (BsMdh; green circle), B. methanolicus Mdh2 (BmMdh2; red triangle) and empty vector control (Empty; blue square) was assessed in vivo by analyzing formaldehyde formation in E. coli ΔfrmA in M9 minimal media supplemented with 60 mM methanol. (B) Activity of B. methanolicus RuMP (BmRuMP; red bars), M. gastri RuMP (MgRuMP; green bars) and empty vector control (Empty; blue bars) was assessed in vivo by analyzing formaldehyde consumption in E. coli ΔfrmA in M9 minimal media supplemented with 0.5 mM formaldehyde. (C) Mdh in vitro activities were compared between lysates of recombinant E. coli strains expressing methanol utilization genes and Methylomonas L3. (D) Hps in vitro activities were compared between lysates of recombinant E. coli strains expressing methanol utilization genes and Methylomonas L3. Error bars indicate standard deviation (n≥3).
3.3. *in vitro* Mdh and Hps activities in the engineered *E. coli* ΔfrmA strain are similar to those of a fast-growing native methylotroph, *Methylomonas L3*

We cloned the codon optimized genes for the *B. stearothermophilus* Mdh or *B. methanolicus* Mdh2 and *B. methanolicus* RuMP pathway onto the pM6tac vector using a monocistronic configuration (Supplementary Fig. 2). These vectors were transformed into the *E. coli* ΔfrmA strain. Cultures of these strains were grown for 4 hours in M9 minimal medium containing 1 g/L yeast extract and 60 mM methanol. After 4 hours, cells were harvested, lysed and Mdh and Hps activities were determined (Figs. 2c-d). *E. coli* expressing the *B. stearothermophilus* Mdh alone or along with the RuMP pathway exhibited Mdh activities of 17.0 ± 0.9 and 19.8 ± 2.3 mU/mg, respectively. *E. coli* expressing the *B. methanolicus* Mdh2 alone or with the RuMP pathway exhibited Mdh activities of 7.2 ± 0.1 and 3.3 ± 0.0 mU/mg, respectively. *E. coli* expressing both the *B. stearothermophilus* Mdh and RuMP pathway was observed to have 4.2 ± 0.5 U/mg of Hps activity, and the strain expressing the *B. methanolicus* Mdh2 and RuMP pathway was observed to have 2.9 ± 1.3 U/mg of Hps activity. These values are similar to those of a fast-growing native methylotroph, *Methylomonas L3* (28 ± 5 mU/mg for Mdh and 5.9 ± 0.8 U/mg for Hps, respectively (Figs. 2c-d)) (Bussineau and Papoutsakis, 1986; Hirt et al., 1978). Additionally, the Mdh activities of our *E. coli* strains are similar to, if not better than, previously engineered strains of *E. coli* expressing MdhS from *B. methanolicus* (1.4 – 10 mU/mg) (Muller et al., 2015). These data indicate that the methylotrophic enzymes required for methanol utilization are functionally expressed in our engineered *E. coli* strain and that the activities are similar to those of native methylotrophs.
3.4. Effective methanol assimilation by the engineered E. coli strain can be enhanced in the presence of small amounts of yeast extract or tryptone

Our engineered E. coli strain (BW25113 ΔfrmA expressing B. stearothermophilus Mdh and B. methanolicus RuMP) could not initiate growth on methanol alone in a defined medium. Thus, we hypothesized that small amounts of defined or undefined supplements might enable initiation of growth on methanol. To test this hypothesis, we utilized M9 minimal medium with 60 mM methanol supplemented with 1 g/L of either yeast extract, tryptone, glucose or xylose. As controls, the strain was also grown on the aforementioned substrates in the absence of methanol. Thus, any improvements observed when methanol was present would derive from the contribution of the assimilated methanol into biomass formation. Additionally, we analyzed the growth of a ΔfrmA strain containing the empty pM6tac vector with and without methanol to account for methanol evaporation and any possible native methanol oxidation by E. coli.

