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Optimization of the IPP-bypass mevalonate pathway and fed-batch fermentation for the production of isoprenol in *Escherichia coli*

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Highlights

1. The IPP-bypass pathway was optimized to substantially improve isoprenol titer.
2. PMD mutant was introduced for MVAP conversion with high efficiency.
3. Isoprenol titer reached 3.7 g/L in batch cultures at 44% of the theoretical yield.
4. The highest isoprenol titer (10.8 g/L) was achieved in fed-batch fermentations.
5. Use of a solvent overlay improved titer by removing the toxic final product.
Abstract

Isoprenol (3-methyl-3-buten-1-ol) is a drop-in biofuel and a precursor for commodity chemicals. Biological production of isoprenol via the mevalonate pathway has been developed and optimized extensively in *Escherichia coli*, but high ATP requirements and isopentenyl diphosphate (IPP) toxicity have made it difficult to achieve high titer, yield, and large-scale production. To overcome these limitations, an IPP-bypass pathway was previously developed using the promiscuous activity of diphosphomevalonate decarboxylase, and enabled the production of isoprenol at a comparable yield and titer to the original pathway. In this study, we optimized this pathway, substantially improving isoprenol production. A titer of 3.7 g/L (0.14 g isoprenol per g glucose) was achieved in batch conditions using minimal medium by pathway optimization, and a further optimization of the fed-batch fermentation process enabled an isoprenol titer of 10.8 g/L (yield of 0.105 g/g and maximum productivity of 0.157 g L\(^{-1}\) h\(^{-1}\)), which is the highest reported titer for this compound. The substantial increase in isoprenol titer via the IPP-bypass pathway in this study will facilitate progress toward commercialization.

Keywords: Isoprenol; IPP-bypass; mevalonate pathway; biofuel; fermentation; bioconversion
1. Introduction

Increasing concerns about the cost and environmental impact of petroleum-derived fuels (Baral et al., 2019) has motivated the development of microbial hosts for the production of fuels from renewable carbon sources (Cheon et al., 2016; Liao et al., 2016; Meadows et al., 2018; Rabinovitch-Deere et al., 2013). In particular, 3-methyl-3-buten-1-ol (isoprenol) is a promising alternative to gasoline due to its anti-knocking properties, comparable energy density, and comparable research octane number (Liu et al., 2014; Mack et al., 2014). Isoprenol is also a precursor for isoprene, a polymer building block used in the production of synthetic rubber (Ye et al., 2016). Production of isoprenol at various levels has been demonstrated in *E. coli* by extensive optimization of the mevalonate (MVA) pathway (George et al., 2015; Li et al., 2018; Zada et al., 2018; Zheng et al., 2013). The conventional MVA pathway includes condensation of three acetyl-CoA molecules and reduction to MVA along with two subsequent phosphorylation reactions by mevalonate kinase (MK) and 5-phosphomevalonate kinase (PMK). The product of phosphorylation, mevalonate diphosphate (MVAPP), is decarboxylated to isopentenyl diphosphate (IPP) by a diphosphomevalonate decarboxylase (PMD). Lastly, isoprenol is formed by hydrolysis of the pyrophosphate group from IPP, and overall these reactions consume 3 mol of ATP per mol of isoprenol.

To overcome intrinsic limitations of the conventional MVA pathway, such as lower pathway efficiency and toxicity of an essential intermediate, IPP, an alternative IPP-bypass MVA pathway (Figure 1A) has been developed by taking advantage of the promiscuous activity of PMD toward the non-native substrate mevalonate monophosphate (MVAP) (Kang et al., 2016). The advantages of this alternative pathway
over the conventional MVA pathway include the prevention of IPP toxicity by avoiding formation of IPP and increased robustness under lower aeration culture conditions, as this new pathway requires less ATP than the original MVA pathway (Kang et al., 2016). In a follow-up study, the PMD was engineered to have higher promiscuous activity towards mevalonate phosphate (MVAP), and isoprenol production was further improved to a titer of 1.2 g/L (Kang et al., 2017).

While previous studies used rich media and small-scale batch fermentations to produce isoprenol, further improvements in yield and productivity using inexpensive media in larger volumes are required in order to derisk trials at commercial scale (Balan, 2014; Hollinshead et al., 2014; Wehrs et al., 2019). In this study we optimize the IPP-bypass mevalonate pathway for production of isoprenol in E. coli using several HMGR, HMGS and MK variants as well as engineered PMD mutants to provide optimal levels of the pathway intermediates. As a result of these engineering efforts, isoprenol production in minimal medium reached 3.7 g/L in batch cultures whereas a titer of 10.8 g/L was reached using fed-batch cultures with a solvent overlay in a 2-L bioreactor, which is the highest reported titer for this compound.

2. Materials and Methods

2.1. Plasmids and strains

All plasmids and strains used in this study are listed in Table 1. Strains and plasmids along with their associated information (annotated GenBank-format sequence files) have been deposited in the public version of the JBEI Registry (https://public-registry.jbei.org)
and are physically available from the authors and/or Addgene (http://www.addgene.org) upon request.

2.2. **Batch production of isoprenol in *E. coli***

Isoprenol production in EZ-Rich defined medium was performed as previously described (Kang et al., 2016). Briefly, to prepare seed cultures, each single colony was inoculated in LB (lysogeny broth) medium containing appropriate antibiotics and grown overnight. Seed cultures were diluted to an optical density (OD\textsubscript{600nm}) of 0.05 in EZ-Rich defined medium (Teknova, USA) supplemented with 10 g/L glucose (1 %, w/v) and two antibiotics, namely, 100 μg/mL ampicillin and 30 μg/mL chloramphenicol. Diluted cell cultures (5 mL) were first grown at 37°C with shaking at 200 rpm. When cell density reached an OD\textsubscript{600nm} value of 0.4-0.6, expression of proteins was induced with 0.5 mM IPTG, and the cultures were transferred to an incubator at 30°C with shaking at 200 rpm.

