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METABOLIC ENGINEERING AND SYNTHETIC BIOLOGY

Synthesis of methyl ketones by metabolically engineered *Escherichia coli*

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Abstract Methyl ketones are a group of highly reduced platform chemicals with widespread applications in the fragrance, flavor and pharmacological industries. Current methods for the industrial production of methyl ketones include oxidation of hydrocarbons, but recent advances in the characterization of methyl ketone synthases from wild tomato have sparked interest towards the development of microbial platforms for the industrial production of methyl ketones. A functional methyl ketone biosynthetic pathway was constructed in *Escherichia coli* by over-expressing two genes from *Solanum habrochaites*: *shmks2*, encoding a 3-ketoacyl-ACP thioesterase, and *shmks1*, encoding a beta-decarboxylase. These enzymes enabled methyl ketone synthesis from 3-ketoacyl-ACP, an intermediate in the fatty acid biosynthetic cycle. The production of 2-nonanone, 2-undecanone, and 2-tridecanone by MG1655 pTH-shmks2-shmks1 was initially detected by nuclear magnetic resonance and gas chromatography–mass spectrometry analyses at levels close to 6 mg/L. The deletion of major fermentative pathways leading to ethanol (*adhE*), lactate (*ldhA*), and acetate (*pta*, *poxB*) production allowed for the carbon flux to be redirected towards methyl ketone production, doubling total methyl ketone concentration. Variations in methyl ketone production observed under different working volumes in flask experiments led to a more detailed analysis of the effects of oxygen availability on methyl ketone concentration in order to determine optimal levels of oxygen. The methyl ketone concentration achieved with MG1655 Δ*adhE* Δ*ldhA* Δ*poxB* Δ*pta* pTr-cHis2A-shmks2-shmks1, the best performer strain in this study, was approximately 500 mg/L, the highest reported for an engineered microorganism. Through the establishment of optimal operating conditions and by executing rational metabolic engineering strategies, we were able to increase methyl ketone concentrations by almost 75-fold from the initial confirmatory levels.

Keywords Methyl ketones · Metabolic engineering · *Escherichia coli*

John Park and María Rodríguez-Moyá contributed equally to this work.

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Introduction

Aliphatic methyl ketones, a group of highly reduced platform chemicals, are a class of organic chemicals whose natural origin has been studied and firmly established over many years. The occurrence of methyl ketones in nature was first reported in 1858 when Williams discovered 2-undecanone as the primary constituent of oil from *Ruta graveolens*, commonly known as rue [38]. Watts later found that 2-undecanone was also present in essential oils of other plants [36]. Since then, numerous reports have confirmed methyl ketones as constituents of oils from

cloves, cinnamon, coconut, hops, palm, peanut, and cottonseed, among others, where they mainly acting as natural insecticides [13]. The main methyl ketones that have been identified from plants and seed oils are 2-heptanone, 2-nonanone, 2-undecanone, and 2-tridecanone. In some cases, they occur together with their associated secondary alcohols (i.e., 2-nonanol, 2-undecanol, and 2-tridecanol) which are thought to be the reduced equivalents of their corresponding methyl ketones [20, 37].

Methyl ketones are also produced by different species of bacteria, fungi, and insects [13]. For example, *Mycobacterium rhodochrous* has been found to produce 2-undecanone from *n*-undecane [26], while *Pseudomonas methanica* and *Mycobacterium smegmatis* were found to produce shorter chain methyl ketones (2-butanone, 2-pentanone, and 2-hexanone) from their corresponding alkanes [23, 24, 26]. *Penicillium glaucum* and *Penicillium roqueforti*, two fungi species used to make cheese, have also been identified as producers of 2-undecanone and 2-heptanone, respectively [15, 34]. In addition, several species of *Aspergillus* have been found to produce different types of methyl ketones [1, 25], and methyl ketones have also been found as pheromones in odorous secretions of ants [3, 6, 7, 28] and honeybees [31].

Methyl ketones do not only provide aroma and flavor to dairy products, and their commercial roles extend from additives in the fragrance [5, 18] and flavor [17, 33] industries, to components in pharmacological syntheses [32, 42]. Considering their favorable cetane numbers, some methyl ketones could complement diesel and be used as biofuels [16], but exploiting their potential as platform chemicals for the fragrance, flavor and pharmacological industries seems more convenient due to the lower concentrations needed for these applications.

Methyl ketones are currently produced mainly by the oxidation of hydrocarbons, often with air, but recent advances in the characterization of wild tomato (*Solanum habrochaites* and *S. lycopersicum*) genes involved in the production of methyl ketones [2, 14, 40] have sparked research interest towards the development of microbial platforms for the production of methyl ketones in industrial environments. To this end, the bacterium *Escherichia coli* is a suitable microorganism due to the extensive body of knowledge about its genome, the availability of tools for genetic manipulations and its low-cost and efficient growth under industrially relevant conditions. As *E. coli* does not produce methyl ketones natively, we engineered a new pathway that incorporates genes encoding methyl ketone synthases from wild tomato into the industrially attractive *E. coli* host in order to enable the production of methyl ketones in a renewable manner and exploit their natural potential and applications.

Materials and methods

Strains and plasmids

All strains, plasmids, and primers used in this study are listed in Tables 1 and 2. All of the strains used are derivatives of *E. coli* K-12 strain MG1655 (F- λ- ilvG- rfb-50 rph-1), obtained from the University of Wisconsin *E. coli* Genome Project (www.genome.wisc.edu) [21]. Mutant alleles were moved into this background via standard P1 transduction as previously described [9].

