

Enhancing Limonene production by probing the metabolic network through time-series metabolomics data

Jasmeet Kaur Khanijou^{1,*}, Clement P. M. Scipion^{1,2}, Shreyash Borkar¹, Xixian Chen¹, Wee Chew^{1,*}

¹ Singapore Institute of Food and Biotechnology Innovation (SIFBI), Agency for Science, Technology and Research (A*STAR), Singapore 138673, Singapore.

² CNRS@CREATE, 1 Create Way, #08-01 Create Tower, Singapore 138602

*Authors to whom correspondence should be addressed. E-mail address: Khanijou_Jasmeet_Kaur@sifbi.a-star.edu.sg; chewwee@gmail.com

Abstract

Introduction

Limonene is a monoterpene with diverse applications in food, medicine, fuel, and material science. Recently, engineered microbes have been used to biosynthesize target biochemicals such as limonene.

Objective

Metabolic engineering has shown that factors such as feedback inhibition, enzyme activity or abundance may contribute to the loss of target biochemicals. Incorporating a hypothesis driven experimental approach can help to streamline the process of improving target yield.

Method

In this work, time-series intracellular metabolomics data from *Escherichia coli* cultures of a wild-type strain engineered to overproduce limonene (EcoCTs3) was collected, where we hypothesized having more carbon flux towards the engineered mevalonate (MEV) pathway would increase limonene yield. Based on the topology of the metabolic network, the pathways involved in mixed fermentation were possibly causing carbon flux loss from the MEV pathway. To prove this, knockout strains of lactate dehydrogenase (LDH) and aldehyde dehydrogenase-alcohol dehydrogenase (ALDH-ADH) were created.

Results

The knockout strains showed 18 to 20 folds more intracellular mevalonate accumulation over time compared to the EcoCTs3 strain, thus indicating greater carbon flux directed towards the MEV pathway thereby increasing limonene yield by 8 to 9 folds.

Conclusion

Ensuring high intracellular mevalonate concentration is therefore a good strategy to enhance limonene yield and other target compounds using the MEV pathway. Once high intracellular mevalonate concentration has been achieved, the limonene producing strain can then be further modified through other strategies such as enzyme and protein engineering to ensure better conversion of mevalonate to downstream metabolites to produce the target product limonene.

36 **Keywords:** Intracellular Metabolomics, Quantitative Metabolomics, Metabolic Network, Limonene,
37 Mevalonate Pathway

38 1. Introduction

39 Monoterpenoids are a class of structurally varied natural compounds with a strong presence in
40 industrial applications (1). Limonene, a cyclized monoterpene, is assumed as GRAS (generally
41 recognized as safe) by the US Food and Drug Administration (2). Limonene and its derivatives, such as
42 carveol, menthol, and α -terpineol, have broad applications in food and beverage, pharmaceuticals,
43 cosmetics, biomaterials, and biofuels due to their pleasant fragrance and physicochemical properties
44 (3-5). Limonene has typically been extracted from plant biomass, where availability could be affected
45 by variations in climate and agricultural land (6). As limonene's structure has a chiral centre, it is found
46 in nature as two enantiomers, (D)-limonene and (L)-limonene. (D)-limonene is usually obtained
47 through cold pressing citrus peels and pulps (7) while (L)-limonene is found in essential oils of other
48 plant species (8). With advancements in metabolic engineering and synthetic biology, microbes can be
49 utilised as an alternative approach to sustainably produce high-value natural products, including
50 limonene. Microbes including yeast, bacteria, and mould have been effectively used for limonene
51 biosynthesis and derivatisation (9-11). However, the carbon yields for limonene biosynthesis are
52 relatively low for economically feasible bioprocesses (12). The low productivity in limonene
53 biosynthesis is due to various challenges such as: the inefficiency of enzymes for biosynthesis
54 pathways, minimal metabolic fluxes directed towards limonene, intrusion of the native metabolism of
55 microbes with complicated heterologous biosynthesis pathways, the absence of appropriate
56 conditions for the heterologous expression of optimized enzymes and the cytotoxicity of limonene
57 affecting microbial chassis (13). The model organisms *Escherichia coli*, *Saccharomyces cerevisiae*, and
58 cyanobacteria have emerged as leading candidates for limonene production due to the availability of
59 metabolic information and tools for genetic engineering (13). *E. coli* is ideal for terpenoid production
60 due to its high growth rate, genetic traits, and presence of genetic engineering tools (14).

61 Terpenoids are produced from the metabolite isopentenyl pyrophosphate (IPP) and its isomer,
62 dimethylallyl pyrophosphate (DMAPP), which are then condensed into geranyl pyrophosphate (GPP),
63 farnesyl pyrophosphate (FPP), or geranylgeranyl pyrophosphate (GGPP), which are respective
64 substrates to synthesize monoterpenes, sesquiterpenes, and diterpenes (15). IPP and DMAPP
65 production can be achieved through two main isoprenoid pathways: the mevalonate (MEV) pathway
66 and the deoxyxylulose 5-phosphate (DXP) pathway (Figure 1). Eukaryotes utilise the MEV pathway
67 (except for plants which utilise both pathways) while most prokaryotes utilise the DXP pathway (16,
68 17). High terpenoid titres have been achieved by engineering *E. coli* with the heterologous MEV
69 pathway (18). Previously, Alonso-Gutierrez *et. al.* (19) constructed a plasmid system for limonene
70 production by utilising heterologous enzymes to compose the MEV pathway. In this study, *E. coli* was
71 engineered to produce L-limonene through the heterologous expression of the MEV pathway, a GPP
72 synthase, and a limonene synthase as illustrated in Figure 2 (19). Previous attempts to obtain high
73 terpenoid yields, required a good balance of the availability of co-factors, energy demands, and carbon
74 flux in the DXP and/or MEV pathways in *E. coli* (20-22). Furthermore, as the DXP and MEV pathways
75 obtain their carbon from central carbon metabolism, these pathways compete with many other
76 biochemical reactions to produce required precursors towards limonene production (23).

77 The major biochemical reactions in *E. coli* leading towards eventual limonene production are
78 illustrated in Figure 1, which include competing pathways for carbon flux such as the tricarboxylic acid
79 (TCA) cycle, mixed fermentation pathways, and pentose phosphate pathway (PPP). By understanding

such a metabolic network, in conjunction with intracellular metabolomics data, attempts could be made to improve limonene synthesis in bacterial bio-factories. Furthermore, as illustrated in Figure 1, there are various co-factors involved in the biochemical reactions in the metabolic network, which could also be sources of potential bottlenecks when the co-factors become limiting. Previous work has shown that limonene biosynthesis using the DXP pathway was limited by the low concentrations of terpenoid precursors produced, particularly GPP (24). The overproduction of IPP and DMAPP could be easily executed through the alteration of prenyl transferase (PT) and terpene synthase (TS). However, the production of monoterpenes using microbes has been restricted as many monoterpenes are toxic and volatile (25), and the heterologous expression of GPP synthase and monoterpene synthases have been poor (24, 26). In one study, a dedicated GPP synthase was unable to produce farnesyl diphosphate (FPP) that was instrumental in enhancing the yield of limonene (19). These strategies to improve limonene yield have focused mainly on the downstream enzymes or metabolites of the metabolic network.