Isoprenol production in defined minimal media was performed with M9-MOPS minimal medium (M9 medium (33.9 g/L Na\textsubscript{2}HPO\textsubscript{4}, 15 g/L KH\textsubscript{2}PO\textsubscript{4}, 5 g/L NH\textsubscript{4}Cl, 2.5 g/L NaCl) supplemented with 75 mM MOPS, 2 mM MgSO\textsubscript{4}, 1 mg/L thiamine, 10 nM FeSO\textsubscript{4}, 0.1 mM CaCl\textsubscript{2}, and micronutrients including 3 × 10\textsuperscript{-8} M (NH\textsubscript{4})\textsubscript{6}Mo\textsubscript{7}O\textsubscript{24}, 4×10\textsuperscript{-6} M boric acid, 3×10\textsuperscript{-7} M CoCl\textsubscript{2}, 1×10\textsuperscript{-7} M CuSO\textsubscript{4}, 8×10\textsuperscript{-7} M MnCl\textsubscript{2}, and 1×10\textsuperscript{-7} M ZnSO\textsubscript{4}) with glucose (10-30 g/L, depending on production conditions) as a sole carbon source.

Strains used for production in minimal medium were first adapted in the medium by serially diluting cell cultures in fresh minimal medium. Briefly, each single colony was inoculated in LB overnight and diluted 50-fold (v/v) in M9-MOPS minimal medium. Cultures were grown for another 24 h and re-diluted 50-fold (v/v) in fresh M9-MOPS medium.
medium. These steps were repeated four times, and the final cell cultures adapted to M9-MOPS medium were stored as frozen glycerol stocks at -80°C. For batch production, a loopful of glycerol stock was inoculated in 2 mL M9-MOPS minimal medium with 1% glucose, and seed cultures were grown overnight at 37°C with shaking at 200 rpm and diluted 50-fold in 5 mL M9-MOPS medium with appropriate antibiotics and glucose at concentrations indicated.

2.3. Isoprenol production in fed-batch fermenter

Fed-batch fermentation was performed in a 2-L bioreactor (Sartorius BIOSTAT B plus) with control for dissolved oxygen (DO), pH, and temperature. A 5-mL culture was inoculated from a frozen glycerol stock, grown for 24 hours, and then used to inoculate a 50-mL culture in a 250-mL flask; this culture was then used to inoculate the bioreactor to an OD_{600nm} of 0.1. The medium for batch phase was M9 minimal medium supplemented with 2 mM MgSO_4, 1 mg/L thiamine, 10 µM FeSO_4, 0.1 mM CaCl_2, additional NH_4Cl (if needed), glucose (2% or 3% w/v), 10 g/L yeast extract (if needed), appropriate antibiotics and micronutrients including 3 \times 10^{-8} \text{M} (\text{NH}_4)_6\text{Mo}_7\text{O}_{24}, 4 \times 10^{-6} \text{M} boric acid, 3 \times 10^{-7} \text{M} \text{CoCl}_2, 1.5 \times 10^{-7} \text{M} \text{CuSO}_4, 8 \times 10^{-7} \text{M} \text{MnCl}_2, and 1 \times 10^{-7} \text{M} \text{ZnSO}_4. The pH of the culture was maintained at 7.0 by supplementation with a base solution (10 N KOH).

Temperature, DO, and airflow were set to 30°C, 30%, and a rate of 1 VVM (volume of air per volume of liquid per minute), respectively, throughout the fermentation run. Protein expression was induced with 0.5 mM IPTG when the culture reached an OD_{600nm} of 0.4-0.6. Glucose feeding started when the initial amount of glucose was depleted (indicated by a sharp increase in DO or HPLC analysis) with a feed solution containing
200 g/L glucose, 15 g/L MgSO$_4$·7H$_2$O, 5 g/L yeast extract (if needed), micronutrients according to previous descriptions (Korz et al., 1995) and appropriate antibiotics; also, antifoam B was added to the bioreactor when required. Glucose feeding was carried out using a Watson-Marlow DU520 peristaltic pump; for constant feeding, the flow rate was selected to closely match the glucose consumption rate at the end of the batch phase. For exponential feeding, the feeding rate was changed every hour (for a total of 12 hours) and calculated according to the following equation (Korz et al., 1995):

\[
m(t) = \left( \frac{\mu}{Y_{X/S}} + m \right) V_{t_F} X_{t_F} e^{\mu(t-t_F)},
\]

where \( m(t) \) is the mass flow of the substrate (g/h), \( \mu \) is the specific growth rate (0.1 h$^{-1}$), \( Y_{X/S} \) is the biomass/substrate yield coefficient (0.5 g/g), \( m \) is the specific maintained coefficient (0.025 g g$^{-1}$ h$^{-1}$), \( V_{t_F} \) is the cultivation volume at the time of feeding (\( t_F \)) and \( X_{t_F} \) is the biomass concentration (g/L). After 12 hours of exponential feeding, the feeding rate was set constant and glucose was continuously measured in the medium, and the feeding rate was adjusted to prevent its accumulation at more than 2 g/L. For the two-phase cultivation, 20% (v/v) oleyl alcohol was added to the fermenter at the time of induction. During the fed-batch phase, additional oleyl alcohol was added so its volume never decreased below 10% of the total volume. For OD$_{600nm}$ measurement and isoprenol quantification during the two-phase cultivation, the sample was first separated by centrifugation (8 min, 4000 x g) and the aqueous phase was used to quantify isoprenol as described above (except that up to 1:3 dilutions with ethyl acetate were used for isoprenol concentrations greater than 2 g/L) as well as OD$_{600nm}$ and DCW (dry cell weight) measurements; in order to quantify isoprenol from the organic phase, 10 µL of the oleyl alcohol was added to 990 µL ethyl acetate containing 1-butanol as an internal standard.
and the total isoprenol was calculated based on the actual culture volume at the time of the sampling.