The genes *shmks2* and *slmks2*, encoding 3-ketoacyl-ACP thioesterases from *S. habrochaites* and *S. lycopersicum* respectively, and *shmks1*, encoding a beta-decarboxylase from *S. habrochaites* [40], were cloned in the medium-copy vector pTrcHis2A (Invitrogen, Carlsbad, CA). All three genes were amplified from expression vectors carrying full open reading frames (ORFs) [2] and cloned into pTrcHis2A using the In-Fusion Cloning System from Clontech (Mountain View, CA). In the case of the single-expression plasmids, a cloning site was chosen within the multi-cloning site upstream of the promoters and ribosome binding sites as the insertion site for the gene of interest (*Kpn*I and *Sal*I), and the plasmid was linearized. In the case of the double-expression plasmids, the procedure was the same, with the exception that the primers used for the amplification of the second gene (*shmks1*) involved an addition of a ribosome binding site consensus sequence (GAGGAG) along with six dummy base pairs between the 15-bp homology to the vector and the gene-specific priming sequence [19]. The PCR inserts derived from these primer sets were cloned downstream of the ORF of the first gene (*shmks2* or *slmks2*). Proper cloning of the inserts was checked through colony-PCR, and plasmids mini-prepped from the final strains were verified through both restriction analysis and sequencing (Lone Star Labs, Houston, TX).

The manufacturer's protocols (Qiagen, Venlo, the Netherlands) and standard methods [27, 30] were followed for DNA purification, plasmid isolation, and electroporation. The strains were kept in 32.5 % glycerol stocks at −80 °C. Plates were prepared using Luria–Bertani (LB) medium containing 1.5 % agar and the antibiotics chloramphenicol and kanamycin at concentrations of 34 and 50 μg/mL, respectively.

Culture medium and cultivation conditions

All strains were tested using Neidhardt MOPS Minimal Medium supplemented with 20 g/L glucose, 1.32 mM Na_2HPO_4 (in place of K_2HPO_4), 5 mM $(NH_4)_2SO_4$, and 30 mM NH_4Cl, unless otherwise stated [29]. All chemicals for culture media were obtained from Fisher Scientific (Pittsburgh, PA) and Sigma-Aldrich Co. (St. Louis, MO).

Table 1 Plasmids used in this study

Plasmid	Relevant information	Source
pZSblank	Blank plasmid created by removing *Citrobacter freundii dhaKL* fragment from pZSKLcf and self-ligating the plasmid (tetR, oriR SC101*, cat)	Yazdani and Gonzalez [39]
pZS-shmks2	*Solanum habrochaites shmks2* gene under control of $P_{Ltet0-1}$ (tetR, oriR SC101*, cat)	This study
pZS-slmks2	*S. lycopersicum slmks2* gene under control of $P_{LtetO-1}$ (tetR, oriR SC101*, cat)	This study
pZS-shmks1	*S. habrochaites shmks1* gene under control of $P_{LtetO-1}$ (tetR, oriR SC101*, cat)	This study
pTrcHis2A-shmks2	*S. habrochaites shmks2* gene under control of P_{lacZ} (lacR, oriR BR322)	This study
pTrcHis2A-slmks2	*S. lycopersicum slmks2* gene under control of P_{lacZ} (lacR, oriR BR322)	This study
pTrcHis2A-shmks1	*S. habrochaites shmks1* gene under control of P_{lacZ} (lacR, oriR BR322)	This study
pTrcHis2A-shmks2-shmks1	*S. habrochaites shmks2* and *S. habrochaites shmks1* genes under control of P_{lacZ} (lacR, oriR BR322)	This study
pTrcHis2A-slmks2-shmks1	*S. lycopersicum slmks2* and *S. habrochaites shmks1*genes under control of P_{lacZ} (lacR, oriR BR322)	This study
pCR-shmks2	*S. habrochaites shmks2* gene under control of P_{lacZ} (oriR pUC)	This study
pCR-slmks2	*S. lycopersicum slmks2* gene under control of P_{lacZ} (oriR pUC)	This study
pCR-shmks2-shmks1	*S. habrochaites shmks2* and *S. habrochaites shmks1* genes under control of P_{lacZ} (oriR pUC)	This study
pCR-slmks2-shmks1	*S. lycopersicum slmks2* and *S. habrochaites shmks1*genes under control of P_{lacZ} (oriR pUC)	This study
pZS-adhF1	*Pseudomonas fluorescens adhF1* gene under control of $P_{Ltet0-1}$ (tetR, oriR SC101*, cat)	This study
pTrcHis2A-adhF1	*Pseudomonas fluorescens adhF1* gene under control of P_{lacZ} (lacR, oriR BR322)	This study

Table 2 Strains used in this study

Strain	Relevant information	Source
MG1655	F-l-ilvG-rfb-50 rph-1	Kang et al. [22]
Δ*adhE*	MG1655 Δ*adhE*::FRT-Kan-FRT	This study
Δ*ldhA*	MG1655 Δ*ldhA*::FRT-Kan-FRT	This study
Δ*poxB*	MG1655 Δ*poxB*::FRT-Kan-FRT	This study
Δ*pta*	MG1655 Δ*pta*::FRT-Kan-FRT	This study
Δ*adhE* Δ*pta*	MG1655 Δ*adhE*::FRT Δ*pta*::FRT-Kan-FRT	This study
Δ*ldhA* Δ*poxB*	MG1655 Δ*ldhA*::FRT Δ*poxB*::FRT-Kan-FRT	This study
Δ*ldhA* Δ*poxB* Δ*pta*	MG1655 Δ*ldhA*::FRT Δ*poxB*::FRT Δ*pta*::FRT	This study
Δ*adhE* Δ*ldhA* Δ*poxB* Δ*pta*	MG1655 Δa*dhE*::FRT Δ*ldhA*::FRT Δ*poxB*::FRT Δ*pta*::FRT	This study

Calcium carbonate (5 % w/w) was used for controlling the pH of the flask cultures, unless otherwise stated.