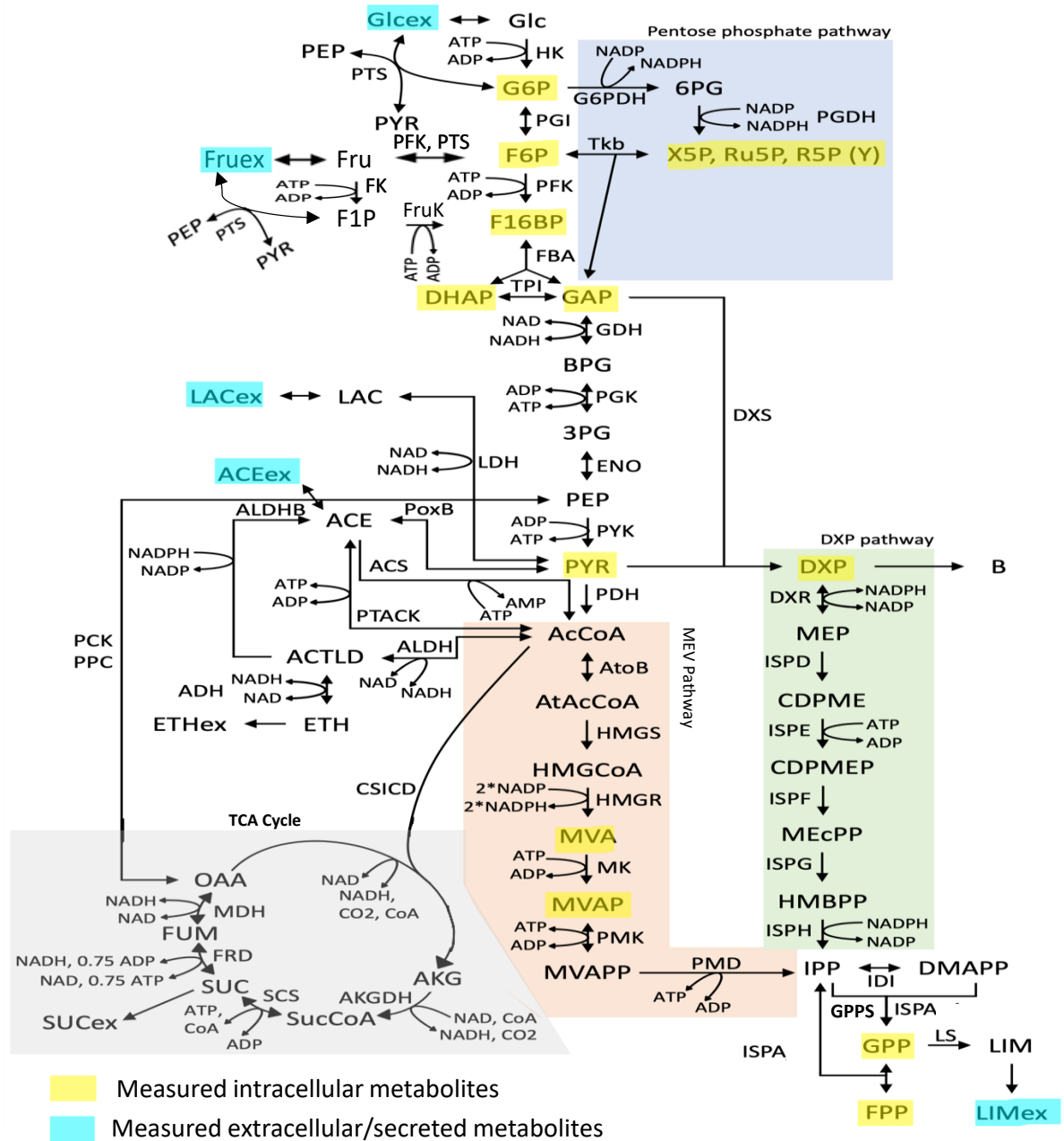


Figure 1. Illustration of various pathways involved in limonene production in *E. coli* engineered with the mevalonate (MEV) pathway (in orange). The other pathways include the glycolytic, pentose-phosphate pathway (PPP, in blue), deoxyxylulose 5-phosphate (DXP) pathway (in green) and tricarboxylic acid (TCA) cycle (in grey). Cofactor consumption is represented by curved arrows. Intermediates: Glcex, glucose extracellular; Glc, glucose; Fruex, fructose extracellular; Fru, fructose; F1P, fructose-1-phosphate; G6P, glucose-6-phosphate; 6PG, 6-phosphogluconate; X5P, xylulose-5-phosphate; Ru5P, ribulose-5-phosphate; R5P, ribose-5-phosphate; F6P, fructose-6-phosphate; F16BP, fructose-1,6-biphosphate; GAP, glyceraldehyde-3-phosphate; DHAP, dihydroxyacetone phosphate; BPG, 1,3-bisphosphoglycerate; 3PG, 3-phosphoglycerate; PEP, phosphoenolpyruvate; PYR, pyruvate; DXP, 1-deoxy-D-xylulose-5-phosphate; B, flux to vitamin B6 pathway; MEP, 2-C-methylerythritol-4-phosphate; CDPME, 4-diphosphocytidyl-2-C-methylerythritol; CDPMEP, 4-diphosphocytidyl-2-C-

methylerythritol-2-phosphate; MEcPP, 2-C-Methylerythritol-2,4-cyclodiphosphate; HMBPP, hydroxymethylbutenyl 4-diphosphate; IPP, isopentenyl pyrophosphate; DMAPP, dimethylallyl diphosphate; GPP, geranyl diphosphate; FPP, farnesyl diphosphate; LIM, limonene; LIMex, extracellular limonene; AcCoA, acetyl coenzyme A; AtAcCoA, acetoacetyl-CoA; HMGCoA, hydroxymethylglutaryl-CoA; MVA, mevalonate; MVAP, 5-phosphomevalonate; MVAPP, 5-diphosphomevalonate; ACE, acetic acid; ACEex, acetic acid extracellular; ACTLD, acetaldehyde; ETH, ethanol; ETHex, extracellular ethanol; LAC, lactic acid; LACex, extracellular lactic acid; AKG, α -ketoglutarate; SucCoA, succinyl CoA; SUC, succinate; SUCex, extracellular succinate; FUM, fumarate; OAA, oxaloacetate. Enzymes: PTS, phosphotransferase system; HK, hexokinase; FK, fructokinase; FruK, fructose-1-kinase; PFK, phosphofructokinase; G6PDH, lumped reactions of glucose-6-phosphate dehydrogenase and 6-phosphogluconolactonase; PGDH, 6-phosphogluconate dehydrogenase; Tkb, transketolase; PGI, phosphoglucose isomerase; PFK, phosphofructokinase; FBA, fructose-1,6-biphosphate aldolase; GDH, glutamate dehydrogenase; PGK, phosphoglycerate kinase; ENO, enolase; PYK, pyruvate kinase; DXS, DXP synthase; DXR, DXP reductase; ISPD, CDPME synthase; ISPE, CDPME kinase; ISPF, MecPP synthase; ISPG, HMBPP synthase; ISPH, HMBPP reductase; IDI, isopentenyl diphosphate isomerase; ISPA, farnesyl diphosphate synthase; GPPS, geranyl pyrophosphate synthase; LS, limonene synthase; PDH, pyruvate dehydrogenase; AtoB, acetyl-CoA acetyltransferase; HMGS, HMGCoA synthase; HMGR, HMGCoA reductase; MK, mevalonate kinase; PMK, phosphomevalonate kinase; PMD, diphosphate mevalonate decarboxylase; LDH, lactate dehydrogenase; PoxB, pyruvate oxidase; PCK, phosphoenolpyruvate carboxykinase; PPC, phosphoenolpyruvate carboxylase; ACS, acetyl-CoA synthetase; PTACK, lumped reactions of phosphate acetyltransferase and acetate kinase; ALDHB, aldehyde dehydrogenase B; ALDH, aldehyde dehydrogenase; ADH, alcohol dehydrogenase; CSICD, lumped enzymatic reactions of citrate synthase, aconitate hydratase A, aconitate hydratase B and isocitrate dehydrogenase; AKGDH, α -ketoglutarate dehydrogenase; SCS, succinyl-CoA synthetase; FRD, fumarate reductase; MDH, malate dehydrogenase.

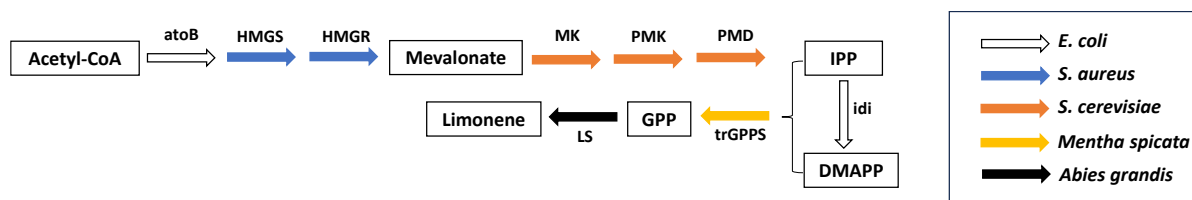


Figure 2. Enzymes from other microbes and some intermediate metabolites involved in limonene production through the heterologous mevalonate (MEV) pathway engineered in *E. coli*.

Previous literature focused more on downstream metabolites of the MEV pathway to improve limonene yield (19, 24, 26). There exists a gap in knowledge regarding the impact of upstream metabolites in improving limonene yield by understanding the metabolic network. In this study, we hypothesized the need to unblock carbon flux channelling towards MEV pathway by optimising upstream metabolic fluxes to enhance limonene yield in *E. coli* cell cultures. A wild-type *E. coli* strain (K-12 MG1655) was engineered to overproduce L-limonene by heterologous expression of the MEV pathway, a GPP synthase, and a limonene synthase through plasmid pJBEI-6409 (19), resulting in an EcoCTs3 strain, based on which knockout strains were created. Following a rationale experimental design approach, we traced the metabolic topology of the key *E. coli* pathways involved from glycolysis, TCA cycle, PPP down to the MEV and DXP pathways, by measuring time-series concentrations of

important intracellular and extracellular metabolites. By studying the intracellular metabolomics data obtained from the EcoCTs3 strain and the metabolic network topology, attempts were made to improve the limonene yield through a systematic hypothesis driven approach. First, the carbon source was changed from glucose to fructose to improve intracellular metabolite concentrations in upstream fluxes. Second, to enhance carbon flux flow towards the MEV pathway to improve limonene production, two mutant strains were created, with the gene knockouts of *adhE* and *ldh*, which are genes involved in the mixed fermentation pathways. The mutant strains increased limonene yield by 8 to 9 folds through the elimination of pathways competing for the carbon flux.

2. Materials and Methods

All chemicals, standards, solvents, and media components used in this study were obtained from Sigma-Aldrich (St. Louis, MO), Fisher Scientific (Fair Lawn, NJ, USA), or VWR (Radnor, PA, USA) unless otherwise noted. Water was deionised and filtered by Sartorius Arium Pro VF Type 1 water system (18.2 MΩ, 0.2 µm).