2.4. Isoprenol quantification by gas chromatography (GC)

For isoprenol quantification, an aliquot of cell culture (250 μL) was combined with an equal volume of ethyl acetate (250 μL) containing 1-butanol (30 mg/L) as an internal standard and vigorously mixed for 15 min. Mixtures of cell cultures and ethyl acetate were centrifuged at 20,000 x g for 3 min, and 50 or 100 μL of the ethyl acetate layer was diluted 10-fold or 5-fold in ethyl acetate containing 1-butanol (30 mg/L). An aliquot (1 μL) of each of the diluted samples was analyzed by gas chromatography – flame ionization detection (Thermo Focus GC) equipped with a DB-WAX column (15-m, 0.32-mm inner diameter, 0.25-μm film thickness, Agilent, USA), the oven temperature program was as follows: started at 40°C, a ramp of 15°C/min to 100°C, a ramp of 40°C/min to 230°C and held at 230°C for 3 min.

2.5. Quantification of metabolites, sugars and fermentation acids.

For analysis of metabolites from the IPP-bypass pathway, 0.5-1 mL of cell culture was centrifuged at 14,000 x g for 3 min at 4°C, the cell pellets were resuspended in 250 μL of methanol and stored at 20°C. Pellets were thawed on ice and combined with 250 μL water, the methanol-water mixtures were centrifuged at 15,000 rpm for 10 min at 4°C and the supernatant was filtered through a Millipore™ Amicon Ultra 3kD MW cut-off filter at 14,000 x g for 45 min at 4°C. Filtered solutions were diluted with an equal volume of acetonitrile (final 50% (v/v) ACN) and analyzed via liquid chromatography-mass
spectrometry (LC-MS; Agilent Technologies 1200 Series HPLC system and Agilent
Technologies 6210 time-of-flight mass spectrometer) on a ZIC-HILIC column (150-mm
length, 4.6-mm internal diameter, and 5-µm particle size) (Baidoo et al., 2019).
Concentrations of intracellular metabolites were calculated assuming cell volume of 1
OD$_{600}$ nm/mL as 3.6 µL (Volkmer and Heinemann, 2011).
For quantification of glucose and organic acids, 5 µL of filtered supernatant (0.45-µm
centrifugal filter) was analyzed by isocratic elution with 4 mM sulfuric acid through an
HPLC system equipped with an Aminex HPX-87H column (Bio-Rad, Richmond, CA,
USA) and a refractive index detector (Agilent Technologies). The sample tray and
column compartment were set to 4 and 50°C, respectively, and the flow rate was
maintained at 0.6 mL/min. Data acquisition and analysis were performed via Chemstation
software (Agilent Technologies).

3. Results and Discussion
3.1. Pathway optimization for the biosynthesis of MVAP
For isoprenol production via the IPP-bypass mevalonate pathway, we initially expressed
*atoB* (*E. coli*), *HMGS*$_{Sc}$ _o_ (*S. cerevisiae*), *HMGR*$_{Sc}$ _o_ (*S. cerevisiae*), and *MK*$_{Sc}$ _co_ (*S.
cerevisiae*, codon optimized) from a medium-copy plasmid and the PMD (*S.
cerevisiae*) from a high-copy plasmid (Kang et al., 2016) (plasmid arrangements are
shown in Figure 1B); isoprenol production reached 513 mg/L after 48 hours using EZ-
Rich medium supplemented with 1% glucose (strain AK01, Figure 2A). Using this strain
as a baseline, we tested different expression levels of MK, HMGR, and HMGS in order
to identify limiting steps for the biosynthesis of MVAP in the IPP-bypass pathway.
First, we tested whether increasing the expression of the MK would affect isoprenol production. We expressed MK (i.e., MK\textsubscript{Sc\_co}) from a high-copy plasmid (ColE1-origin) with a Trc promoter instead of the medium-copy plasmid (p15A origin, Figure 1C), which increased the isoprenol titer significantly, reaching 1.05 g/L (strain AK02, Figure 2A), suggesting that isoprenol production might be limited by MVAP levels. To test whether the changes in expression of the enzymes upstream of the MK such as HMGS and HMGR would also improve isoprenol production, we tested variants of these enzymes from different organisms (Table 1). The three HMGS variants tested were from \textit{S. cerevisiae} (HMGS\textsubscript{Sc\_o}, native sequence; and HMGS\textsubscript{Sc\_co}, codon-optimized sequence) and from \textit{S. aureus} (HMGS\textsubscript{Sa}), and the five HMGR variants tested were from \textit{S. cerevisiae} (HMGR\textsubscript{Sc\_co}, codon-optimized), \textit{S. aureus} (HMGR\textsubscript{Sa}), \textit{Bordetella petrii} (HMGR\textsubscript{Bp}), \textit{Delftia acidovorans} (HMGR\textsubscript{Da}), and \textit{Pseudomonas mevalonii} (HMGR\textsubscript{Pm}) (Ma et al., 2011). Both the HMGR\textsubscript{Sc\_co} and HMGR\textsubscript{Sa} use NADPH as co-factor and have preference for the forward reaction (conversion of HMG-CoA to mevalonate), whereas the HMGR\textsubscript{Bp}, HMGR\textsubscript{Da} and HMGR\textsubscript{Pm} preferentially use NADH as cofactor (Ma et al., 2011). Different combinations of the HMGS and HMGR variants were tested while MK\textsubscript{Sc\_co} was expressed from the high copy plasmid. Figure 2B shows that isoprenol production was improved when the HMGR\textsubscript{Sc\_co} and HMGS\textsubscript{Sc\_co} were used (1.34 g/L, strain AK07) and when HMGR\textsubscript{Sa} was used in combination with either the HMGS\textsubscript{Sc\_co} (1.35g/L, strain AK08) or HMGS\textsubscript{Sa} (1.26 g/L, strain AK09). In general, lower isoprenol levels were observed when the NADH-dependent HMGRs were used; since these HMGRs have relatively higher activity for the reverse reaction (conversion of mevalonate to HMG-CoA) (Ma et al., 2011), it is possible
that less carbon flux is being directed to the formation of mevalonate and downstream intermediates. Higher isoprenol titers were observed when the MK_Sc_co and the HMGRs with higher forward reaction activity were overexpressed, which suggests that production of isoprenol could be limited by the availability of mevalonate and mevalonate phosphate (MVAP). Several MK variants were also tested but titers were not improved (Figure 2C). We also tested if higher HMGR expression would positively affect carbon flux towards mevalonate as HMGR is known as a rate-limiting enzyme of the top pathway (acetyl-Co to mevalonate), but expression of an additional copy of the HMGR from a high-copy plasmid did not improve titers either (Figure 1D); in fact, a decrease in isoprenol production was observed compared to the control strain regardless of the HMGR variant used (Figure 2D). Further analysis of one of these HMGR-overexpressing strains (strain AK19) showed that expression of this additional enzyme from the high-copy plasmid resulted in a lag phase after induction (Supplementary Figure 1A), which indicates that growth of the strain was inhibited; the growth recovered after 40 hours but this increase in cell biomass did not result in an increase of isoprenol production (Supplementary Figure 1B). Quantification of intermediate metabolites showed an increase in MVAP levels when the additional HMGR was expressed but no accumulation of other potentially toxic intermediates was observed (Supplementary Figure 1C). We also tested whether overexpression of the HMGR itself was toxic to the cells, but overexpression of only the HMGR_Sc_co from a pTrc promoter from a high-copy number plasmid did not inhibit growth (data not shown).