Prior to inoculating starter cultures, LB plates (with appropriate antibiotics if required) were streaked from frozen glycerol stocks (stored as glycerol stocks at −80 °C) and incubated overnight at 37 °C. A single colony was used to inoculate 10 mL working volume in 25-mL Erlenmeyer flasks (Corning, Corning, NY), which contained LB with the appropriate antibiotics. The flasks were then incubated at 37 °C and 200 rpm in an NBS C24 Benchtop Incubator Shaker (New Brunswick Scientific, Edison, NJ) until an OD_{550} of approximately 0.5 was reached. An appropriate volume of this actively growing preculture was centrifuged, and the pellet was washed and used to inoculate flasks or fermentors (see below), with a target initial optical density at 550 nm of 0.05.

The experimental flask cultures were cultivated in minimal media with appropriate antibiotics at various working volumes in 125-mL Erlenmeyer flasks (Corning). The operating conditions were as described in the previous paragraph. Cultivations in fermentors were performed using a SixFors Multi-fermentation System (Infors HT, Bottmingen, Switzerland) with control of dissolved oxygen (DO), pH, and temperature at specified values. The pH was adjusted with either 1.5 M sulfuric acid or 3 M sodium hydroxide. DO was controlled by adjusting the rpm to between 200 and 800 via the Sixfors microcontroller.

Methyl ketone extraction

Aliquots of culture (10 mL) were placed into 14-mL round-bottom polystyrene Falcon tubes (Becton–Dickinson

Labware, Franklin Lakes, NJ). Each aliquot, while in an ice bath, was sonicated for 5 min using a Branson Sonifier 450 (Emerson Industrial Automation, Danbury, CT) set at a duty cycle of 30 % and a power setting of 1.

Once sonication was complete, each aliquot was transferred to a Corning CentriStar centrifuge tube (Corning) for liquid–liquid extraction, and 1 mL of benzene [deuterated, if preparing samples for nuclear magnetic resonance (NMR) analysis] was added, following by vortexing for 2 min. The solution was placed on a Thermo Scientific Barnstead Labquake rotisserie (Thermo Fisher Scientific, Asheville, NC) for 1 h to maximize methyl ketone transfer and to allow the aqueous and organic droplets to settle. Finally, the solution was centrifuged for 15 min at 12,000*g* to separate the phases, and the organic phase was recovered. The recovered extract was placed in a 2 mL screwtop PTFE glass vial and stored at −20 °C for future gas chromatography–mass spectrometry/flame ionization detection (GC–MS/FID) or NMR analysis.

Identification and quantification of methyl ketones

The GC–MS analysis was conducted by injecting 1 μL of sample through a Shimadzu GC–MS-QP2010S (Shimadzu Scientific Instruments, Kyoto, Japan) equipped with an Agilent DB-5 5 % phenyl-methylpolysiloxane, non-polar, high-temperature column (Agilent Technologies, Foster City, CA). Helium was used as the carrier gas. The injector was programmed to hold at 250 °C for 0.5 min, ramp to 280 °C at 50 °C/min, and hold for 1 min. The column oven was programmed to initialize at 50 °C, hold for 5 min, then ramp to 275 °C at a rate of 3.75 °C/min. Identification of the compounds was conducted using the similarity search algorithm through built-in libraries of Shimadzu's GCMS Solution software. Peaks identified as methyl ketones in the similarity search were further confirmed by comparing their retention times with those from methyl ketone standards.

NMR analysis was conducted using a Varian Inova 500 MHz NMR system equipped with an HCNP quad probe (Varian, Palo Alto, CA) to obtain the one-dimensional (1D) proton spectra of 600-μL aliquots of extract in glass NMR tubes. The parameters used were: sample temperature, 25 °C; sweep width, 8,000 Hz; acquisition time, 2.8 s; number of acquisitions 256; pulse width, 6.3 μs; pulse repletion delay, 1.2 s; presaturation 2 s. Prior to running the samples, methyl ketone standards at a concentration of 50 mg/L were used to identify the signature peaks and their associated J-coupling and chemical shift values.

Methyl ketone concentration was determined using a Varian CP-3800 GC system (Varian) with a flame ionization detector. The sample was injected through a Varian VF-5ht 5 % phenyl-methyl dimethylpolysiloxane, non-polar, high-temperature column. Prior to sample analysis, standards of three methyl ketones (2-nonanone, 2-undecanone, 2-tridecanone) at concentrations of 5, 10, 20, 50, and 100 mg/L were injected. The peak areas were calculated using the Varian GC software, and calibration curves were constructed to relate peak area to methyl ketone concentration in benzene. Helium was used as the carrier gas. The injector was programmed to hold at 250 °C for 0.5 min, ramp to 280 °C at 50 °C/min, and hold for 1 min. The column oven was programmed to initialize at 50 °C, hold for 5 min, then ramp to 275 °C at a rate of 3.75 °C/min.

Analysis of protein expression via sodium dodecyl sulfate–polyacrylamide gel electrophoresis

Pellets for sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) analysis of protein expression were analyzed using NuPAGE® Novex 12 % Bis–Tris 10-well gels (Invitrogen), as described by the manufacturer. Once electrophoresis was completed, the gel was washed and stained with SimplyBlue™ SafeStain (Invitrogen) according to the manufacturer's protocol.