2.1. Strain construction

The limonene producing engineered wild-type strain was created by transforming pJBEI-6409 plasmid which was a gift from Taek Soon Lee (Addgene plasmid #47048 ; <http://n2t.net/addgene:47048> ; RRID:Addgene_47048) (19) by heat shock in *E. coli* K-12 MG1655 (F- lambda- *ilvG*- *rfb*-50 *rph*-1) forming the strain EcoCTs3. The strain details are listed in Table 1. To create the ALDH-ADH and LDH knockouts, the deletion of *adhE* (UniProt P0A9Q7) and *ldhA* (UniProt P52643) genes from *E. coli* MG1655 strain was executed using CRISPR Cas9-assisted recombineering method as described previously (27). For the successful knockout strains, the *ldhA* and *adhE* regions were PCR amplified and sequenced to confirm the deletion of the genes. The strains were stored in 40% glycerol at -80 °C before further use. For limonene production experiments, the pJBEI-6409 plasmid was transformed by heat shock in *E. coli* K-12 MG1655 Δ *ldhA* and Δ *adhE* strains. Competent cells were prepared using the Mix & Go! *E. coli* Transformation Kit (Zymo Chem) from the *ldhA* and *adhE* deletion strains, following manufacturer's specifications. pJBEI-6409 transformed strains (*E. coli* K-12 MG1655, Δ *ldhA* and Δ *adhE*) were each plated on a chloramphenicol selective Luria-Bertani agar plate and incubated overnight at 37 °C.

Table 1. Bacterial strains used in this study

Name	Description	Reference	Remarks
<i>E. coli</i> DH5a (ECOS 101)	endA1 recA1 relA1 deoR gyrA96 nupG hsdR17(rk-, mk+) supE44 thi-1 Δ (lacZYA-argF)U169 Φ 80 lacZ Δ M15 λ - F-	Yeastern Biotech	Used for cloning.
<i>E. coli</i> K-12 MG1655	F- lambda- <i>ilvG</i> - <i>rfb</i> -50 <i>rph</i> -1	ATCC 700926	Used for limonene production.
EcoCTs3	<i>E. coli</i> K-12 MG1655 strain transformed with pJBEI-6409 (Addgene #47048). Plasmid carries ecAtoB, saHMGS, saHMGR, scMK, scPMK, scPMD, ecIDI, abTrGPPS, msTrLS genes and chloramphenicol resistance gene.	(28)	Strain for limonene production.

<i>E. coli</i> K-12 MG1655 $\Delta ldhA$	<i>E. coli</i> K-12 MG1655 with deletion of <i>ldhA</i> gene	(28)	Used for limonene production.
<i>E. coli</i> K-12 MG1655 $\Delta adhE$	<i>E. coli</i> K-12 MG1655 with deletion of <i>adhE</i> gene	(28)	Used for limonene production.
LDH KO	<i>E. coli</i> K-12 MG1655 $\Delta ldhA$ strain transformed with pJBEI-6409	(28)	Strain for limonene production.
ALDH-ADH KO	<i>E. coli</i> K-12 MG1655 $\Delta adhE$ strain transformed with pJBEI-6409	(28)	Strain for limonene production.

2.2 Growth conditions

After the prepared *E. coli* strains were streaked onto plates and incubated overnight, one colony was picked from each plate. Each colony was inoculated into 5 mL of Luria-Bertani medium consisting of 10 g/L tryptone, 5 g/L yeast extract, and 10 g/L NaCl, where 30 µg/mL chloramphenicol was added and grown overnight at 37°C and 220 rpm. Each cell pellet was washed and re-suspended in 50 mL M9 medium adapted from a previous study (29), where the M9 medium consisted of 12.7 g/L Na₂HPO₄·7H₂O, 3.1 g/L KH₂PO₄, 1 g/L NH₄Cl, 0.5 g/L NaCl, 0.25 g/L MgSO₄·7H₂O, 15 mg/L CaCl₂·2H₂O, 8.1 mg/L FeCl₃, 0.89 mg/L MnCl₂·4H₂O, 1.7 mg/L ZnCl₂, 0.34 mg/L CuCl₂, 0.6 mg/L CoCl₂·6H₂O, 0.51 mg/L Na₂MoO₄, 10 g/L glucose, and incubated overnight at 30 °C and 220 rpm. Each strain was stored in 40% glycerol at – 80 °C. Pre-cultures of each strain were then prepared by adding 100 µL of each strain stored in glycerol to 50 mL M9 medium in 250 mL flasks, which were left overnight at 30 °C at 220 rpm. For experiments using mixtures of fructose and glucose as carbon sources, the carbon source was made up to 10 g/L using 0%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% and 100% fructose. To collect time-series metabolomics data, multiple cell culture flasks were prepared for duplicate biological samples for each time-point. Cell cultures of each strain were prepared by adding 100 µL of pre-cultures to 50 mL M9 medium in 250 mL flasks with incubation at 30 °C and 220 rpm. Upon reaching an optical density of 1 at 600 nm, isopropyl β-d-1-thiogalactopyranoside (IPTG) was added, resulting in a concentration of 25 µM. A dodecane overlay of 5 mL was added to cell cultures to allow trapping of the volatile secreted limonene (19). The flasks of cell cultures were left at 30 °C and 220 rpm. The flasks were sacrificed in duplicates at 2 h, 3 h, 6 h, 7 h, 24 h, and 25 h post-IPTG induction to profile the concentrations of the intracellular, extracellular, and secreted limonene.

2.3. Metabolomics extraction and analysis

2.3.1 Secreted Limonene and Extracellular Metabolites

Dynamic sampling at time points 2 h, 3 h, 6 h, 7 h, 24 h and 25 h post IPTG induction was performed. Specifically, duplicate *E. coli* cell cultures of 50 mL were sacrificed and centrifuged for 10 mins at 3000 rpm. The dodecane layers containing secreted limonene were removed and stored at -80 °C prior to quantitative analysis. Limonene samples were diluted in ethyl acetate and run on an Agilent 7890B gas chromatography mass spectrometry (GC-MS) system with a DB-5 column (5%-phenyl-methylpolysiloxane, 30 m x 0.25 mm ID x 0.25 µm, Agilent Technologies, USA). For each run, 1 µL sample was injected with a 10:1 split ratio and 10 mL/min split flow. The GC oven temperature program

was set at 40 °C for 3 min, followed by a 10 °C/min ramp to 100 °C and another 60 °C/min ramp to 220 °C with a hold time of 2 min. The injector and MS transfer line temperatures were at 250 °C and 280 °C, respectively. The MS was operated in selected ion-monitoring (SIM) mode using ions of m/z 136, 68 and 93, representing the molecular ion and two abundant fragmental ions of limonene. Calibration standards were run for limonene prepared in ethyl acetate from 0.05 µg/mL to 10 µg/mL. Limonene concentrations were determined using the Agilent Quantitative software.

The media supernatants after centrifugation were stored at -80 °C prior to extracellular metabolites quantitative analysis. Subsequently after thawing these, 1 mL of each supernatant sample was filtered using a polyamide filter prior to analysis using an Agilent 1200 high performance liquid chromatography (HPLC) system with a Bio-rad Aminex HPX-87H column (300 x 7.8 mm) and 1260 Infinity II Refractive Index Detector (RID). For each run, 5 µL of sample was injected with an isocratic gradient using 0.01 N sulphuric acid and a 0.6 mL/min flow rate for 28 min. The column temperature was set at 35 °C, while the RID temperature was at 30 °C with positive polarity. Calibration mixtures of the following range: glucose (0.5 to 80 g/L), lactic acid (0.125 to 8 g/L), acetic acid (0.125 to 80 g/L), and ethanol (0.5 to 80 g/L) were used to construct calibration curves to establish linearity and the concentrations of each extracellular metabolite in the prepared samples. Cell pellets were dried in the oven overnight at 80 °C prior to weighing with the Mettler Toledo XSE105 precision balance.

2.3.2 Intracellular Metabolites

To measure intracellular metabolites, *E. coli* cell cultures were sacrificed in duplicates at 2 h, 3 h, 6 h, 7 h, 24 h, and 25 h after IPTG induction. Cell cultures were subjected to a modified protocol from the fast filtration and quenching in liquid nitrogen previously described (30, 31). In brief, 10 mL of cell culture were filtered through a 0.2 µm polyamide membrane filter (Sartorius, Goettingen, Germany) and washed with 5 mL of wash solution (12.7 g/L Na₂HPO₄·7H₂O, 3.1 g/L KH₂PO₄, 1 g/L NH₄Cl, 0.5 g/L NaCl). The membrane filter containing the cells was folded in aluminum foil and rapidly plunged into liquid nitrogen for rapid quenching of biochemical reactions. The aluminum foil packages with membrane filters were stored at -80 °C until intracellular metabolites extraction. For the extraction of intracellular metabolites, membranes were removed from the aluminum foil packages and placed into 5 mL of extraction solvent consisting of methanol, acetonitrile, and water (4:4:2) and left in an ice bath for 10 mins. After this, the membranes were vortexed for 1 min followed by 3 rounds of sonication for 3 mins each time, with placement in an ice bath after each round of sonication for 1 min. The extracts were decanted into glass tubes and spiked with 20 µL internal standard mixture consisting of 50 µg/mL mevalonic acid-d₃ (MVA-d₃) and 10 µg/mL thymolphthalein monophosphate (TMP) prepared in methanol: 10 mM ammonium hydroxide mixture (7:3). The extracts were then subjected to a vacuum concentrator and reconstituted with 200 µL methanol: 10 mM ammonium hydroxide mixture (7:3) and filtered prior to analysis via liquid chromatography mass spectrometry.

