Gram-scale production of the sesquiterpene α-humulene with Cupriavidus necator

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Abstract
Terpenoids have an impressive structural diversity and provide valuable substances for a variety of industrial applications. Among terpenes, the sesquiterpenes (C₁₅) are the largest subclass with bioactivities ranging from aroma to health promotion. In this article, we show a gram-scale production of the sesquiterpene α-humulene in final aqueous concentrations of 2 g L⁻¹ with the recombinant strain Cupriavidus necator pKR-hum in a fed-batch mode on fructose as carbon source and n-dodecane as an extracting organic phase for in situ product removal. Since C. necator is capable of both heterotrophic and autotrophic growth, we additionally modeled the theoretically possible yields of a heterotrophic versus an autotrophic process on CO₂ in industrially relevant quantities. We compared the cost-effectiveness of both processes based on a production of 10 t α-humulene per year, with both processes performing equally with similar costs and gains. Furthermore, the expression and activity of 3-hydroxymethylglutaryl-CoA reductase (hmgR) from Myxococcus xanthus was identified as the main limitation of our constructed C. necator pKR-hum strain. Thus, we outlined possible solutions for further improvement of our production strain, for example, the replacement of the hmgR from M. xanthus by a plant-based variant to increase α-humulene production titers in the future.

KEYWORDS
Cupriavidus necator, mevalonate pathway, process design, sustainable economy, terpene production, α-humulene

1 | INTRODUCTION

Terpenoids are the largest group of natural products with remarkably high structural diversity, deriving only from the two different precursor molecules isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP). Among the terpenoids, sesquiterpenes (C₁₅ molecules) are the largest subgroup, with more than 7000 individual compounds identified (Breitmaier, 2006; Fraga, 2013). This subgroup derives from farnesyl pyrophosphate (FPP), which is formed by a reaction of one molecule of DMAPP and two molecules of IPP. From FPP, the addition of a terpene synthase results in a one-step transformation to the desired natural sesquiterpene. IPP and DMAPP can be generated by one of two pathways: the mevalonate pathway (MVA), which is ubiquitous to eukaryotes and can also be found in archaea and some Gram-positive bacteria (Miziorko, 2011), or the methylerythritol 4-phosphate (MEP) pathway, found in eukaryotes and in most bacteria, as in Cupriavidus.
necator (Eisenreich et al., 2004). However, there are examples of the expression of the MVA pathway in C. necator (Hyeok-Won et al., 2019; Krieg et al., 2018). C. necator can grow on heterotrophic carbon sources, for example, fructose or glycerol, as well as litoautotrophically on CO2 and H2. Two hydrogenases catalyze the oxidation of H2— one membrane-bound hydrogenase transfers electrons into the electron transport chain, and one cytosolic hydrogenase generates reducing power by NADH regeneration for CO2 fixation. The growth on CO2 is mediated by the Calvin–Benson–Bassham cycle (Bowien & Schlegel, 1981). Under nutrient, for example, nitrogen limitation, C. necator directs most part of its carbon flux to the synthesis of the storage compound polyhydroxybutyrate (PHB), a biopolymer which is stored in granules in the cell (Reinecke & Steinbüchel, 2009). C. necator is able to store up to 70% of its own weight in PHB (Ishizaki et al., 2001) while growing autotrophically. However, even values up to 90% were reported (Aragno et al., 1977). The natural pathway of PHB synthesis in C. necator has acetocetyl-CoA as a key intermediate. A PHB-deficient C. necator strain (Raberg et al., 2014) was utilized to use the flux toward acetocetyl-CoA and redirect it to the recombinant MVA pathway combined with the α-humulene synthase from Zingiber zerumbet for the production of α-humulene. The original pathway was expressed in Methylobacterium extorquens (Sonntag et al., 2015). Due to a similar codon usage, the heterologous pathway for M. extorquens could be used directly in C. necator. Therefore, the genes coding for the mevalonate pathway, the farnesyl pyrophosphate synthase (erg20), the IPP isomerase (fni), and the α-humulene synthase (zssI) were amplified, and introduced into a previously constructed and characterized vector for expression in C. necator pKRha (Sydow, Pannek, et al., 2017) to form the plasmid pKRhum. The resulting strain C. necator pKR-hum was already shown to be able to produce α-humulene under heterotrophic and autotrophic conditions as well as in an electrochemical system (Krieg et al., 2018).

In this article, we compare the theoretically possible industrial-scale production of α-humulene with the C. necator pKR-hum strain under autotrophic and heterotrophic conditions in terms of cost-efficiency and investigate the actual production limits in a heterotrophic fermentation. In addition, we identify the limitations of the system and propose an optimization strategy to increase the α-humulene production with C. necator to industrially relevant production titers.

2 MATERIALS AND METHODS

2.1 Modeling of heterotrophic versus autotrophic production of α-humulene

The economic efficiency of the heterotrophic and autotrophic production processes of α-humulene with C. necator was calculated based on simulations with literature data. For the modeling of the different production systems and their respective unit operations, SuperPro Designer 11 (Intelligen, Inc.) was applied. The exact description and the applied parameters can be found in the supplementary information.