When the engineered E. coli strain was grown in M9 minimal medium with 1 g/L yeast extract, we observed a maximum OD₆₀₀ of 0.674 ± 0.018 from a starting OD₆₀₀ of 0.097 ± 0.006. However, in the presence of 60 mM methanol, the cell concentration reached a maximum OD₆₀₀ of 0.872 ± 0.035 after 72 hours of growth from a starting OD₆₀₀ of 0.096 ± 0.007, representing a 30% increase in OD₆₀₀ (Fig. 3c; Table 1). The control culture, i.e. yeast extract only, reached stationary phase at ca. 8 hours of growth due to exhaustion of nutrients in the yeast extract. When 60 mM methanol was present, the culture did not reach stationary phase until ca. 72 hours, indicating that between 8 to 72 hours, growth was sustained by the assimilation of methanol. During this period, ca. 10 mM of methanol was consumed at a rate of 0.019 ± 0.001 gMeOH/gCDW·h (Fig. 3c; Table 1). Furthermore, no difference in growth was observed in the strains harboring either the empty vector or the B. methanolicus Mdh2 and RuMP (Figs. 3a-b),
and minimal methanol consumption/loss was observed for these cultures as well (0.0008 gMeOH/L·h or 0.0034 gMeOH/gCDW·h for the empty vector). Given that we could quantify the amount of methanol consumed by the engineered E. coli strain, we determined the amount of biomass derived from methanol to be 0.289 ± 0.028 gCDW/gMeOH (Table 1), which is within the typical range of biomass yields reported for native methylotrophs (Chu and Papoutsakis, 1987; Pluschkell and Flickinger, 2002). We also used bioreactor experiments to examine if this finding could be sustained at a larger scale. In this system, we observed a similar, significant increase in biomass produced by the engineered E. coli strain grown in the presence of the yeast extract and methanol (Fig. 3d; Table 1). Biomass yields on methanol were similar to the shake flask experiments, indicating that the observable methanol contribution to biomass will scale-up.

We also found that 1 g/L tryptone could stimulate growth on methanol and increase OD$_{600}$ by nearly 20% (Table 1), resulting in a biomass yield of 0.294 ± 0.022 gCDW/gMeOH. While not as effective a supplement as yeast extract, tryptone components, which are practically free amino acids, are also capable of initiating and promoting growth on methanol.
Fig 3. Growth characteristics and methanol consumption of E. coli ΔfrmA strains expressing methanol utilization genes in pM6tac. The ΔfrmA strains harboring either the empty vector, pM6tac (A), pM6tac_BmRuMP_BmMdh2 (B) or pM6tac_BmRuMP_BsMdh (C, D) were grown in M9 minimal medium supplemented with 1 g/L yeast extract (OD; blue square) or M9 minimal medium supplemented with 1 g/L yeast extract and methanol (OD; green circle) in baffled flasks (A-C) or bioreactors (D). Methanol (red triangle) was quantified via HPLC analysis of the culture supernatant and biological consumption was determined by subtracting out evaporation in bioreactors. Error bars indicate standard deviation (n≥3) for flask cultures and standard error of the mean (n=2) for bioreactor cultures.

Table 1. Biomass yields and methanol uptake rates of E. coli ΔfrmA strains expressing methanol utilization genes in pM6tac and grown in M9 minimal medium supplemented with 1 g/L yeast extract in the absence (-MeOH) or presence (+MeOH) of methanol. Data correspond to Fig. 3. Standard deviation (n≥3) is presented for flask cultures and standard error of the mean (n=2) is presented for bioreactor cultures. Statistical significance of $Y_{SX}$ differences was determined using a two-tailed unpaired t-test. *, p<0.05; **, p<0.01.
<table>
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<th>Y&lt;sub&gt;SS&lt;/sub&gt; (gCDW/gMeOH)</th>
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*, p<0.05; **, p<0.01