Analytical methods

Optical density was measured at 550 nm (Genesys 20; Thermo Fisher Scientific, Waltham, MA) and used as an estimate of cell mass (1 $OD_{550} = 0.34$ g dry weight/L) [11]. After centrifugation, supernatants were stored at −20 °C for further analysis. The quantification of glucose, organic acids, and ethanol was conducted by analyzing supernatants via ion-exclusion high-performance liquid chromatography (HPLC). A Shimadzu Prominence SIL 20 system (Shimadzu Scientific Instruments) equipped with an HPX-87H organic acid column (Bio-Rad, Hercules, CA) was used, with the operating conditions as previously described [10, 11]. Data for glucose consumption and product synthesis were used to calculate average product yields (g product/g of glucose) for specified cultivation times, as previously described [12, 39].

Results and discussion

Design and analysis of a pathway to synthesize methyl ketones from glucose in *E. coli*

The knowledge of the two biochemical reactions leading to the synthesis of methyl ketones from acyl-ACP intermediates, as described in the literature [2, 14, 40], allowed the design of a pathway from glucose to the end product methyl ketone in *E. coli* (Fig. 1a). The glycolytic pathway

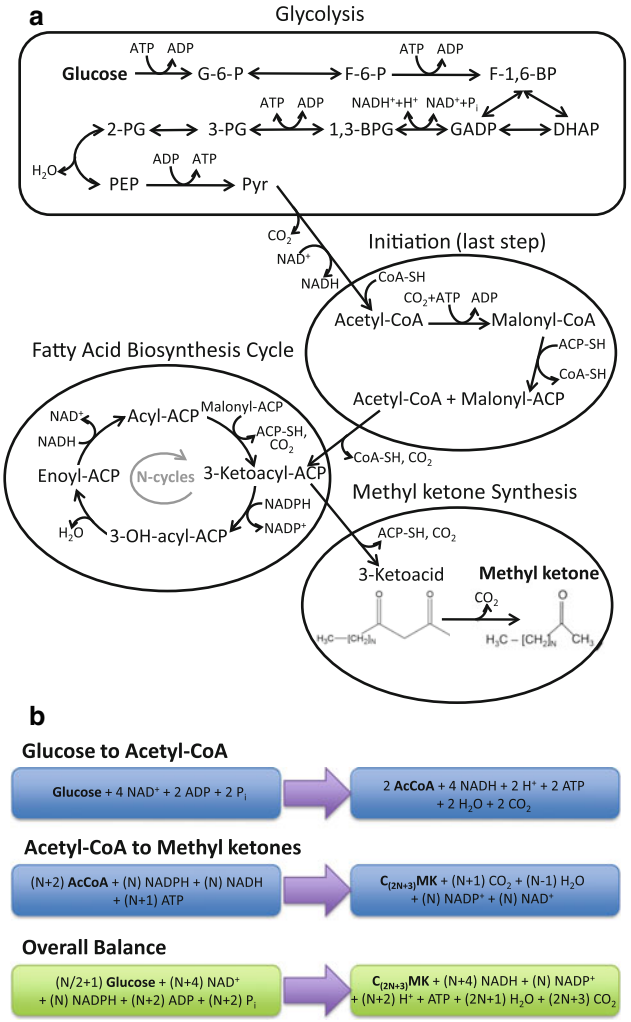

Fig. 1 **a** *Echerichia coli* pathways involved in glycolysis and the fatty acid biosynthetic cycle leading to methyl ketone synthesis. *G-6-P* Glucose-6-phosphate, *F-6-P* fructose-6-phosphate, *F-1,6-BP* fructose-1,6-bisphosphate, *DHAP* dihydroxyacetone-phosphate, *GADP* glyceraldehyde-3-phosphate, *1,3-BPG* 1,3-bisphospho-glycerate, *3-PG* 3-phospho-glycerate, *2-PG* 2-phospho-glycerate, *PEP* phosphoenol pyruvate, *Pyr* pyruvate. **b** Carbon and redox balance during methyl ketone production. *AcCoA* Acetyl-CoA

converts glucose to pyruvate, the precursor to acetyl-CoA, which eventually becomes the indirect building block of fatty acids and methyl ketones. Reactions involved in the initiation of fatty acids (and methyl ketone) biosynthesis need to take place for both the first molecule that enters the fatty acid cycle (acetoacetyl-ACP) and the 2-C donor for chain elongation (malonyl-ACP). The fatty acid synthesis/elongation cycle and the termination reactions that lead to the synthesis of methyl ketones from 3-ketoacyl-ACP are also shown in Fig. 1a. The stoichiometric balance from glucose to acetyl-CoA, from acetyl-CoA to methyl ketones of various chain lengths, and a combined balance from glucose to methyl ketones of various chain lengths was obtained by tracing the metabolites along the pathway and is shown in Fig. 1b.

The calculated maximum theoretical yield using the aforementioned balances is approximately 0.31 g methyl ketone/g glucose for methyl ketones ranging from five to 17 carbons. Assuming that pyruvate is being converted to acetyl-CoA by the pyruvate dehydrogenase complex, the overall balance also indicates a net generation of two molecules of reducing equivalents and one molecule of ATP per molecule of methyl ketone synthesized, regardless of the length of the carbon chain. The redox-generating nature of the overall conversion of glucose to methyl ketones suggests the requirement of an external electron acceptor such as oxygen.

As *E. coli* does not natively produce methyl ketones, we selected well-characterized beta-ketoacyl-ACP thioesterases and beta-ketoacid decarboxylases [termed "methyl ketone synthases (MKSs)" by the authors characterizing the enzymes] [2]. Previous studies by the same authors confirmed the hypothesis that SHMKS2 (beta-ketoacyl-ACP thioesterase from *S. habrochaites*) and SLMKS2 (beta-ketoacyl-ACP thioesterase from *S. lycopersicum*) catalyze the thioester hydrolysis step and that SHMKS1 (beta-ketoacid decarboxylase from *S. habrochaites*) catalyzes the decarboxylation step. In our study, we codon-optimized the DNA sequences encoding these enzymes to ensure adequate expression in *E. coli*.