### 3.2. Pathway optimization with PMD mutants

Based on the results from the previous section, we hypothesized that the conversion of MVAP to IP might also be a bottleneck in the pathway. Therefore, in order to improve the conversion of MVAP to IP, we tested a PMD mutant (R74G) that was reported to have higher $K_i$ than the wild-type PMD (MVAP is a non-competitive inhibitor of PMD) (Kang et al., 2017). Initially, both the wild-type PMD (strain AK07) and the R74G mutant (strain AK21) were expressed with enzyme variants described in the previous section containing the HMGS\_Sc\_co, HMGR\_Sc\_co, and MK\_Sc\_co. The maximum isoprenol titers of two strains were similar, but there was a significant improvement in rate of production with the R74G mutant (strain AK21) compared to the wild-type PMD (strain AK07), reaching a maximum titer of 1.6 g/L after 30 hours instead of 40 hours (Figure 3A). The strain AK21 also showed faster growth (Figure 3B) and faster glucose consumption (Supplementary Figure 2A). Acetate accumulation peaked at 6 hours after induction reaching 0.8 g/L in both strains, but it decreased to 0.5 g/L and 0.3 g/L in the AK07 and AK21 strains, respectively, at 30 hours after induction (Supplementary Figure 2B). Slower assimilation of acetate for strain AK07 might explain the slower glucose consumption rate at the early stage after induction. In order to test how the use of the R74G PMD mutant affected the conversion of MVAP, we measured MVA, MVAP and IP over time (Figure 3B). Both the intracellular and extracellular concentrations of MVAP were significantly lower in the strain with the R74G mutant (strain AK21) compared to those of the strain with the wild-type PMD (strain AK07), whereas the IP levels were higher, suggesting that using the R74G mutant increases the conversion of MVAP to IP. The strain AK21 also showed lower mevalonate concentrations, suggesting that mevalonate was converted to MVAP more efficiently in strain AK21 than in strain
AK07, where mevalonate increased over time and reached a final concentration four
times higher than in strain AK21 after 24 hours (Figure 3B).

In addition to the R74G mutant, two other PMD mutants with higher $k_{cat}$ or higher $K_i$ for
MVAP were identified in our previous study, and these mutants could increase the
conversion efficiency of MVAP to IP and improve isoprenol production (Kang et al.,
2017); one contains a single R74H mutation and the other contains three mutations,
R74H, R147K and M212Q (abbreviated as HKQ). The PMD with these mutations were
also tested with the HMGS_Sc_co-HMGR_Sc_co-MK_Sc_co system (strains AK22 and
AK23, respectively) but lower isoprenol titers were observed (Figure 4A). We
hypothesized that other HMGS, HMGR, and MK variants could provide different
metabolite levels for the upstream intermediates, which could have synergistic effects on
isoprenol production for the PMD mutants. Therefore, two additional strains containing
the HMGS_Sa-HMGR_Sa-MK_mm or the HMGS_Sa-HMGR_Sa-MK_Sc_co (which
showed high isoprenol titers in the previous section) were also tested with the PMD
mutants (Figure 4A). Interestingly, very different isoprenol titers were observed for each
PMD mutant depending on the HMGS-HMGS-MK system used; these differences were
more significant with the R74H mutant, where the titer ranged from 784 mg/L for strain
AK22 to 1.84 g/L for strain AK28 (Figure 4A). In general, higher titers were observed
using the HMGS_Sa-HMGR_Sa-MK_Sc_co system, reaching a maximum titer of 1.84
g/L with the R74H mutant (strain AK28) and 1.81 g/L with the HKQ mutant (strain
AK29).