For quantitation of intracellular metabolites, an Agilent 6230 time of flight-mass spectrometer (TOF-MS) with a Dual Agilent Jet Stream (AJS) ion source was coupled with an Agilent ultra-performance liquid chromatography (UPLC) 1290 system and a Waters Acquity UPLC BEH C18 (2.1 x 150 mm, 1.7 µm) column with a VanGuard pre-column (2.1 x 5 mm). The chromatographic method used was adapted from previous studies (30, 31), with appropriate modification. For each run, 2 µL of sample was injected and a dual mobile phase system was utilized with mobile phase A as 5 mM ammonium formate in water (pH 9.5) and mobile phase B as 5 mM ammonium formate (pH 9.5) in acetonitrile:

water (9:1). The following solvent gradient was used: start with 100% mobile phase A from 0-3.5 min with 0.1 mL/min flow rate to 100% mobile phase B at 12 min and held for 8 min with increased flow rate to 0.5 mL/min. At 20 min, the system was recalibrated back to 100% mobile phase A for 5 min and held for another 5 min. The column temperature was kept constant at 35 °C. Negative electrospray ionisation was used with the following TOF settings: Gas temperature, 325 °C; Gas flow, 11 L/min; Nebuliser pressure, 35 psi; Sheath gas temperature, 375 °C; Sheath gas flow, 11 L/min; Vcap voltage, 3500 V; Nozzle voltage, 500 V; Skimmer, 65; OctopoleRFPeak, 750; Scan rate, 2 spectra/s. The fragmentor voltage was varied throughout each 35 min sample analysis: 2 – 7.5 min, 140 V; 7.5 – 15 min, 100 V, 140 V and 150 V. UPLC flow diversions were as follows: 0 – 2 min to waste, 2 – 15 min to TOF-MS, and 15 – 35 min to waste.

Standards for quantitation of intracellular metabolites were prepared in methanol: 10 mM ammonium hydroxide mixture (7:3). The following intracellular metabolites were measured DXP (1-deoxy-D-xylulose-5-phosphate), DHAP+GAP (pool of dihydroxyacetone phosphate and glyceraldehyde-3-phosphate), F16BP (fructose-1,6-biphosphate), FPP (farnesyl diphosphate), GPP (geranyl diphosphate), MVA (mevalonate), MVAP (5-phosphomevalonate), PYR (pyruvate), R5P+Ru5P+X5P (pool of ribose-5-phosphate, ribulose-5-phosphate, and xylulose-5-phosphate) and G6P+F6P (pool of glucose-6-phosphate and fructose-6-phosphate). Different concentrations of intracellular intermediates were used to prepare calibration mixtures for construction of calibration curves: DHAP (DHAP + GAP pool) – 0.04 to 5 µg/mL; DXP – 0.04 to 10 µg/mL; F6P (F6P + G6P pool) – 0.04 to 10 µg/mL; F1,6BP – 0.04 to 6 µg/mL; MVA – 0.04 to 10 µg/mL; R5P (R5P + Ru5P + X5P pool) – 0.04 to 10 µg/mL; PYR – 0.04 to 1.5 µg/mL; MVAP – 0.01 to 0.3 µg/mL; GPP – 0.05 to 1.5 µg/mL; FPP – 0.05 to 1.5 µg/mL. Each calibration mixture was made up to 100 µL with 10 µL of internal standard mixture (50 µg/mL MVA-d3 and 10 µg/mL TMP) added. Metabolite quantitation was performed using the Agilent Masshunter Workstation Quantitative Analysis software for TOF. Normalization of quantitative metabolomics data to g DCW (dry cell weight) enabled comparisons across the various time points after IPTG induction.

3. Results and Discussion

3.1 Time-series intracellular metabolites from engineered wild-type strain EcoCTs3

The wild-type *E. coli* strain engineered to overproduce L-limonene (EcoCTs3) was cultured and harvested at aforesaid time-points after IPTG induction where the metabolomics data from their targeted intra-/ extra-cellular metabolite analyses via GC-MS and LC-ToF-MS were collated. Figures 3A and 3B show the data from the EcoCTs3 strain grown in glucose where it was observed that many of the intracellular metabolites such as G6P and F6P (pooled), R5P, Ru5P and X5P (pooled), DHAP and GAP (pooled), PYR, DXP, MVA, FPP were found to generally decrease from 2 h to 7 h post-IPTG induction followed by an accumulation 24 h to 25 h after IPTG induction. The decreased concentrations of such intracellular metabolites corresponded to increased limonene production from 2 h to 7 h post-IPTG induction as shown in Figure 4A. From 24 h to 25 h post-IPTG induction, the dry cell weights of the cell pellets from 50 mL cell cultures were relatively constant at 83.3 ± 2.2 mg, indicating the stationary phase with negligible culture growth (Figure S1, Supplementary section). This could explain the observed accumulation of intracellular metabolites due to diminished cell growth and possibly reduced efficiencies of the biochemical reactions occurring in the metabolic network to produce limonene.

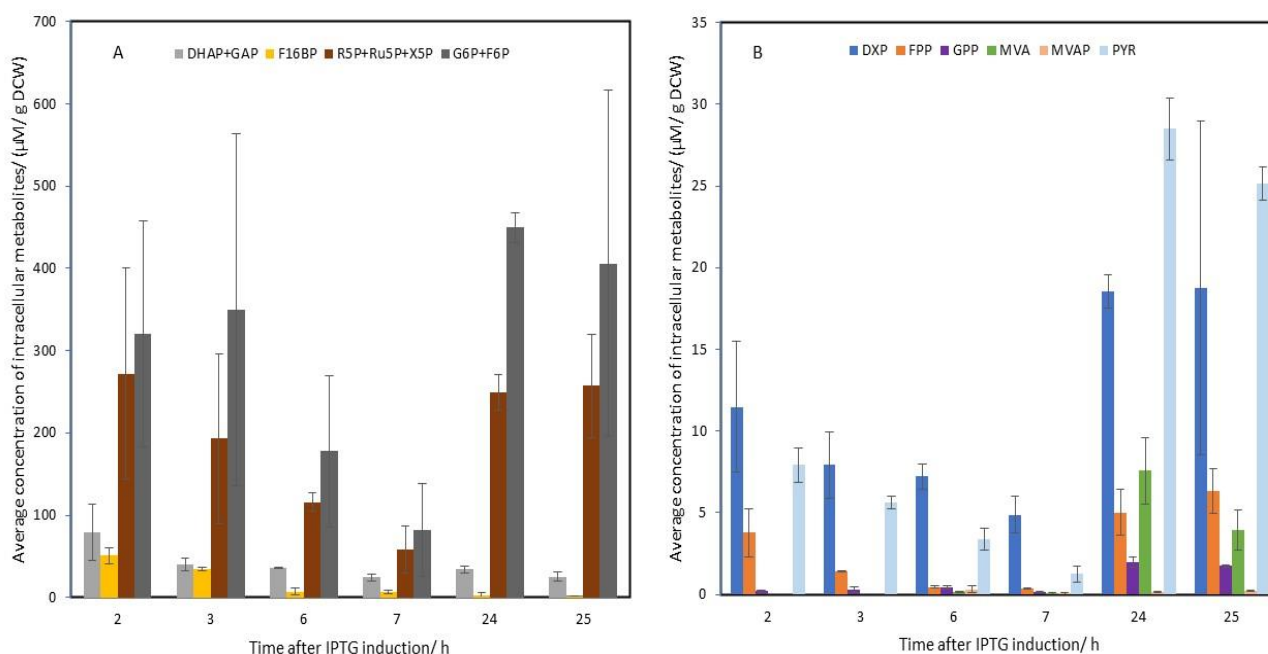


Figure 3. Time-series concentrations of intracellular metabolites from wild-type *E. coli* engineered to overproduce limonene (EcoCTs3) grown in glucose after IPTG induction. Figure 3A shows the intracellular metabolites of higher concentration DHAP+GAP (pool of dihydroxyacetone phosphate and glyceraldehyde-3-phosphate), F16BP (fructose-1,6-biphosphate), R5P+Ru5P+X5P (pool of ribose-5-phosphate, ribulose-5-phosphate, and xylulose-5-phosphate), and G6P+F6P (pool of glucose-6-phosphate and fructose-6-phosphate) while Figure 3B illustrates the measured intracellular metabolites DXP (1-deoxy-D-xylulose-5-phosphate), FPP (farnesyl diphosphate), GPP (geranyl diphosphate), MVA (mevalonate), MVAP (5-phosphomevalonate), and PYR (pyruvate).