Over-expression of methyl ketone synthases and identification of methyl ketone synthesis

Methyl ketone production was enabled by the expression of the methyl ketone synthases SHMKS2 and SHMKS1 in wild-type *E. coli* MG1655. Different levels of protein expression were evaluated in order to maximize methyl ketone production while concomitantly minimizing the physiological burden on the cells and toxic effects due to plasmid replication and the production of heterologous proteins, respectively. Even though low- (pZSblank, Table 1) and high-copy (pCR2.1, Table 1) vectors were tested, it was the medium-copy pTrcHis2A that gave the most promising results (data not shown). The poor results obtained by the low- and high-copy vectors may be due to insufficient protein expression or to toxic amounts of the enzymes, respectively. Different levels of expression of the methyl ketone synthase from *S. lycopersicum*, SLMKS2, were also evaluated, but none of the strains expressing this enzyme produced detectable amounts of methyl ketones, possibly due to hindered activity in vivo in the non-native environment of *E. coli* (data not shown).

The expression of proteins SHMKS2 and SHMKS1 was verified by SDS–PAGE gel analysis of whole cell extracts (Fig. 2), and methyl ketone production was confirmed via

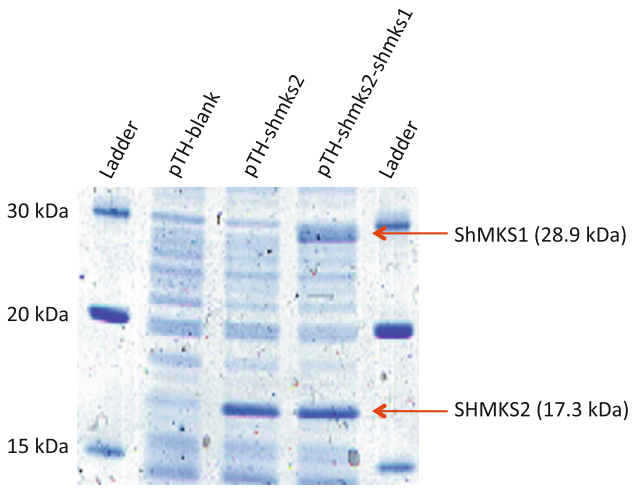

Fig. 2 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis verifying the expression of methyl ketone synthases SHMKS1 and SHMKS2 (methyl ketones from *Solanum habrochaites*)

1D proton NMR and GC–MS. NMR analysis provided structural verification by confirming the presence of the first five methyl ketone carbons, while GC–MS provided total mass information, revealing the chain lengths of the methyl ketones present in the sample. Methyl ketone quantification was achieved via GC–FID analysis.

Figure 3 shows the mass spectra for 2-nonanone, 2-undecanone, and 2-tridecanone, the primary methyl ketones being produced. The calculated concentrations of methyl ketones were in agreement with those calculated from the GC–FID analysis. According to these analyses, 2-tridecanone was the major product, followed by 2-undecanone, and 2-nonanone, respectively. Although 2-nonanone was detected via GC–MS, it was not detected with the GC-FID, probably because of differences in instrument sensitivities. Controls containing the blank pTrcHis2A plasmid were also analyzed, with no methyl ketones being detected. NMR results, as shown in Fig. 4, agree with the results from the GC–MS analysis by confirming the presence of methyl ketones in the samples. Subsequent product quantification was done by integrating the GC–FID peaks after the peak identities were confirmed via NMR and GC–MS.

Elimination of fermentative pathways leading to the synthesis of competing byproducts

After confirmation of methyl ketone synthesis by MG1655 [pTricHis2A-shmks2-shmks1], additional genetic modifications were introduced to strain MG1655 in order to reduce the presence of secondary products and, thereby, increase the final concentration and yield. Under anaerobic and microaerobic conditions, *E. coli* converts glucose into a mixture of metabolic products that consist primarily of organic acids (acetate, formate, lactate, succinate) and ethanol [8, 12] (Fig. 5). Since these fermentative pathways consume acetyl-CoA (the building block of fatty acids) and pyruvate (precursor to acetyl-CoA), they divert carbon away from the synthesis of methyl ketones. Therefore, it was hypothesized that knocking out several or all of these pathways would result in increased methyl ketone yields.

Several mutants that combined the deletion of genes mediating the synthesis of ethanol (*adhE*), acetate (*poxB* and *pta*), and lactate (*ldhA*) were constructed in order to block the routes that could divert carbon flux away from methyl ketone synthesis (Table 2). Plasmid