3.5. 13C-methanol incorporation into intracellular metabolites and biomass

13C-labeling experiments were performed to further confirm methanol assimilation by the recombinant E. coli strain. Complete lists of analyzed metabolites can be found in Supplementary Tables 4, 5 and 6. Initially, the strain expressing the B. stearothermophilus Mdh and B. methanolicus RuMP genes was cultured in M9 minimal medium with 13C-methanol as the sole carbon source for 48 hours. While under this condition, no net growth was observed, though we detected 13C-labeled intracellular metabolites derived from methanol in these cultures (Supplementary Table 4). After 24 hours, we measured that ca. 18% of malate, an anaplerotic intermediate at the interphase of glycolysis and the TCA cycle, received labeled carbon from methanol. Likewise, we measured that ca. 20% of the glycolytic intermediate, 3-phosphoglycerate (3PG), pool contained labeled carbon originating from methanol after 48 hours. Additionally, we observed ca. 11% labeling in the TCA cycle intermediate succinate and ca. 9% labeling in the amino acid glutamate, which is synthesized from the TCA-cycle intermediate α-ketoglutarate, after 48 hours. These results show that carbon from methanol
passes through glycolysis and the TCA cycle and is able to enter metabolic pathways branching off of these core pathways.

Furthermore, we detected multiple carbons labeled for all of the aforementioned metabolites, with approximately 10% and 6% of the malate pool exhibiting M+2 and M+3 labeling, respectively. We measured that approximately 12% of the 3PG pool contains M+2 labeling and 4% of the pool is fully labeled. Also, approximately 7% of the succinate pool contains M+2 labeling, 3% contains M+3 labeling and 2% is fully labeled. Similarly, 6% of the glutamate pool contains M+2 labeling and 2% contains M+3 labeling. Since we observed multiple carbon labeling, these data show that the RuMP pathway is indeed “cycling” in the recombinant E. coli strain.

We repeated the methanol labeling experiments with the recombinant strain grown on 1 g/L yeast extract and 60 mM methanol. Under these conditions, we observed an increase in the labeling percentage of intracellular metabolites that had incorporated labeled carbon from $^{13}$C-methanol. For example, the labeled succinate pool increased from 11% in methanol alone to 37% in yeast extract plus methanol (Fig. 4a). Under these conditions, 22% of the succinate pool exhibited M+2 labeling, 22% contained M+3 labeling and 5% of the pool was completely labeled (Fig. 4b). We also observed similar labeling patterns for the other TCA cycle intermediate, malate (Figs. 4e-f). Glutamate labeling increased from 9% in methanol alone to 15% in yeast extract plus methanol (Fig. 4c). Again, we could observe that 12% of the glutamate pool contained M+2 labeling, 7% contained M+3 labeling, 4% contained M+4 labeling and 1% of the pool was completely labeled (Fig. 4d). The lower labeling in glutamate compared to other metabolites can be attributed to the presence of yeast extract, which contains glutamate (Sezonov et al., 2007), that dilutes the pool of labeled glutamate. The labeling in 3PG, which characterizes
labeling in the lower part of the EMP, or glycolytic, pathway, increased from 20% in methanol alone to 53% in yeast extract plus methanol (Fig. 4g). Again, we observed that 27% of the 3PG pool contained M+2 labeling and that 30% of the pool was completely labeled (Fig. 4h).

We also analyzed a recombinant strain of E. coli expressing the Mdh2 from B. methanolicus along with the RuMP pathway. This enzyme had previously been shown to yield high amounts of intracellular labeling using 1 M methanol as a substrate (Muller et al., 2015). Under the growth condition of 1 g/L yeast extract and 60 mM methanol, very little labeling in intracellular metabolites was observed with this strain (Figs. 4a, c, e and g). This is likely due to the high K_m this enzyme possesses for methanol, limiting its ability to oxidize methanol at concentrations (ca. 60 mM) that are more conducive for E. coli growth. Lastly, no significant methanol incorporation into intracellular metabolites was observed in the E. coli strain harboring only the empty vector, further confirming that methanol oxidation was a result of recombinant gene expression (Figs. 4a, c, e and g).