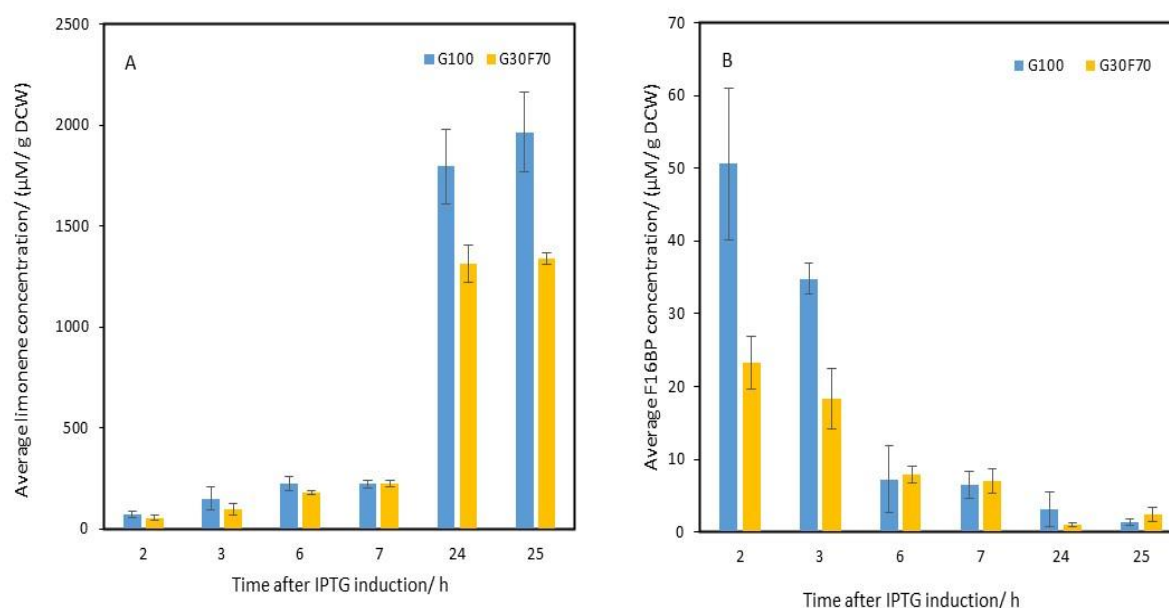


Figure 4. Comparison of metabolites from cell cultures of wild-type *E. coli* engineered to overproduce limonene (EcoCTs3) grown on different substrates, 100% glucose (G100), and a mixture of 30% glucose and 70% fructose (G30F70). Figure 4A shows the concentration of secreted limonene while Figure 4B shows the concentration of intracellular fructose-1,6-biphosphate (F16BP) over the time points 2 h to 25 h post-IPTG induction.

The presence and quantitation of intracellular FPP (Figure 3B) showed that there was a loss of carbon flux towards limonene production, as this metabolite is formed from GPP and IPP (Figure 1). With less carbon contribution to the immediate precursor metabolite to limonene, GPP, the limonene yield would not be optimal. The presence of both intracellular GPP and FPP as observed in Figure 3B could be due to their formations being catalyzed by the same prenyl diphosphate synthase, the enzyme farnesyl diphosphate synthase (IspA) (32, 33). FPP production is favoured in *E. coli* as it is required in the formation of peptidoglycans which are important constituents of cell wall (34) and components of the respiratory chain (35, 36), where its accumulation 24 h to 25 h after IPTG induction further indicates diminished cell growth in the cultures. To prevent the loss of carbon flux towards FPP, Alonso-Gutierrez *et. al.* (19) improved the titre of limonene through the expression of a heterologous dedicated GPP synthase which produced only GPP, unlike the native IspA, producing both GPP and FPP. The redirection of carbon flux towards GPP and preventing its loss to FPP helped in improving the yield of the target. Using this reported finding, it is therefore possible to redirect carbon flux towards the target by eliminating competing biochemical reactions, whether such reactions occur upstream or downstream of the MEV pathway.

The metabolic network in Figure 1 shows the importance of the metabolite pyruvate (PYR) in relation to the other biochemical pathways. PYR is formed as the end product of glycolysis, and is channelled into the fermentation fluxes, the DXP pathway, and it is the precursor to the intermediate metabolite, acetyl coenzyme A (AcCoA) involved in the TCA and MEV pathway. The cultures of the EcoCTs3 strain

had a drastic decrease in the intracellular concentration of PYR by about 41 folds (concentration of PYR at 2 h post IPTG induction was 7.92 $\mu\text{M/g DCW}$), when compared to the pool of the first two metabolites in the glycolysis pathway (320.31 $\mu\text{M/g DCW}$), *i.e.* G6P and F6P at the same time point of 2 h post IPTG induction (Figures 3A and 3B). With such a significant concentration reduction from the G6P+F6P pool to PYR, we deduced that there would be a low amount of carbon flux entering the MEV pathway, resulting in the low production of limonene observed in the EcoCTs3 strain thus far. In principle, improvements in limonene production could be achieved by increasing metabolite concentrations upstream of the metabolite PYR in the metabolic network. Comparing F16BP at 2 h and 25 h post IPTG shows its concentration decreased by six times compared to the pool of G6P and F6P at 2 h point, and it steadily decreased until 25 h (Figure 3A). Therefore, we hypothesized increasing the concentrations of F16BP by channelling more flux from G6P+F6P could be a strategy to eventually improve the carbon flux going towards limonene production.

3.2 Attempting to improve intracellular F16BP concentration by changing carbon source

In our attempts to increase the intracellular F16BP concentration, fructose was used as a possible carbon source alternative to glucose. From the metabolite network in Figure 1, and based on previous work (37), fructose can be converted to either F1P (fructose-1-phosphate) or F6P prior to forming F16BP. When varying proportions of fructose (0% to 100%) to glucose were tested in the culture medium, the highest amount of limonene was produced by growing the EcoCTs3 *E. coli* strain in 100% glucose (G100) followed by a mixture of 30% glucose and 70% fructose (G30F70), as shown in Figure 4A (Figure S2 represents the full dataset, Supplementary section). However, attempts with higher proportion of fructose to improve the initial intracellular F16BP concentration did not occur (Figure 4B), as glucose was still the main driver of F16BP production with possibly diauxic growth. Glucose passively diffuses into *E. coli*, where inner membrane transport followed by phosphorylation occurs (38). This process utilises the phosphoenolpyruvate:carbohydrate phosphotransferase system (PTS), which consists of multiple proteins in the phospho-relay process that are involved in the import and concurrent phosphorylation of carbohydrates (39). With the presence of glucose in the cell culture medium, there is enhanced glucose transport activity by bacteria due to induced expression of *ptsG* from the phosphotransferase system (40, 41). This corroborates with the findings in Figure 4, where glucose was still the best substrate for limonene production. With no improvement in intracellular F16BP and therefore limonene concentrations through mixtures of glucose and fructose as carbon source, we next hypothesized potential improvement of re-directing carbon flow towards MEV pathway by curtailing the loss of carbon flux to other pathways, and in so doing, enhance limonene yield.

3.3 Generating mutant strains to direct carbon flux towards MEV pathway

The second hypothesis tested for upstream flux optimisation to improve limonene yield is to direct more carbon flux into the MEV pathway. One approach is to allow more carbon flux to flow towards PYR since it is a key metabolite involved in numerous pathways, and at the same time curtail PYR losses through pathways that do not lead to limonene production. Mixed fermentation is one common route where there is loss of carbon flux due to the formation of lactic acid and ethanol. To eliminate the mixed fermentation pathways, two mutant strains were engineered. One strain had the *ldh* gene removed, thereby representing a LDH knockout, while a second strain had the *adhE* gene removed,

resulting in an ALDH-ADH knockout, as the *adhE* gene encodes for both enzymes ADH and ALDH (42, 43). Plasmid pJBEI-6409, which overexpresses MEV enzymes, as well as GPPS and LS, was transformed in each knockout strain. These strains were then assessed for limonene production, and the outcome is shown in Figure 5. There was loss of carbon flux in the EcoCTs3 strain as it was found to produce extracellular lactic acid and acetic acid (Figure S3, Supplementary section), however, the amount of ethanol produced was below the limit of quantitation for the HPLC-RID system. The LDH and ALDH-ADH knockouts did not produce extracellular lactic acid and ethanol, respectively.