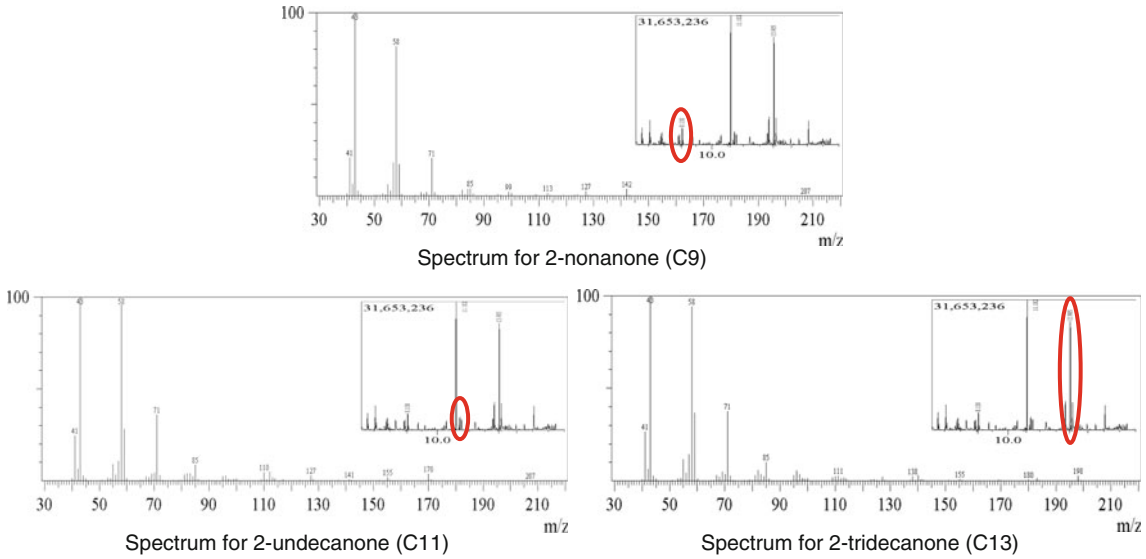

Fig. 3 Gas chromatography–mass spectrometry of 2-nonanone, 2-undecanone, and 2-tridecanone. *Red circles* in *inset* chromatograms Peak from which the mass spectra were obtained

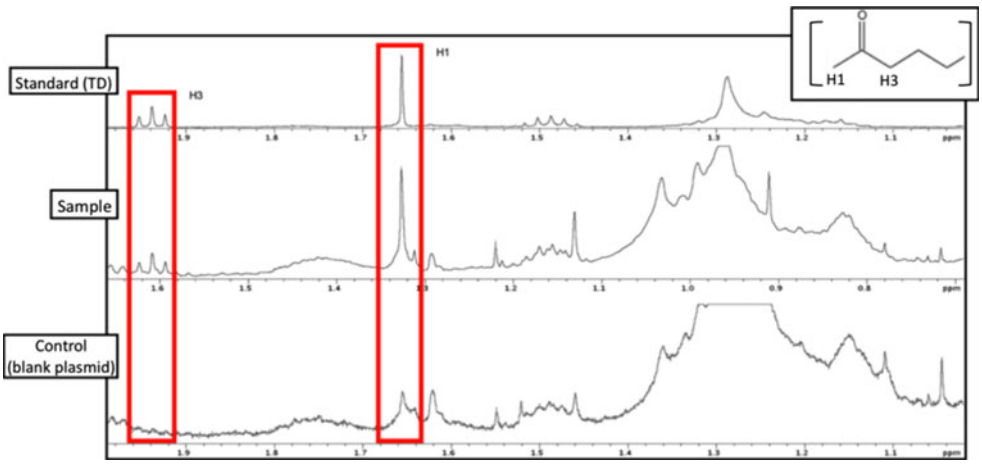

Fig. 4 Nuclear magnetic resonance (NMR) spectra of 2-tridecanone structure (*top*), sample from methyl ketone-producing strain (*middle*), and control sample (*bottom*). *Red boxes* Signature peaks aligned across the NMR spectra. *H1* corresponds to the chemical shift of the protons on the first carbon (methyl next to ketone group); *H3* corresponds to the chemical shifts of the protons of the third carbon (represented in *inset* of chemical structure)

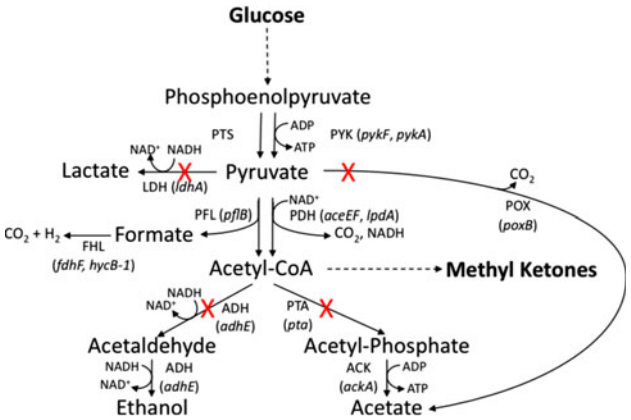

Fig. 5 *Escherichia coli* fermentative pathways. *Broken lines* illustrate multiple steps. Relevant reactions are represented by the enzyme names with the gene(s) coding for the enzymes given *in parenthesis*: *PTS* phosphotransferase system, *PYK* pyruvate kinase, *LDH* lactate dehydrogenase, *PFL* pyruvate formate-lyase, *PDH* pyruvate dehydrogenase, *POX* pyruvate oxidase, *FHL* formate hydrogenlyase, *ADH* acetaldehyde/alcohol dehydrogenase, *PTA* phosphate acetyltransferase, *ACK* acetate kinase. *Red crosses* indicate gene deletions