### 3.3. Isoprenol production in minimal media
Because of its greater batch-to-batch consistency and lower cost, chemically defined minimal medium is usually employed for production in bench-scale fermentations; however, since all metabolites need to be synthesized de novo, growth in minimal medium can result in major shifts in cell resources and affect production (Singh et al., 2017). Therefore, we tested our previously optimized strains for production on minimal medium supplemented with 1% glucose; isoprenol production was approximately half of that obtained with rich medium (Supplementary Figure S3). Increasing the glucose concentration to 2% generally resulted in higher titers, except for a few strains (e.g., AK28 and AK29), but the degree of improvement varied depending on the MK, HMGS, HMGR, and PMD variants used (Figure 4B). In general, strains with lower isoprenol production showed slower growth and higher acetate accumulation (data not shown). The highest isoprenol titer was achieved when the R74G PMD mutant was expressed with the HMGS_Sc_co-HMGR_Sc_co-MK_Sc_co system (strain AK21), reaching titers of 2.74 g/L. Comparable titers were also observed with strains AK27, AK25 and AK26. Interestingly, more than a 10-fold difference in isoprenol titer was observed for the R74G mutant depending on the HMGS-HMGR-MK system used (ranging from 246 mg/L for strain AK24 to 2.74 g/L for strain AK21; Figure 4B). The initial glucose concentration affected not only the titer but also the yield; for example, for strain AK26, the yield from 1% glucose was 0.095 g isoprenol per g glucose, whereas from 2% glucose the yield was 0.14 g isoprenol per g glucose; the latter is close to 44% of the theoretical yield using the MVA pathway (Dugar and Stephanopoulos, 2011). In both cases, the stationary phase was reached after 20 hours (Figure 5A), therefore, it is possible that the higher yield with 2% glucose was due to additional isoprenol produced during stationary phase when
production could occur without cell growth. The glucose consumption rates of AK26 in minimal medium supplemented with 2% glucose were slower (0.37 g glucose per hour) than those in the medium with 1% glucose (0.49 g glucose per hour; Figure 5B), but there was no significant difference in the isoprenol production rate or biomass production estimated by OD_{600nm} while glucose was being consumed (Figure 5C). Although both the glucose consumption and isoprenol accumulation increased linearly until the glucose was fully consumed, it is interesting to note that no increase in growth (OD_{600nm}) was observed after 22 hours regardless of the initial glucose concentration. This halted growth could be due to a limiting nutrient or the accumulation of growth inhibitors in the medium. After biomass production stopped at ~ 22 hr, isoprenol yield did not increase, suggesting that a portion of the remaining glucose was used for byproduct formation (e.g., acetate and ethanol): both fermentation byproducts showed a gradual increase during stationary phase, when ethanol accumulated to 1.2 g/L (Supplementary Figure S4). Three of the strains with the highest titers (AK21, AK26 and AK27) were further tested for production using minimal medium supplemented with 3% glucose (Figure 6). Interestingly, a significant improvement in titer was observed for strain AK26, reaching 3.71 g/L of isoprenol after 63 hours (Figure 6A), but not for strains AK27 and AK21. A higher glucose consumption rate and less acetate accumulation were observed for strain AK26, which might explain the difference in titers (Figure 6B and 6C). The yield for strain AK26 using 3% glucose was the same as when 2% glucose was used (0.14 g isoprenol per g glucose), whereas ethanol accumulated to 1.9 g/L (Figure 6D) and acetate was not produced at significant levels (0.3 g/L, Figure 6B). Since the mevalonate pathway generates 6 mol of NADH per mol of isoprenol produced (Dugar and
Stephanopoulos, 2011), accumulation of ethanol suggests that glucose might be used for production of ethanol to regenerate NAD$^+$ from excessive accumulation of NADH generated as a result of higher isoprenol production. When the glucose concentration was further increased to 4% and 5%, lower isoprenol titers, lower glucose consumption rate, and significant acetate accumulation were observed (data not shown), which is a signature feature of overflow metabolism (Basan et al., 2015; Szenk et al., 2017; Wolfe, 2005).

3.4. Fed-batch fermentation

3.4.1. Media and feeding optimization

To test if higher titers and yields of isoprenol could be achieved in a bioreactor by feeding additional glucose, the optimized strain presented in the previous section (strain AK26) was used for fed-bath fermentations in a 2-L bioreactor. M9 minimal medium with 2% glucose (see Material and Methods for full description of the medium components) was used during the batch phase, and glucose was continuously added at a constant rate when the initial glucose was depleted, indicated by a sharp increase in DO or by HPLC analysis of the glucose level. This initial fermentation is referred to as Ferm 1 and subsequent fermentations are numbered accordingly (for a description of all cultivation conditions and a summary of titers, yields, and productivities, see Supplementary Table S1). Figure 7A shows that production of isoprenol for Ferm 1 continuously increased during the fermentation, reaching a titer of 3.55 g/L and a yield of 0.048 g/g glucose at 150 hours, which is lower than what we observed under shake flask-conditions (yield of 0.14 g/g glucose). The growth profile of Ferm 1 (Figure 7B) showed
that the OD$_{600nm}$ reached 6.2 at 22 hours and that the glucose in the batch medium was depleted after 48 hours of cultivation (Supplementary Figure S5). This result suggests that growth was halted even before all glucose was consumed. Therefore, we hypothesize that nutrients other than glucose might be limiting growth during the batch phase. When we increased the concentration of the nitrogen source (NH$_4$Cl) in the batch medium, from a C/N ratio of 18 in Ferm 1 to a ratio of 10 (Ferm 2), a maximum OD$_{600nm}$ of 7.5 was reached at 22 hours (see Ferm 2 in Figure 7B). Also, there was a significant reduction in the time needed for the complete consumption of the initial glucose in the batch culture from 55 hours to 30 hours (see Supplementary Figure S5), reflecting a higher glucose consumption rate for Ferm 2 compared to that of Ferm 1. Despite the higher biomass accumulation during the batch phase, the maximum isoprenol titer for Ferm 2 was 3.44 g/L at 119 hours, which is similar to that of Ferm 1 (Figure 7A).