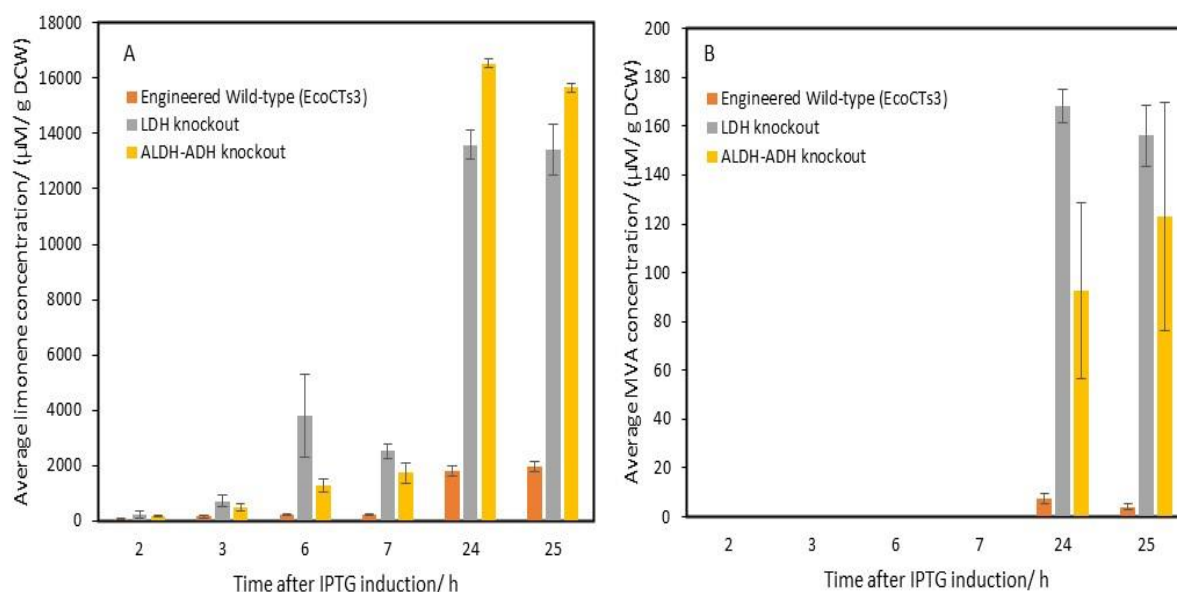
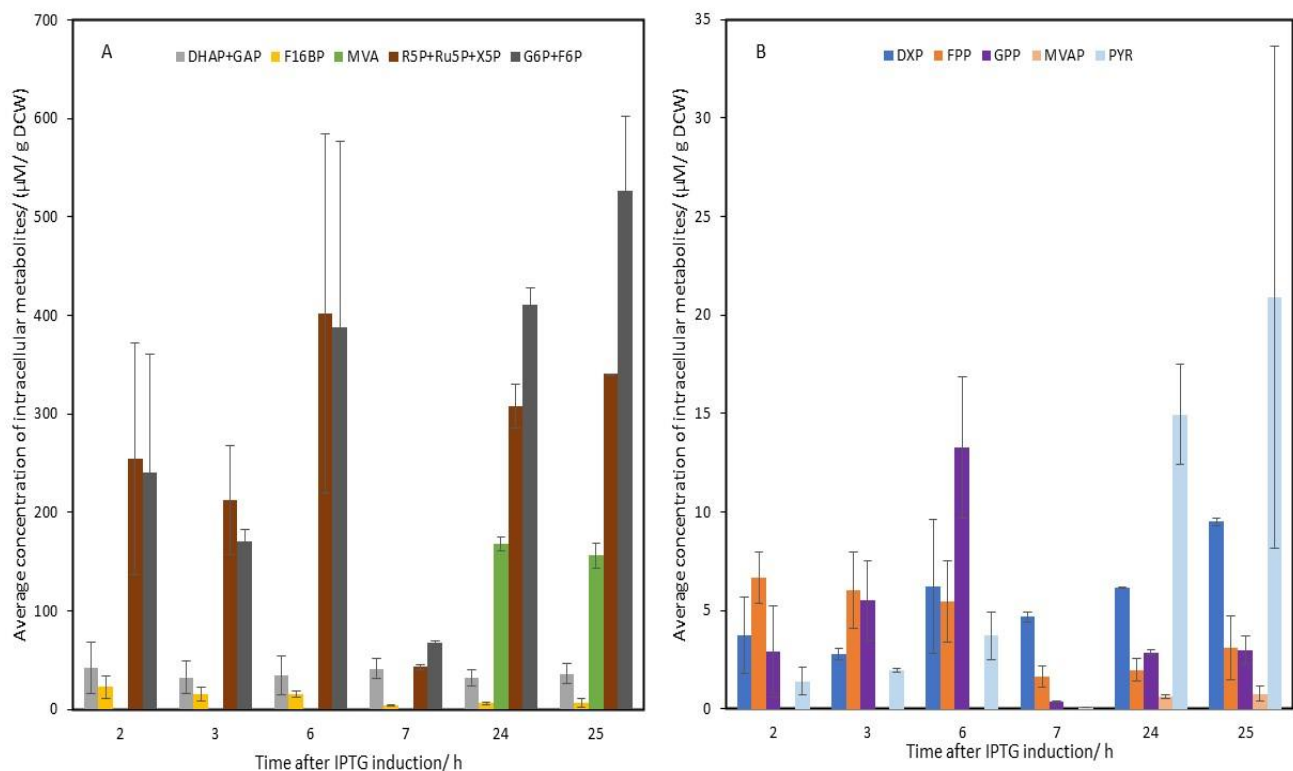


Figure 5. Comparison of metabolites from cell cultures containing different *E. coli* strains: engineered wild-type, LDH (lactate dehydrogenase) knockout and ALDH-ADH (aldehyde dehydrogenase-alcohol dehydrogenase) knockout. Figure 5A shows the concentration of limonene produced while Figure 5B shows the concentration of intracellular mevalonate (MVA) over the time points 2 h to 25 h post-IPTG induction.

When compared to the EcoCTs3 strain, these knockout strains were found to substantially improve limonene yield by 8 to 9 folds (Figure 5A). This enhanced limonene production was correlated to the 18 to 20 fold increase in intracellular MVA measured in the knockout strains, as illustrated in Figure 5B. There was also greater accumulation of intracellular MVA compared to some other metabolites at 24 h to 25 h post-IPTG for these mutant strains, as observed in Figures 6A and 6B for the LDH knockout strain and Figures 7A and 7B for the ALDH-ADH knockout strain. MVA was observed at the later time points (Figures 6A and 7A) likely because the enzyme mevalonate kinase (MK) could not efficiently convert MVA to MVAP. Since this biochemical reaction requires the presence of ATP (adenosine triphosphate), it could suggest the availability of ATP becomes a limiting factor in the biochemical synthesis of limonene. Such reduced ATP availability has also been observed in previous studies in various microbes, thereby affecting the productivity of the MEV pathway in generating isoprenoids (21, 22). Furthermore, the observation of high levels of intracellular MVA could be explained by previous work, in which downstream intermediates such as IPP, DMAPP, FPP, and GPP have been found to inhibit MK activity through competitive binding to the ATP-binding site of MK (44-47). With the

competitive inhibition of MK, there would be less MVAP produced and more intracellular MVA accumulated, as observed in Figures 6A and 6B for the LDH knockout strain and Figures 7A and 7B for the ALDH-ADH knockout strain. Furthermore, in another study using targeted proteomics, the production of enzymes MK and phosphomevalonate kinase (PMK) were found to be poor (48). These compounding factors make it difficult for the accumulation of intracellular metabolites downstream of MVA such as MVAP and GPP (Figures 6B and 7B) and explains the poor conversion efficiency of MVA to limonene, as observed from the intracellular data, where the 18 to 20 fold increase in MVA did not result in the same fold increase of limonene (Figure 5). The LDH and ALDH-ADH knockout strains also showed loss of flux to FPP (Figures 6B and 7B) due to the native enzyme IspA catalyzing its formation. Redirecting the flux to GPP and preventing its loss to FPP can be executed through the expression of an improved heterologous GPP synthase (19) and improving limonene synthase activity, which could further improve limonene titres for both the knockout strains.

Targeted metabolomics has been useful in identifying limitations in engineered metabolic pathways for further fine-tuning. In one study, metabolomics was conducted on a recombinant *Saccharomyces cerevisiae* strain producing β -carotene and the parent strain, where most of the metabolites involved in central metabolism were found in lower quantities in the recombinant yeast strain compared to the parent strain (49). This led to a strategy of the exogenous addition of acetate in the exponential phase, which increased the levels of intracellular metabolites and in turn led to increased β -carotene production in the recombinant strain. In another study involving the engineering *E. coli* for the overproduction of coenzyme Q10, the overproduction of key enzymes and the removal of competing pathways were identified through targeted metabolomics which in turn guided strain optimisation (50). In this work, the removal of competing pathways through the engineering of LDH and ALDH-ADH knockout strains led to improved limonene yield. Furthermore, the metabolomics data of these knockout strains showed high amounts of MVA accumulating which can guide further fine-tuning of the knockout strains to improve limonene yield such as increasing ATP availability and improving the catalytic capacities of enzymes such as MK and PMK.



427

428

429

430

431

432

433

434

435

436

Figure 6. Representation of the time-series concentrations of intracellular metabolites from LDH (lactate dehydrogenase) knockout *E. coli* strains grown in glucose after IPTG induction. Figure 6A represents the intracellular metabolites DHAP+GAP (pool of dihydroxyacetone phosphate and glyceraldehyde-3-phosphate), F16BP (fructose-1,6-biphosphate), MVA (mevalonate), R5P+Ru5P+X5P (pool of ribose-5-phosphate, ribulose-5-phosphate, and xylulose-5-phosphate), and G6P+F6P (pool of glucose-6-phosphate and fructose-6-phosphate), which were of higher concentration while Figure 6B displays intracellular metabolites DXP (1-deoxy-D-xylulose-5-phosphate), FPP (farnesyl diphosphate), GPP (geranyl diphosphate), MVAP (5-phosphomevalonate), and PYR (pyruvate).