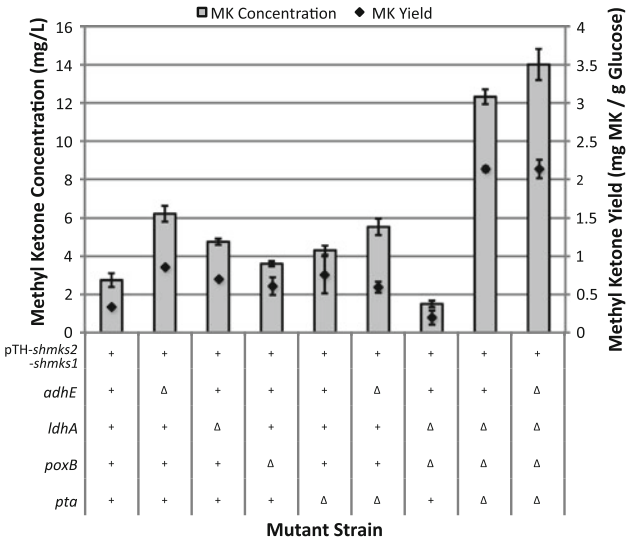

Fig. 6 Effect of the disruption of major fermentative pathways on methyl ketone (*MK*) production in 22 h with a working volume of 30 mL (in 125-mL flasks). Gene over-expression in pTH-shmks2-shmks1 plasmid: *plus* plasmid and/or gene(s) present in strain *triangle* gene deletion. *Error bars*: standard deviation (SD) for a minimum of triplicate measurements

pTrcHis2A-shmks2-shmks1 was then transformed into these mutants to enable methyl ketone production. The effects of these knockouts were evaluated by measuring methyl ketone yield and concentration (Fig. 6).

As expected, the results show that the highest yield and titers were attained through those strain backgrounds with the most fermentative pathways knocked out (Δ*ldhA* Δ*poxB* Δ*pta* and Δ*adhE* Δ*ldhA* Δ*poxB* Δ*pta*). In terms of yield, most knockout strains performed better than the wild-type background. However, the double-mutant Δ*ldhA* Δ*poxB* gave low titers and yields compared to its respective single mutants (Δ*ldhA* and Δ*poxB*), with yield values similar to that of the non-engineered wild-type. This was unexpected, since growth and glucose consumption were similar to those of the other strains, and HPLC analysis revealed a much higher build-up of pyruvate pools than that found in the other mutant strains. This result contradicts the hypothesis that knocking out the competing pathways for pyruvate and consequently increasing the pools of pyruvate would lead to increased flux towards acetyl-CoA and, ultimately, to methyl ketones. However, an additional *pta* deletion relieved this build-up. It is unclear as to why this build-up occurred, although it remained true that the quadruple and triple mutants performed best in terms of both concentration and yield, with the former performing better in terms of these metrics. This latter result was expected since the rationale behind the knockouts was to redirect carbon flux from fermentative pathways that became active at lower oxygen levels. Since the production of other metabolites was significantly reduced, we found that a higher flux was directed to the fatty acid synthesis pathway, subsequently increasing both methyl ketone yield and concentration (Fig. 6).

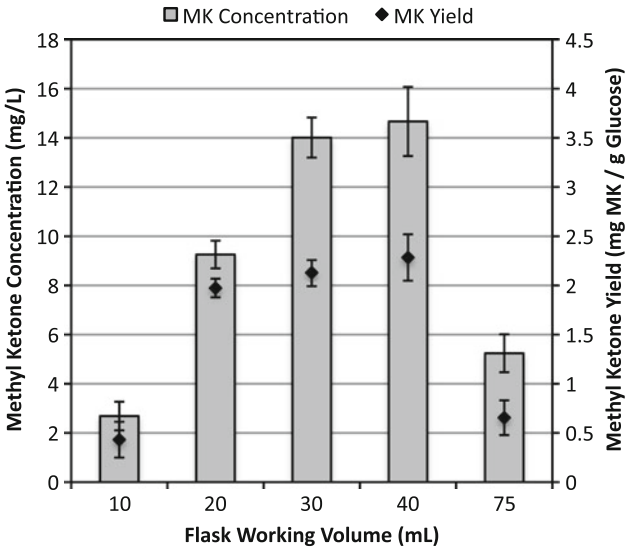

Fig. 7 Effect of different levels of aerobicity on methyl ketone production by strain MG1655 pTH-*shmks2-shmks1* Δ*adhE* Δ*ldhA* Δ*poxB* Δ*pta*. Methyl ketone production in working volumes of 10 and 20 mL (in 125-mL flasks) was measured at 18 h; production in working volumes of 30, 40, and 75 mL (in 125-mL flasks) was measured at 22 h. *Error bars*: SD for a minimum of triplicate measurements

Effect of oxygen availability on methyl ketone synthesis

In addition to studying different knockout mutants, we also assessed the effect of aerobicity for each knockout combination. Methyl ketone concentration and yield profiles were obtained for the wild-type and knockout mutants at different working volumes (10, 20, 30, 40, and 75 mL media volume in 125-mL flasks). Figure 7 shows the comparison for the best performer strain selected above (MG1655 Δ*adhE* Δ*ldhA* Δ*poxB* Δ*pta* pTrcHis2A-shmks2-shmks1). Most strains showed significant differences in methyl ketone production under different working volumes, with 30 and 40 mL being the most beneficial for the top performer strains.

The differences in methyl ketone yield and concentration observed under different degrees of aerobicity in shake flasks led to the study of the effect of DO concentration on methyl ketone synthesis in order to find the best culture conditions in a controlled bioreactor vessel. To quantify the specific DO percentage (DO%) that optimized yield and titer and to obtain a better grasp on how these metrics change with specific changes in DO, several bioreactor fermentations were conducted with different DO set-points. The best performing strain from the shake flask experiments (MG1655 Δ*adhE* Δ*ldhA* Δ*poxB* Δ*pta* pTrcHis2A-shmks2-shmks1), detailed in the previous section, was cultured at DO concentrations between 2 and 20 % of saturation with air. The strain was cultivated in a 500-mL