Compared to previously published data (Muller et al., 2015), we present labeling in TCA cycle intermediates and amino acids, indicating that carbon derived from methanol passes through glycolysis and is being used for biomass generation and growth. Additionally, our data show higher labeling than previously reported efforts to engineer Corynebacterium glutamicum for the utilization of methanol (Leßmeier et al., 2015; Witthoff et al., 2015). For example, phosphoenolpyruvate (PEP), a key glycolytic intermediate, was reported to reach an average carbon labeling of 3.5% by Leßmeier et al. and 15% M+1 labeling by Witthoff et al. Our results show above 19% average carbon labeling and nearly 23% M+1 labeling in PEP, indicating that there is high flux through glycolysis (Supplementary Table 4). These results are similar to those reported previously for an engineered methanol-utilizing E. coli, which demonstrated ca. 25%
average carbon labeling in PEP (Muller et al., 2015). To further confirm that the recombinant E. coli strain is utilizing methanol for growth, biomass samples were hydrolyzed and analyzed for $^{13}$C-labeling. Labeling was detected in several proteinogenic amino acids, demonstrating incorporation of methanol-derived carbon into proteins (Supplementary Table 6). Some amino acids showed no labeling, suggesting that there remains a limiting factor for growth on methanol alone.

Taken together, these data show that methanol is being assimilated into intracellular metabolites and that the RuMP pathway is cycling in the engineered E. coli strain. Furthermore, addition of yeast extract to the media increases the amount of methanol assimilation, indicated by the higher depth of labeling observed in the intracellular metabolites.

![Fig 4. Intracellular metabolite profiles of E. coli ΔfrmA strains expressing methanol utilization genes. The ΔfrmA strains harboring either the empty vector, pM6tac (Empty; blue square), pM6tac_BmRuMP_BmMdhh2 (BmMdhh2; red triangle) or pM6tac_BmRuMP BsMdhh (BsMdhh; green circle) were grown in M9 minimal medium supplemented with 1 g/L yeast extract and 60 mM $^{13}$C methanol. Average carbon labeling and relative abundance of mass isotopomers for succinate (A, B), glutamate (C, D), malate (E, F) and 3PG (G, H) are shown. Error bars indicate standard error of the mean (n=2).](image-url)
3.6. Transcriptional optimization results in enhanced growth on methanol

To further improve the efficiency of our methanol utilization module, we applied the recently published ePathOptimize method for transcriptional optimization (Jones et al., 2015b). We randomly incorporated one of six promoters of varied strength (tac, G6, H9, H10, C4 and consensus T7) to control transcription of each gene in the methanol utilization pathway from a monocistronic operon configuration. The relative strengths of these promoters are: tac (0.54), G6 (0.21), H9 (0.30), H10 (0.71), C4 (1.54) consensus T7 (1.00) as determined by endpoint mCherry expression in LB media. These transcriptionally optimized mutants were then transformed into E. coli BL21star™(DE3) and screened for growth enhancement in the presence of M9 minimal medium supplemented with 1 g/L yeast extract and 60 mM methanol (Supplementary Fig. 3). Four lead mutants from the original screen were further analyzed with additional controls (Fig. 5). When growth was normalized to each of the respective No MeOH/No IPTG controls, several of the selected mutants outperformed both control strains with all tac or all consensus T7 transcriptional control, respectively, up to 37 ± 3%. Furthermore, additional controls of MeOH/No IPTG and No MeOH/IPTG demonstrated growth similar to that of the No MeOH/No IPTG case indicating tight transcriptional control of the operon. Some degree of false positives did result from the initial screening; upon testing, the apparently false positive, 3G2 mutant (Fig. 5), demonstrated only marginal growth improvements when compared to the all tac or all T7 controls and resulted in slightly less $^{13}$C flavanone labeling (see next section) than the all tac control at both 30 and 37°C. Growth experiments with $^{13}$C methanol revealed little difference between a representative optimized strain (2G1) and the control strain (Supplementary Table 7). It is likely that the positive cell growth results are due to
gene expression changes leading to a decrease in metabolic burden rather than increased methanol assimilation (Wu et al., 2016).

**Fig 5.** Normalized growth data for transcriptionally optimized lead mutants. Growth was performed in M9 minimal medium supplemented with 1 g/L yeast extract in the absence or presence of methanol and IPTG, respectively. Mutants 4A3, 2G1 and 3E2 show significant growth advantage in the presence of methanol. Error bars indicate standard error of the mean (n≥2).