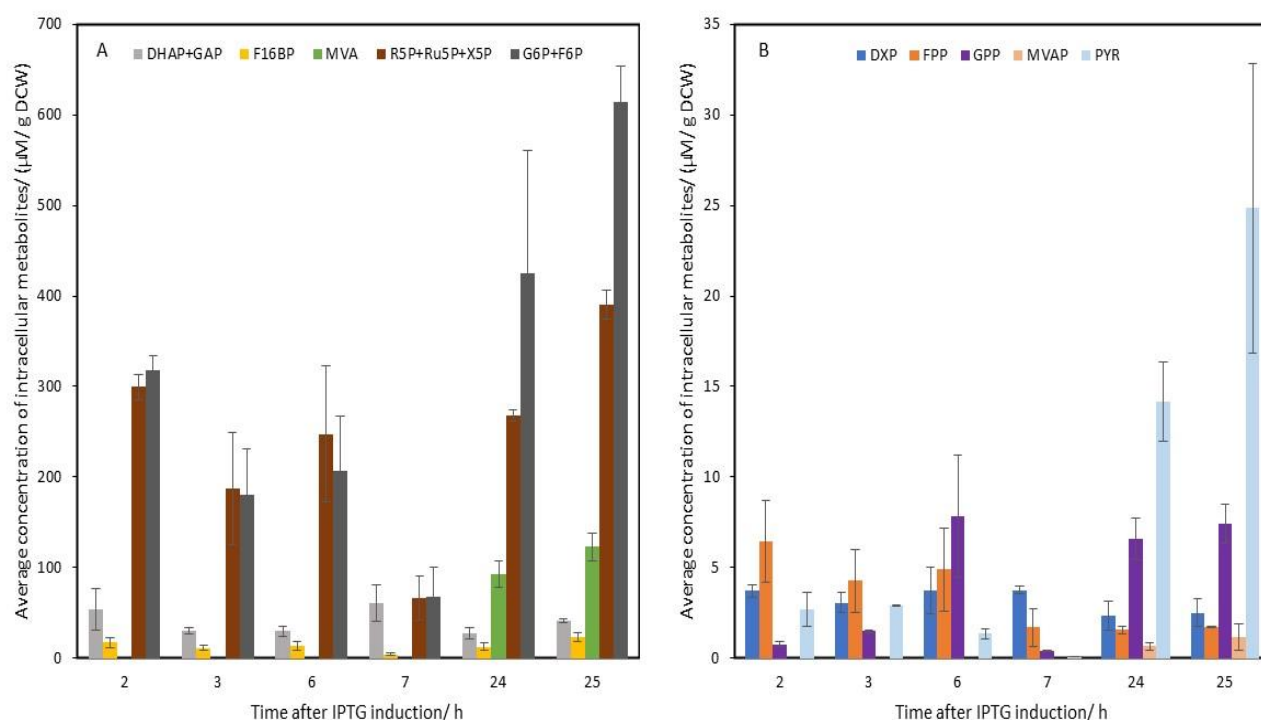


Figure 7. Representation of the intracellular concentrations of metabolites obtained at various time-points from ALDH-ADH (aldehyde dehydrogenase-alcohol dehydrogenase) knockout *E. coli* strains grown in glucose after IPTG induction. Figure 7A illustrates the intracellular metabolites DHAP+GAP (pool of dihydroxyacetone phosphate and glyceraldehyde-3-phosphate), F16BP (fructose-1,6-biphosphate), MVA (mevalonate), R5P+Ru5P+X5P (pool of ribose-5-phosphate, ribulose-5-phosphate, and xylulose-5-phosphate), and G6P+F6P (pool of glucose-6-phosphate and fructose-6-phosphate), which were of higher concentration while Figure 7B shows intracellular metabolites DXP (1-deoxy-D-xylulose-5-phosphate), FPP (farnesyl diphosphate), GPP (geranyl diphosphate), MVAP (5-phosphomevalonate), and PYR (pyruvate).

4. Conclusion

When engineering bacterial strains to enhance the yield of target compounds, combining intracellular time-series metabolomics data together with the understanding of the metabolic network can be very useful. Incorporating a hypothesis-driven experimental approach with the idea of using different strategies such as changing the carbon source and eliminating competing pathways that result in flux loss can aid in achieving the desired goal of improving target yield whilst streamlining the workflow.

From this study, enhancing the concentration of intracellular MVA is a key step in improving the yield of limonene and possibly other target compounds in the MEV pathway. Once a strain producing high intracellular MVA has been identified, further fine-tuning of the strain can be executed to improve the target yield. The presence of bottlenecks downstream of MVA in the MEV pathway could possibly be due to the lack of ATP to metabolize MVA to MVAP and the feedback-inhibition of MK. To further minimise the bottlenecks observed downstream of the metabolite MVA in the engineered MEV pathway, enzymes homologous to those in the engineered MEV pathway from alternative organisms could be utilised instead (51). For instance, feedback-resistant MK homologs have been found to uphold high activity in the presence of DMAPP, IPP, GPP, FPP, and MVAPP (47, 52, 53). These homologs

can reduce MVA accumulation, thereby improving titres. Furthermore, protein engineering can also enhance substrate affinity of feedback-resistant MK (54) and it has been used on enzyme isopentenyl diphosphate isomerase (IDI) to improve its catalytic activity and thereby increase titre levels (55).

Author contributions

J. K. K. carried out culturing and metabolomics experiments on the EcoCTS03 and modified strains. C. P. M. S., S. B., and X. C. designed and prepared the modified strains. J. K. K. and W. C. designed the study and wrote the paper.

Conflict of interest

The authors declare no competing interests.

Acknowledgements

This work was supported by the Intra-Create Thematic Grant “Cities” (grant number: NRF2019-THE001-0007) under the EcoCTs project. The EcoCTs research project is supported by the National Research Foundation, Prime Minister’s Office, Singapore, under its campus for Research Excellence and Technological Enterprise (CREATE) programme. In addition, we are thankful to Dr. Florian Bellvert, Hanna Kulyk, and Cecilia Berges from MetaToul (Metabolomics & Fluxomics Facilities, Toulouse, France) for their experimental guidance and insights. We would also like to acknowledge Dr. Kumar Selvarajoo from the Bioinformatics Institute (BII), A*STAR, Singapore for technical advice and discussion.

References

1. Wojtunik-Kulesza KA, Kasprzak K, Oniszczyk T, Oniszczyk A. Natural Monoterpenes: Much More than Only a Scent. *Chem Biodivers*. 2019;16(12):e1900434.
2. Mamidipally PK, Liu SX. First approach on rice bran oil extraction using limonene. *European Journal of Lipid Science and Technology*. 2004;106(2):122-5.
3. Hąc-Wydro K, Flasiński M, Romańczuk K. Essential oils as food eco-preservatives: Model system studies on the effect of temperature on limonene antibacterial activity. *Food Chem*. 2017;235:127-35.
4. Felipe LdO, Oliveira AMd, Bicas JL. Bioaromas – Perspectives for sustainable development. *Trends in Food Science & Technology*. 2017;62:141-53.
5. Thomsett MR, Moore JC, Buchard A, Stockman RA, Howdle SM. New renewably-sourced polyesters from limonene-derived monomers. *Green Chemistry*. 2019;21(1):149-56.
6. Ciriminna R, Lomeli-Rodriguez M, Demma Carà P, Lopez-Sanchez JA, Pagliaro M. Limonene: a versatile chemical of the bioeconomy. *Chem Commun (Camb)*. 2014;50(97):15288-96.
7. Ibáñez MD, Sanchez-Ballester NM, Blázquez MA. Encapsulated Limonene: A Pleasant Lemon-Like Aroma with Promising Application in the Agri-Food Industry. A Review. *Molecules*. 2020;25(11).
8. Kvittingen L, Sjursnes BJ, Schmid R. Limonene in Citrus: A String of Unchecked Literature Citings? *Journal of Chemical Education*. 2021;98(11):3600-7.