Instead of feeding glucose at a constant rate, more efficient feeding strategies have been developed in order to prevent overfeeding or underfeeding of the substrate; in particular, feeding the limiting substrate at an exponentially increasing rate results in constant specific growth rates (Korz et al., 1995). Therefore, we changed the feeding strategy from constant feeding to exponential feeding (see Materials and Methods for details). As shown in Figure 7B the exponential feeding strategy (Ferm 3) resulted in a continuous increase in OD$_{600nm}$ during the first 50 hours of the fermentation (c.f. batch phase ended ca. 30 hours), a maximum OD$_{600nm}$ of 11.1, and a significant increase in isoprenol titer, reaching a maximum titer of 4.86 g/L.

It has been shown that *E. coli* growth on minimal medium containing ammonium salts as a sole nitrogen source can be significantly improved by supplementing the medium with
an organic nitrogen source such as yeast extract, as it provides amino acids that can be
directly used for enzyme synthesis (Hugo and Lund, 1968). Therefore, we supplemented
the cultivation medium with yeast extract (10 g/L in the batch phase and 5 g/L during the
fed-bath phase) to test its effect on growth and production of isoprenol. As can be seen in
Figure 7B (Ferm 4), supplementing the medium with yeast extract resulted in higher OD
values compared to previous fermentations, reaching a maximum OD$_{600nm}$ of 16.7.
Moreover, the isoprenol titer also improved, reaching a maximum of 5.42 g/L after 94
hours. Since exponential feeding and the addition of yeast extract (which constituted less
than 10% of the total carbon added) resulted in significant improvements in OD$_{600nm}$ and
titer, these conditions were used for further fermentations.

3.4.2. Reduction of acetate formation

During the optimization of the medium and feeding strategy, it was observed that the
OD$_{600nm}$ of the culture decreased after 46 hours (see Ferm 4 in Figure 7B), and a
significant amount of acetate (1.5 g/L) was observed at 54 hours; acetate concentration
continued to increase to more than 6.6 g/L by the end of the fermentation (see Ferm 4 in
Figure 8A). Acetate accumulation not only represents a loss of carbon but it can also be
detrimental to cell growth at concentrations as low as 1 g/L, affecting the stability of
intracellular proteins and acting as proton conductor that can reduce proton motive force
(Eiteman and Altman, 2006; De Mey et al., 2007). Two main pathways are responsible
for acetate production in *E. coli*, the pyruvate oxidase and the acetate kinase -
phosphotransacetylase pathway, which are encoded by the *poxB* and *ackA/pta* genes,
respectively (see Figure 1A). To minimize acetate production during the fermentation, a
strain in which these acetate-pathway genes were deleted (strain AK30) was used for isoprenol production in the fermenter. No acetate was detected during the fermentation when this mutant strain was used (Ferm 5, Figure 8A); moreover, there was no significant decrease in OD_{600nm} during the cultivation as was previously observed for the wild-type strain (Ferm 4, Figure 7B).

The glucose consumption rate for the mutant strain was similar to that of the wild-type strain (Figure 8D) but the isoprenol titer increased 14%, reaching 6.15 g/L after 95 hours (Figure 8C). This result suggests that removal of acetate pathway prevented the loss of carbon to acetate, improving the conversion of glucose in Ferm 5. When the glucose concentration in the batch phase was increased from 2% to 3%, the isoprenol titer was further improved, reaching a maximum of 6.84 g/L at 79 hours (yield of 0.084 g/g); no significant accumulation of acetate was detected (see Ferm 6 in Figure 8) as was the case for Ferm 5. The maximum OD_{600nm} was greater with 3% glucose in which additional NH_4Cl was added to maintain a C/N = 10, reaching a value of 25 at 33 hours. This result suggests that the conversion of glucose into isoprenol was further improved by increasing biomass available during fed-batch phase. Based on these results, we used the strain with the acetate-pathway genes deleted for further optimization of the isoprenol production with 3% initial glucose.

3.4.3. Use of a solvent overlay: two-phase cultivation

As shown in Figure 8B, there was no significant increase in OD_{600nm} after 35 hours regardless of acetate accumulation or higher glucose in the batch phase (Ferm 4-6). In fact, most of the acetate accumulation in Ferm 4 was observed after 50 hours, suggesting that acetate toxicity may not be the reason for the halted growth after 35 hours. Besides
acetate accumulation, there was an observed relationship between the time at which the
culture stopped growing and the time at which higher isoprenol titers were reached. As
shown in Figures 7 and 8, no significant increase in OD$_{600nm}$ values was observed after
isoprenol titers reached more than 3 g/L, suggesting that high isoprenol concentration
might be a reason for the halted growth. It is known that exposure to short-chain alcohols
can result in compromised cell membranes, causing uncontrolled transport of solutes,
leakage of important cofactors, and inactivation of membrane and cytosolic enzymes,
resulting in a decline in growth rate and cell viability (Huffer et al., 2011; Ingram, 1986).
In the case of *E. coli*, it has been reported that isoprenol can be toxic at concentrations as
low as 2.4 g/L (Foo et al., 2014). Two-phase cultivations, in which an organic solvent is
used to continuously extract a product *in situ*, can be used as a strategy to reduce the toxic
effects of the product and to increase its yield (Malinowski, 2001). In particular, oleyl
alcohol has been shown to be an effective solvent for the extraction of short-chain
alcohols in two-phase cultivations (Connor et al., 2010; Roffler et al., 1987).
Therefore, oleyl alcohol (20% v/v) was added to the bioreactor at the time of induction
and the cultivation was carried out as described in the previous section, except that
isoprenol was measured both in the aqueous and the organic phases. When the two-phase
cultivation with oleyl alcohol (Ferm 7) was used, significantly higher OD values were
achieved compared to those of the one-phase cultivation (Ferm 6; Figure 9A); moreover,
the OD$_{600nm}$ from two-phase cultivation (Ferm 7) increased throughout cultivation and
reached a maximum value of 44.6 at 95 hours, which is a substantial improvement in
growth compared to Ferm 6. Accumulation of greater biomass during the two-phase
cultivation (Ferm 7) was confirmed by DCW measurements (see Supplementary Figure
S6A). Isoprenol production was also significantly higher when the two-phase cultivation strategy was used (Figure 9B), reaching a maximum titer of 10.8 g/L at 95 hr with a yield of 0.105 g/g glucose and a maximum productivity of 0.157 g L⁻¹ hr⁻¹. Further analysis showed that the amount of isoprenol coming from the aqueous phase accounts for ca. 62% (6.7 g/L) of the total isoprenol produced and the amount of isoprenol coming from the oleyl alcohol phase accounts for ca. 38% (4.1 g/L) of the total isoprenol produced (Supplementary Figure S6C). It is noteworthy that the maximum aqueous-phase concentration of isoprenol for the two-phase cultivation was very similar to the maximum concentration reached in the fermentation without an overlay (see Figure 9B and Supplementary Figure S6D). This result shows that a continuous product extraction using an overlay can be an effective strategy for producing isoprenol at high titers and reducing its detrimental effect on growth by maintaining a low concentration of the toxic product in the producing culture.