3.7. Production of and $^{13}$C methanol incorporation into naringenin, a high-value flavonoid product

To effectively demonstrate that carbon from methanol was capable of generating useful metabolite precursors and thus could be used to generate products of industrial relevance, we engineered the pathway for the flavanone naringenin in our methylotrophic E. coli strain. Naringenin biosynthesis is a three step process that utilizes the added precursor p-coumaric acid and the native E. coli intermediate malonyl-CoA to synthesize naringenin (Fig. 1 and Fig. 6a) (Jones et al., 2016; Santos et al., 2011). Thus, if carbon from methanol is used to synthesize
acetyl-CoA (and subsequently malonyl-CoA), it should be possible to synthesize naringenin from methanol. After screening eight plasmid and strain combinations, the best producing combination was selected for further analysis (Supplementary Fig. 4). This E. coli BL21star™(DE3) strain contains both the methanol utilization module (Mdh, Hps and Phi), expressed from pM6tac, as well as the flavonoid production module consisting of 4-coumaroyl-CoA Ligase (4CL) and chalcone synthase (CHS), expressed from pCDM4 (Fig. 5a). We observed a 650% improvement in flavonoid production from M9 minimal medium containing 1 g/L yeast extract and 60 mM methanol when compared to the strain containing the empty vector control for the methanol utilization module (Fig. 6b). Enhancement in naringenin titer was observed between the no methanol and empty vector controls, but this improvement in titer did not result in 13C labeling enrichment and is presumably due to incorporation of formaldehyde naturally found in E. coli cultures (Gutheil et al., 1992; Yurimoto et al., 2005). Additionally, LCMS analysis revealed an average carbon labeling of ca. 2.8 ± 0.1% in naringenin with ca. 11 ± 0.5% (1 - M+0) of the total naringenin pool containing at least one carbon labeled when cultures were supplemented with 13C-methanol and an active methanol utilization module was present (Fig. 6c and Supplementary Fig. 5a). Improvements in the average labeling and abundance of the labeled fraction were realized by increasing the fermentation temperature to 37°C, which resulted in an improvement over the 30°C condition and led to an average carbon labeling of ca. 3.5 ± 0.1% with ca. 15 ± 0.4% (1 - M+0) of the total naringenin pool having at least one labeled carbon (Fig. 6c).

To further improve the flux from methanol to naringenin, we coupled our transcriptionally optimized methanol utilization pathways with our previously described flavanone production module. Screening under identical conditions, optimized methanol
utilization modules resulted in up to ca. 4.7 ± 0.3% average carbon labeling with ca. 18 ± 1.3% of the total naringenin pool labeled with at least one carbon, representing a further improvement over the non-optimized control at 37°C (Fig. 6d and Supplementary Fig. 5b). Three of the four lead mutants (4A3, 2G1 and 3E2) resulted in enhanced labeling when compared to the all tac control, while one mutant (3G2) resulted in slightly reduced labeling (Fig. 6d). Labeling in four out of the possible six carbons was detected in the 2G1 and 4A3 mutants, providing further support that the RuMP pathway is cycling during the latter methanol-dependent growth regime.
**Fig 6.** Naringenin production in methylotrophic E. coli. (A) Flavanone production from phenylpropanoic acids requires two exogenously expressed enzymes, 4-coumaroyl CoA ligase (4CL) and chalcone synthase (CHS), and three moles of malonyl-CoA per mole of flavanone produced. (B) Naringenin production from natural abundance methanol ($^{13}$C), $^{13}$C methanol ($^{13}$C) or no methanol (-) for a variety of genetic conditions. (C) $^{13}$C labeling data for naringenin production at 30 and 37°C. A statistically significant increase in labeling from 30 to 37°C is shown. (D) $^{13}$C labeling data for naringenin production with transcriptionally optimized mutants used for methanol assimilation pathway at 30 and 37°C. Several mutants demonstrate significant increases in labeling at both 30 and 37°C compared to their respective all tac controls. Error bars indicate standard error of the mean (n≥2).