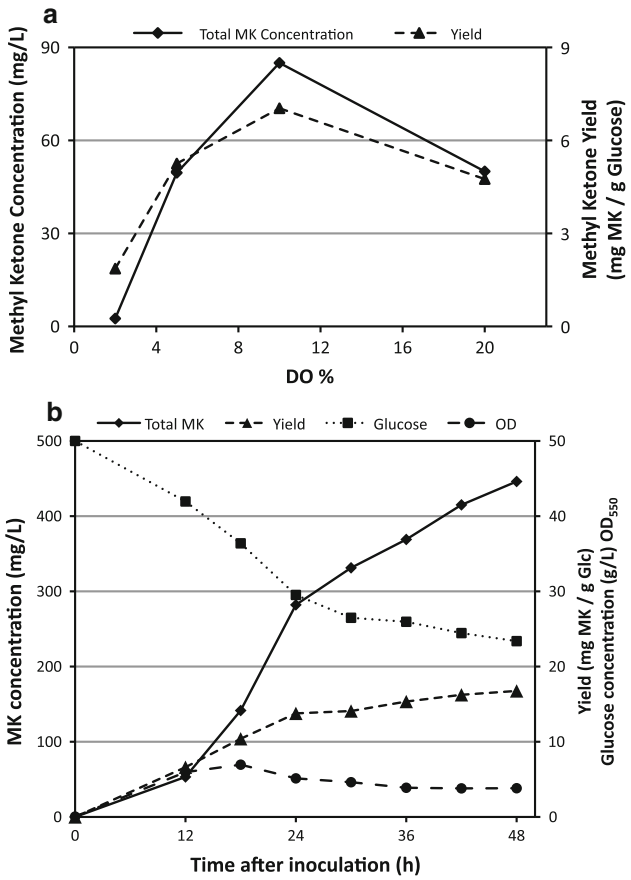

Fig. 8 **a** Effect of dissolved oxygen (*DO*) on methyl ketone production by strain MG1655 pTH-*shmks2-shmks1* Δ*adhE* Δ*ldhA* Δ*poxB* Δ*pta*. Fermentations in a 500-mL bioreactor vessel for 12 h. **b** Kinetics of methyl ketone production by strain MG1655 pTH-*shmks2-shmks1* Δ*adhE* Δ*ldhA* Δ*poxB* Δ*pta*. Experiment was conducted in a fully controlled fermentor at pH 7.0 and 10 % DO. Profile shows total methyl ketone concentration (*diamond*) and yield (*triangle*), glucose concentration (*rectangle*), and optical density (*circle*). Data represent the average of three measurements. *DO%* Dissolved oxygen percentage

bioreactor vessel in which pH and DO were tightly controlled. MOPS minimal media, prepared as explained in the "Materials and Methods" section, was supplemented with 20 g/L glucose. The pH was set at 7 and the temperature at 37 °C, and the cultures were harvested at 12 h.

A major outcome from this study was a marked increase in both the concentration and yield of methyl ketones over those obtained from shake flasks (Fig. 8a). Several parameters that changed from the shake flasks to the bioreactors may be responsible for this drastic improvement in methyl ketone production. The first is a more consistent oxygen availability. A study by Tolosa et al. [35], which measured DO content over the course of *E. coli* and yeast fermentations in 250-mL shake flasks with respect to culture volume and agitation, showed a high variability of DO during the fermentations. Specifically, the authors encountered the

onset of oxygen limitation (DO measured at approx. 0 % of saturation) for both 50-mL and 75-mL culture volumes and at shaker speeds of 150, 250, and 350 rpm. During late exponential and early stationary phase, the DO content rapidly increased back to almost 100 % of saturation. Conversely, DO in the reactor was monitored and kept steady at specified set-points above 0 % of saturation. In other words, the increased availability of oxygen in the media, however low the saturation level, may have greatly improved the production of methyl ketones. Secondly, there may have been losses of methyl ketones through evaporation in the shake flasks, as methyl ketones have been detected as volatiles when extracting volatiles from *Lycopersicon hirsutum* leaves [14] and microbiologically contaminated canned tomatoes [4]. In the bioreactor setup, a condenser is placed between the headspace and the headspace outlet to minimize losses of volatile compounds.

According to strain performance at 12 h, the maximum yield and concentration values were attained when the culture conditions were set at a DO of 10 % of saturation (Fig. 8a). To further characterize the best performer strain under these optimal growth conditions, the strain was cultivated in the bioreactor using a DO setting of 10 % of saturation and glucose supplementation of 50 g/L. The results of this fermentation (Fig. 8a) show methyl ketone titers approaching 500 mg/L by 48 h and a yield leveling off at 17 mg methyl ketone/g glucose. Both metrics are the highest recorded for any of the constructed strains or any microorganism reported thus far for methyl ketone production. Growth entered late exponential to stationary phase after 12 h, at which point the rate of glucose consumption steadily decreased. If total glucose consumption were to be increased, this final strain could achieve even higher methyl ketone titers.

Conclusions

In this study, *E. coli* was engineered by a series of gene deletions and expression of heterologous genes from wild-tomato plants to produce methyl ketones. The methyl ketone concentration achieved with MG1655 Δ*adhE* Δ*ldhA* Δ*poxB* Δ*pta* pTrcHis2A-shmks2-shmks1, the best performer strain in this study, was approximately 500 mg/L, the highest reported for an engineered microorganism. Through the establishment of optimal operating conditions and by executing rational metabolic engineering strategies, we were able to increase methyl ketone concentrations by almost 75-fold—from the initial confirmed levels of 6 mg/L to significant levels of 450 mg/L. In addition to the gene deletions discussed in this work, the flux through the fatty acid biosynthesis pathway, and hence methyl ketone synthesis, can be increased by over-expression of acetyl-CoA carboxylase or cofactor engineering leading to a higher availability of NADPH, as previously reported. These strategies have been found to be viable for increasing fatty acid synthesis from acyl-ACP intermediates [41].

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