Since the use of two-phase fermentations at larger scales could be challenging in terms of the cost and the downstream processing, production of isoprenol at high titers and at larger scales might require the development of strategies to alleviate the toxicity of isoprenol without the use of organic solvents. Improving E. coli’s tolerance to isoprenol by expressing efflux pumps or transporters (Foo et al., 2014) or by directed evolution is a promising strategy that could allow production at high titers without the need for two-phase fermentation, but further host engineering would be required for this goal.

4. Conclusion
The IPP-bypass pathway was optimized for production of isoprenol in *E. coli* by improving the biosynthesis of MVAP and its conversion to IP, a direct precursor of isoprenol. A series of metabolic engineering efforts improved the isoprenol production substantially, reaching 3.7 g/L (0.14 g isoprenol per g glucose) in batch fermentations using minimal medium. The use of fed-batch fermentation allowed production of isoprenol at 10.8 g/L, which is the highest reported titer for this compound. In addition to medium optimization and the elimination of acetate accumulation, production at high titers required the use of a two-phase cultivation process whereby isoprenol was partially removed from the aqueous phase into an organic overlay. The removal of isoprenol from the aqueous phase contributed to relieving its toxicity, resulting in considerably higher biomass levels in the cultivation with an organic overlay.

**Acknowledgements**

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References


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FIGURE LEGENDS

Figure 1. Isoprenol production pathway and plasmids for heterologous pathway expression (A) Pathway reactions and relevant enzymes for isoprenol production. Heterologous enzymes targeted for engineering are highlighted in filled dark arrows. (B-D) configuration of two plasmids used in this study. (B) Configuration used only in strain AK01. (C) Format used for MK variants, HMGS-HMGR variants, and PMD variants. (D) Format used for the expression of additional HMGR.

Figure 2. Optimization of intermediates for MVAP biosynthesis. (A) Isoprenol titer with MK expressed from a medium-copy plasmid (p15A origin) or high-copy plasmid (ColE1 origin); (B) Production with HMGS and HMGR from different sources; (C) Isoprenol production with MK from various sources; (D) Expression of an additional copy of HMGR. Error bars represent one standard deviation from three biological replicates. Cultures were grown in test tubes with EZ-Rich medium at 30°C, induction at OD_{600nm} = 0.4-0.6 with 0.5 mM IPTG, and isoprenol production was measured at 48 hr. Sc_o: S. cerevisiae wild type, Sc_co: S. cerevisiae codon optimized, Sa: S. aureus, Bp: Bordetella petrii, Da: Delftia acidovorans, Pm: Pseudomonas mevalonii, Mm: M. masei, Sn: Streptococcus pneumoniae, MK: mevalonate kinase, HMGS=HMG-CoA synthase, HMGR= HMG-CoA reductase

Figure 3. Comparison of strains containing wild-type PMD (strain AK07) and the R74G PMD mutant (strain AK21). (A) Isoprenol production and growth; (B) Mevalonate (MVA) and mevalonate phosphate (MVAP) levels. Error bars represent one standard
deviation from three biological replicates of EZ-Rich medium (50 mL in 250-mL flasks) supplemented with 1% glucose at 30°C.

**Figure 4.** Effect of PMD mutants and HMGS-HMGR-MK systems on isoprenol production. (A) Production in EZ-Rich medium; (B) Production in minimal medium. PDM with the single mutations R74G or R74H, or with triple mutation R74H, R147K, and M212Q (HKQ) were tested with different HMGS, HMGR, and MK. Figure shows the average of three biological replicates of cultures grown at 30°C. EZ Rich medium was supplemented with 1% glucose and isoprenol production was measured at 48 hr. Minimal medium was supplemented with 2% glucose and isoprenol production was measured at 63 hr. Sc_co = *S. cerevisiae*, codon-optimized; Sa = *S. aureus*; Mm = *M. mazei*.

**Figure 5.** Data from strain AK26 grown on minimal medium supplemented with 1% and 2% glucose. (A) Growth; (B) glucose concentration in the medium; (C) isoprenol production. Cultures were grown in flasks at 30°C and error bars represent one standard deviation from three biological replicates.