### 4. Discussion

The ability to convert methanol into industrially useful metabolites is an attractive possibility given the decreasing cost of methanol and recent efforts in developing technologies to convert the plentiful natural gas reserves into methanol (Haynes and Gonzalez, 2014). Previous reports have attempted to engineer a methanol utilization strain of E. coli using Mdh and RuMP genes from B. methanolicus (Muller et al., 2015). They were able to demonstrate intracellular metabolite labeling in sugar phosphates and glycolytic intermediates using 1 M $^{13}$C-methanol. However, no methanol consumption or growth data was presented. If this strain was unable to direct carbon flux from methanol to the TCA cycle, this strain would not be able to grow on methanol as the sole carbon and energy source. Here, we demonstrated the engineering of a recombinant methanol utilization pathway in E. coli utilizing a superior Mdh from B. stearothermophilus. Expression of this enzyme with the RuMP pathway from B. methanolicus in an E. coli ΔfrmA background led to a 30% improvement in biomass when methanol was utilized as a co-substrate with 1 g/L yeast extract compared to when yeast extract alone was used for growth. Additionally, we show for the first time in E. coli, labeling from methanol-derived carbon into hydrolyzed proteins, i.e. biomass components, which confirms that our recombinant
E. coli strain utilizes methanol for growth and biomass generation under appropriate culture conditions.

We identified an alternative Mdh from B. stearothermophilus, which exhibits more favorable enzyme kinetics than previously characterized B. methanolicus Mdh enzymes, for engineering a methylotrophic E. coli. We confirmed expression and functionality of this Mdh in E. coli and also demonstrated the improvements this Mdh possesses over B. methanolicus Mdhs. Taken together, the Mdh from B. stearothermophilus allows for faster methanol turnover at concentrations that are well below the point where methanol begins to exert a bactericidal effect. Further strain optimization could be accomplished by sourcing additional Mdhs with desirable characteristics, i.e. low $K_m$ and high $V_{max}$ for methanol, from other organisms or by engineering current Mdhs (Wu et al., 2016).

Importantly, we demonstrated for the first time that E. coli can be engineered for the bioconversion of methanol to high-value chemicals. Through combination of plasmid-based modules for both methanol assimilation and naringenin biosynthesis, we were able to demonstrate improvements in naringenin titer deriving from the use of methanol as a co-substrate. Additionally, we were able to track the carbon flux from methanol to naringenin using $^{13}$C LCMS analysis and demonstrated labeling in up to four of a possible six carbons. Transcriptional balancing techniques made it possible to optimize the expression levels for each of the enzymes in the methanol assimilation module that would result in improvements in both growth on methanol and enhanced $^{13}$C labeling in the naringenin product. These data support the power of transcriptional balancing approaches but also the need for enhanced screening tools to enable high-throughput combinatorial studies (Cress et al., 2016; Cress et al., 2015; Jones et al., 2015a). Finally, further support for the active and functioning methanol utilization system was
demonstrated through temperature-based $^{13}$C labeling experiments. Given the thermophilic source of the Mdh, Hps and Phi enzymes, the enzyme activity should improve with increasing temperature. Furthermore, the thermodynamics for NAD-dependent methanol oxidation favor elevated temperatures. These hypotheses were supported through labeling experiments carried out at 30 and 37°C, which demonstrated significant improvements in $^{13}$C naringenin labeling at elevated temperatures.

In conclusion, an engineered methylotrophic strain of E. coli would be of great value toward the development of biotechnological processes aiming to convert methanol into commodity and specialty chemicals, as well as fuel molecules. While the engineered E. coli strain presented in this study has the ability to convert methanol into biomass and high value chemicals, we hypothesize that further strain engineering will likely lead to a strain capable of growth on methanol as the sole carbon source. Improvements in ribulose-5-phosphate regeneration, methanol uptake rates and developing techniques to study methanol flux in a mixture of methanol and undefined yeast extract will all likely refine and improve upon the synthetic methylotrophic E. coli strain.

Acknowledgements
Funding was provided by the ARPA-E REMOTE contract # DE-AR0000432. The authors would like to thank Dr. Dmitri V. Zagorevski for assistance with LC/MS analysis.

Supplementary material associated with this article can be found in the online version at
(Supplementary Tables 1-7, Supplementary Figures 1-5, Supplementary Methods, Supplementary References)

References


Highlights

- Engineered methanol utilization pathway that allows E. coli to consume methanol.
- Methanol contributes to increased biomass yields in engineered strain.
- Labeled methanol is converted into biomass components.
- Production of naringenin from methanol.