9. Jongedijk E, Cankar K, Buchhaupt M, Schrader J, Bouwmeester H, Beekwilder J. Biotechnological production of limonene in microorganisms. *Appl Microbiol Biotechnol*. 2016;100(7):2927-38.
10. Moser S, Pichler H. Identifying and engineering the ideal microbial terpenoid production host. *Appl Microbiol Biotechnol*. 2019;103(14):5501-16.
11. Lu-lu Z, Gang FAN, Jin HE, Wen H, Jing-nan REN, Si-yi PAN. Advances on Limonene Microbial Biotransformation and Its Related Enzymes. *Science and Technology of Food Industry*. 2019;40(12):317-25,30.
12. Sun C, Theodoropoulos C, Scrutton NS. Techno-economic assessment of microbial limonene production. *Bioresource Technology*. 2020;300:122666.
13. Ren Y, Liu S, Jin G, Yang X, Zhou YJ. Microbial production of limonene and its derivatives: Achievements and perspectives. *Biotechnology Advances*. 2020;44:107628.
14. Wang C, Zada B, Wei G, Kim S-W. Metabolic engineering and synthetic biology approaches driving isoprenoid production in *Escherichia coli*. *Bioresource Technology*. 2017;241:430-8.
15. Zhao L, Chang WC, Xiao Y, Liu HW, Liu P. Methylerythritol phosphate pathway of isoprenoid biosynthesis. *Annu Rev Biochem*. 2013;82:497-530.
16. Boucher Y, Doolittle WF. The role of lateral gene transfer in the evolution of isoprenoid biosynthesis pathways. *Mol Microbiol*. 2000;37(4):703-16.
17. Connolly DM, Winkler ME. Genetic and physiological relationships among the *miaA* gene, 2-methylthio-N⁶-(delta 2-isopentenyl)-adenosine tRNA modification, and spontaneous mutagenesis in *Escherichia coli* K-12. *J Bacteriol*. 1989;171(6):3233-46.
18. Martin VJJ, Pitera DJ, Withers ST, Newman JD, Keasling JD. Engineering a mevalonate pathway in *Escherichia coli* for production of terpenoids. *Nature Biotechnology*. 2003;21(7):796-802.
19. Alonso-Gutierrez J, Chan R, Batth TS, Adams PD, Keasling JD, Petzold CJ, Lee TS. Metabolic engineering of *Escherichia coli* for limonene and perillyl alcohol production. *Metab Eng*. 2013;19:33-41.
20. Ward VCA, Chatzivasileiou AO, Stephanopoulos G. Metabolic engineering of *Escherichia coli* for the production of isoprenoids. *FEMS Microbiology Letters*. 2018;365(10).
21. Gruchattka E, Hädicke O, Klamt S, Schütz V, Kayser O. In silico profiling of *Escherichia coli* and *Saccharomyces cerevisiae* as terpenoid factories. *Microb Cell Fact*. 2013;12:84.
22. Diner BA, Fan J, Scotcher MC, Wells DH, Whited GM. Synthesis of Heterologous Mevalonic Acid Pathway Enzymes in *Clostridium ljungdahlii* for the Conversion of Fructose and of Syngas to Mevalonate and Isoprene. *Applied and Environmental Microbiology*. 2018;84(1):e01723-17.
23. Rohmer M, Seemann M, Horbach S, Bringer-Meyer S, Sahm H. Glyceraldehyde 3-Phosphate and Pyruvate as Precursors of Isoprenic Units in an Alternative Non-mevalonate Pathway for Terpenoid Biosynthesis. *Journal of the American Chemical Society*. 1996;118(11):2564-6.
24. Carter OA, Peters RJ, Croteau R. Monoterpene biosynthesis pathway construction in *Escherichia coli*. *Phytochemistry*. 2003;64(2):425-33.
25. Dunlop MJ, Dossani ZY, Szmids H, Chu HC, Lee TS, Keasling JD, et al. Engineering microbial biofuel tolerance and export using efflux pumps. *Mol Syst Biol*. 2011;7:487.
26. Reiling KK, Yoshikuni Y, Martin VJ, Newman J, Bohlmann J, Keasling JD. Mono and diterpene production in *Escherichia coli*. *Biotechnol Bioeng*. 2004;87(2):200-12.
27. Shukal S, Lim XH, Zhang C, Chen X. Metabolic engineering of *Escherichia coli* BL21 strain using simplified CRISPR-Cas9 and asymmetric homology arms recombineering. *Microbial Cell Factories*. 2022;21(1):19.
28. Khanijou JK, Hee YT, Scipion CPM, Chen X, Selvarajoo K. Systems biology approach for enhancing limonene yield by re-engineering *Escherichia coli*. *npj Systems Biology and Applications*. 2024;10(1):109.
29. Wada K, Toya Y, Banno S, Yoshikawa K, Matsuda F, Shimizu H. 13C-metabolic flux analysis for mevalonate-producing strain of *Escherichia coli*. *Journal of Bioscience and Bioengineering*. 2017;123(2):177-82.

30. Castaño-Cerezo S, Kulyk-Barbier H, Millard P, Portais JC, Heux S, Truan G, Bellvert F. Functional analysis of isoprenoid precursors biosynthesis by quantitative metabolomics and isotopologue profiling. *Metabolomics*. 2019;15(9):115.
31. Ng P, Khoo LW, Thong A, Chew W. Optimization of extraction conditions for LC-ToF-MS analysis of mevalonate pathway metabolites in engineered *E. coli* strain via statistical experimental designs. *Talanta*. 2023;254:124182.
32. Ogura K, Koyama T. Enzymatic Aspects of Isoprenoid Chain Elongation. *Chem Rev*. 1998;98(4):1263-76.
33. Ku B, Jeong J-C, Mijts BN, Schmidt-Dannert C, Dordick JS. Preparation, Characterization, and Optimization of an In Vitro C₃₀ Carotenoid Pathway. *Applied and Environmental Microbiology*. 2005;71(11):6578-83.
34. Apfel CM, Takács B, Fountoulakis M, Stieger M, Keck W. Use of genomics to identify bacterial undecaprenyl pyrophosphate synthetase: cloning, expression, and characterization of the essential *uppS* gene. *J Bacteriol*. 1999;181(2):483-92.
35. Okada K, Minehira M, Zhu X, Suzuki K, Nakagawa T, Matsuda H, Kawamukai M. The *ispB* gene encoding octaprenyl diphosphate synthase is essential for growth of *Escherichia coli*. *Journal of Bacteriology*. 1997;179(9):3058-60.
36. Saiki K, Mogi T, Anraku Y. Heme O biosynthesis in *Escherichia coli*: the *cyoE* gene in the cytochrome bo operon encodes a protoheme IX farnesyltransferase. *Biochem Biophys Res Commun*. 1992;189(3):1491-7.
37. Kornberg HL. Routes for fructose utilization by *Escherichia coli*. *J Mol Microbiol Biotechnol*. 2001;3(3):355-9.
38. Kotrba P, Inui M, Yukawa H. The *ptsI* Gene Encoding Enzyme I of the Phosphotransferase System of *Corynebacterium glutamicum*. *Biochemical and Biophysical Research Communications*. 2001;289(5):1307-13.
39. Postma PW, Lengeler JW, Jacobson GR. Phosphoenolpyruvate:carbohydrate phosphotransferase systems of bacteria. *Microbiological Reviews*. 1993;57:543 - 94.
40. Plumbridge J. Regulation of gene expression in the PTS in *Escherichia coli*: the role and interactions of Mlc. *Current Opinion in Microbiology*. 2002;5(2):187-93.
41. Yao R, Hirose Y, Sarkar D, Nakahigashi K, Ye Q, Shimizu K. Catabolic regulation analysis of *Escherichia coli* and its *crp*, *mlc*, *mgsA*, *pgi* and *ptsG* mutants. *Microbial Cell Factories*. 2011;10(1):67.
42. Bertsch J, Siemund AL, Kremp F, Müller V. A novel route for ethanol oxidation in the acetogenic bacterium *Acetobacterium woodii*: the acetaldehyde/ethanol dehydrogenase pathway. *Environmental Microbiology*. 2016;18(9):2913-22.
43. Kim G, Yang J, Jang J, Choi J-S, Roe AJ, Byron O, et al. Aldehyde-alcohol dehydrogenase undergoes structural transition to form extended spiroosomes for substrate channeling. *Communications Biology*. 2020;3(1):298.
44. Voynova NE, Rios SE, Miziorko HM. *Staphylococcus aureus* Mevalonate Kinase: Isolation and Characterization of an Enzyme of the Isoprenoid Biosynthetic Pathway. *Journal of Bacteriology*. 2004;186(1):61-7.
45. Dorsey JK, Porter JW. The Inhibition of Mevalonic Kinase by Geranyl and Farnesyl Pyrophosphates. *Journal of Biological Chemistry*. 1968;243(18):4667-70.
46. Gray JC, Kekwick RGO. The inhibition of plant mevalonate kinase preparations by prenyl pyrophosphates. *Biochimica et Biophysica Acta (BBA) - General Subjects*. 1972;279(2):290-6.
47. Huang K-x, Scott AI, Bennett GN. Overexpression, Purification, and Characterization of the Thermostable Mevalonate Kinase from *Methanococcus jannaschii*. *Protein Expression and Purification*. 1999;17(1):33-40.
48. Redding-Johanson AM, Batth TS, Chan R, Krupa R, Szmidt HL, Adams PD, et al. Targeted proteomics for metabolic pathway optimization: application to terpene production. *Metab Eng*. 2011;13(2):194-203.

49. Bu X, Sun L, Shang F, Yan G. Comparative metabolomics profiling of engineered *Saccharomyces cerevisiae* lead to a strategy that improving β -carotene production by acetate supplementation. *PLoS One*. 2017;12(11):e0188385.
50. Cluis CP, Ekins A, Narcross L, Jiang H, Gold ND, Burja AM, Martin VJ. Identification of bottlenecks in *Escherichia coli* engineered for the production of CoQ(10). *Metab Eng*. 2011;13(6):733-44.
51. Rinaldi MA, Ferraz CA, Scrutton NS. Alternative metabolic pathways and strategies to high-titre terpenoid production in *Escherichia coli*. *Natural Product Reports*. 2022;39(1):90-118.
52. Primak YA, Du M, Miller MC, Wells DH, Nielsen AT, Weyler W, Beck ZQ. Characterization of a feedback-resistant mevalonate kinase from the archaeon *Methanosarcina mazei*. *Appl Environ Microbiol*. 2011;77(21):7772-8.
53. Kazieva E, Yamamoto Y, Tajima Y, Yokoyama K, Katashkina J, Nishio Y. Characterization of feedback-resistant mevalonate kinases from the methanogenic archaeons *Methanosaeta concilii* and *Methanocella paludicola*. *Microbiology (Reading)*. 2017;163(9):1283-91.
54. Chen H, Liu C, Li M, Zhang H, Xian M, Liu H. Directed evolution of mevalonate kinase in *Escherichia coli* by random mutagenesis for improved lycopene. *RSC Advances*. 2018;8(27):15021-8.
55. Chen H, Li M, Liu C, Zhang H, Xian M, Liu H. Enhancement of the catalytic activity of Isopentenyl diphosphate isomerase (IDI) from *Saccharomyces cerevisiae* through random and site-directed mutagenesis. *Microb Cell Fact*. 2018;17(1):65.