**Figure 6.** Comparison of the optimized strains (AK21, AK26, and AK27) in minimal medium supplemented with 3% glucose at different time points. (A) Isoprenol production; (B) Acetate accumulation; (C) Glucose consumption; (D) Ethanol accumulation. Cultures were grown in test tubes at 30°C and induced at OD_{600nm} 0.4-0.6 with 0.5 mM IPTG.
Figure 7. Media and feeding optimization. (A) Isoprenol titer; (B) optical density (OD$_{600\text{nm}}$). Fermentations were run at 30°C with 2% glucose in the batch medium using strain AK26. Ferm 1: batch medium with C/N=18 and constant feeding, feed started at 54 hours; Ferm 2: batch medium with C/N=10 and constant feeding, feed started at 31 hours; Ferm 3: batch medium with C/N=10 and exponential feeding, feed started at 31 hours; Ferm 4: batch medium with C/N=10, 10 g/L yeast extract and exponential feeding, feed started at 23 hours.

Figure 8. Elimination of acetate production. (A) acetate accumulation; (B) optical density (OD$_{600\text{nm}}$); (C) isoprenol titer; (D) glucose consumption. Fermentations were run at 30°C with 10 g/L yeast extract in the batch medium and exponential feeding. Ferm 4: DH1 strain, batch medium with 2% glucose (strain AK26), feed started at 23 hours; Ferm 5: DH1 $\Delta$poxB $\Delta$ackA $\Delta$pta strain, batch medium with 2% glucose (strain AK30), feed started at 23 hours; Ferm 6: DH1 $\Delta$poxB $\Delta$ackA $\Delta$pta strain, batch medium with 3% glucose (strain AK30), feed started at 25 hours.

Figure 9. Two-phase fermentation. (A) Optical density (OD$_{600\text{nm}}$); (B) isoprenol titer. Fermentations using the DH1 $\Delta$poxB $\Delta$ackA $\Delta$pta strain (AK30), run at 30°C with 3% glucose and 10 g/L yeast extract in the batch medium, exponential feeding. Ferm 6: without overlay; Ferm 7: with 20% (v/v) oleyl alcohol overlay. For both Ferm 6 and 7, feed started at 25 hours.
Figure 1

(A) Glycolysis

Acetate (B) Acetyl-CoA

Acetyl-P (C) Acetyl-CoA

Pyruvate (D) TCA cycle

Isoprenol

\[ \text{AtoB} \rightarrow \text{HMGS} \rightarrow \text{HMGR} \rightarrow \text{MVA} \rightarrow \text{MVAP} \rightarrow \text{IP} \rightarrow \text{Isoprenol} \]

(B) lacUV5

\[ \text{AtoB} \rightarrow \text{HMGS} \rightarrow \text{HMGR} \rightarrow \text{Bba1002} \rightarrow \text{p15A} \]

(C) lacUV5

\[ \text{AtoB} \rightarrow \text{HMGS} \rightarrow \text{HMGR} \rightarrow \text{Bba1002} \rightarrow \text{p15A} \]

(D) lacUV5

\[ \text{AtoB} \rightarrow \text{HMGS} \rightarrow \text{HMGR} \rightarrow \text{Bba1002} \rightarrow \text{p15A} \]

\[ \text{PMD} \rightarrow \text{ColE1} \]

\[ \text{rrnB} \]
Figure 4.

(A) 

(B)
Figure 5

(A) OD at 600nm over time for 1% and 2% glucose.

(B) Glucose concentration over time for 1% and 2% glucose.

(C) Isopreno concentration over time for 1% and 2% glucose.
Figure 7

(A) isoprenol (g/L)

(B) OD600nm
Figure 9

(A) OD$_{600nm}$ vs. Time (hr) for fermentations Ferm 6 and Ferm 7.

(B) Isoprenol (g/L) vs. Time (hr) for fermentations Ferm 6 and Ferm 7.
Supplementary Figure S1. Analysis of additional HMGR expression on (A) growth by OD$_{600nm}$, (B) isoprenol production, and (C) intracellular and extracellular intermediate concentrations.
Supplementary Figure S2. Comparison of strains containing the wild type PMD (strain AK07) and the PMD with the R74G mutation (strain AK21). (A) Glucose remaining in the medium, (B) Acetate
Supplementary Figure S3. Production in minimal medium supplemented with 1% glucose. Production was measured at 24 hours.
Supplementary Figure S4. Comparison of strain AK26 grown on minimal medium supplemented with 1% and 2% glucose. (A) Acetate accumulation; (B) Ethanol accumulation.
Supplementary Figure S5. Effect of NH$_4$Cl concentration on glucose consumption
**Supplementary Figure S6.** Two phase fermentation. (A) comparison of dry cell weight (DCW); (B) glucose consumption; (C) distribution of isoprenol in the aqueous phase and oleyl alcohol phase at different time-points (Ferm 7); (D) isoprenol production in one-phase fermentation (Ferm 6). Ferm 6 corresponds to one-phase fermentation and Ferm 7 corresponds to two-phase fermentation.
**Supplementary Table S1:** Titers, yields and maximum productivities for the different strains and fermentation conditions used in this study. Yield calculations correspond to the time at which the maximum titer was reached (grams of isopentenol /grams of glucose consumed). Productivity corresponds to the maximum productivity reached during the fermentation.

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<td>5.42</td>
<td>0.069</td>
<td>0.079</td>
</tr>
<tr>
<td>Ferm 5</td>
<td>DH1 ΔpoxB ΔackA-pta</td>
<td>2% glucose, yeast extract</td>
<td>exponential</td>
<td>6.15</td>
<td>0.082</td>
<td>0.098</td>
</tr>
<tr>
<td>Ferm 6</td>
<td>DH1 ΔpoxB ΔackA-pta</td>
<td>3% glucose, yeast extract</td>
<td>exponential</td>
<td>6.84</td>
<td>0.073</td>
<td>0.121</td>
</tr>
<tr>
<td>Ferm 7</td>
<td>DH1 ΔpoxB ΔackA-pta</td>
<td>3% glucose, yeast extract, oleyl alcohol overlay</td>
<td>exponential</td>
<td>10.88</td>
<td>0.105</td>
<td>0.157</td>
</tr>
</tbody>
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