2.2 Cultivation media

Lysogeny broth (LB) was used as a complex medium for precultures, if not stated otherwise. LB was composed of (in g L−1): tryptone/peptone 10, yeast extract 5, and NaCl 10. Minimal medium (MM) for C. necator was composed of (in g L−1): NaH2PO4 2.895, NaH2PO4·2 H2O 3.06, K2SO4 0.17, CaSO4·2 H2O 0.097, MgSO4·7 H2O 0.8, (NH4)2SO4 0.943, FeSO4·7 H2O 0.05, and trace elements 1:20,000. Trace elements stock was composed of the following ingredients (in g L−1; in 0.05 M H2SO4): FeSO4·7 H2O 15, MnSO4·H2O 2.4, ZnSO4·7 H2O 2.4, CuSO4·5 H2O 0.48, Na2MoO4·2 H2O 1.8, NiSO4·6 H2O 1.5, and CoSO4·7 H2O 4.02 × 10−2. Fructose was added to the MM to a final concentration of 4 g L−1 (44.4 mM) under heterotrophic conditions, if not stated otherwise. Media were supplemented with 15 µg mL−1 tetracycline hydrochloride (Tc) for recombinant strains. α-L-rhamnose was used as inducer at a final concentration of 11 mM (0.2%) using a 20% (w/v) stock solution for α-humulene production. All basic media components were purchased from Sigma-Aldrich and Carl Roth.

2.3 Plasmid construction and transformation of plasmids into E. coli and C. necator

The construction of the pKRha, the pKR-hum, and the pKR-hum ΔzssI was described previously (Krieg et al., 2018). The construction of the pKR-hum ΔMVA plasmid is described in detail in the supplementary information.

Escherichia coli strains were made chemically competent for plasmid uptake by a standard protocol modified after Hanahan (1983). For details see the supplementary information methods section.

Plasmids were transferred to the recipient C. necator H16 PHB ‘4 (Raberg et al., 2014) by conjugation. The details are given in the supplementary information.

2.4 Heterotrophic cultivation in shake flasks

Strains were cultivated at 30°C and 37°C for C. necator and E. coli, respectively. Liquid cultures were shaken at 180 rpm in the incubation shakers Mintron or Ecotron (Infors AG) with a deflection of 25 mm. LB was used as a complex medium for all strains, if not stated otherwise, and supplemented with 15 µg mL−1 tetracycline for both bacteria, according to the applied plasmids.

2.5 Autotrophic cultivation in injection flasks

Autotrophic cultivation was performed in 200 mL septum flasks containing 20 mL minimal medium without fructose and 5 mL n-dodecane as an organic phase for in situ product removal. A heterotrophic preculture was used as inoculum. The defined gas atmosphere was composed of H2/CO2/O2 (64:16:20). Cultures were grown at 30°C and 180 rpm (Mintron or Ecotron, Infors AG) with a deflection of 25 mm and induced
at exponential growth phase with 11 mM ω-hamnosose. Samples of aqueous and organic phases were taken frequently to follow growth and α-humulene production (via LC-MS/MS), respectively.

2.6 Quantification of fructose in the supernatant with HPLC

Fructose concentration was quantified by HPLC (SCL 10-A, Shimadzu Deutschland GmbH) equipped with the refractive index detector RID-10A and provided with LabSolutions, version 5.57 software. A Rezex ROA organic acid H + (8%) analytical column was used (Phenomenex Inc.). Elution was preceded with 5 mM H2SO4 at a flow of 0.5 mL min⁻¹ and 57°C.

2.7 Quantification of α-humulene production via LC-MS/MS

Due to strongly asymmetric partitioning, α-humulene is detected solely in the organic phase (data not shown). Therefore, only product concentrations in the organic n-dodecane phase were investigated. Higher sensitivity and accuracy were achieved by quantifying α-humulene using a LC-MS/MS system consisting of a triple quadrupole mass spectrometer (LCMS-8040, Shimadzu Deutschland GmbH) equipped with an atmospheric pressure ionization source (APCI; Shimadzu Deutschland GmbH) and an associated UHPLC system (Nexera 30 series, Shimadzu Deutschland GmbH). Details are described in the supplementary information.

2.8 Heterotrophic fed-batch fermentation in DASGIP® parallel fermentation system

C. necator (pKR_hum) was cultivated in a SR1000ODLL bioreactor system connected to the sensor module pH4pO4 for pH and dissolved oxygen (DO) monitoring and control, TC4SC4 module for agitation and temperature control, exhaust gas analyzer GA4, multipump module MP8, and compressed air supplier MX4/4 (DASGIP). For pH control, the pH was maintained at 6.8 with a 4 M KOH solution. The applied feeding solution composition was as follows: 446.4 g L⁻¹ fructose, 111.6 g L⁻¹ (NH₄)₂SO₄, and one-time addition of trace elements as in the regular minimal medium. After 7.5 days of cultivation, the feeding solution was replaced by one without (NH₄)₂SO₄. The reactors were placed into a Bioblock to control the temperature at 30°C. The regulation of the process was performed with DASGIP control, v4.5. The initial settings for gassing were set to six standard liters per hour (sL h⁻¹) and the initial agitation speed was set to 400 rpm. The agitation and gassing were regulated to keep the DO signal at 20%. Initial liquid volume was set to 0.3 L. A heterotrophic preculture grown overnight in MM was centrifuged, resuspended in the bioreactor medium and the reactor was inoculated to an optical density (OD) of 0.1.

Cultures were induced at 0.84 ± 0.03 gCDW L⁻¹ after 13–14 h with 0.2% ω-hamnosose. At induction time, 20% n-dodecane (75 ml) was added to the culture through a port to remove α-humulene in situ. The feed was started after 14 h with the initial flow rate of 0.75 ml h⁻¹ and was increased throughout the fermentation until a maximal feed of 1.10 ml h⁻¹. Increase in biomass, product formation, and concentration of carbon and nitrogen source was followed over time. After the bioreactors had been stopped, the total amount of α-humulene was recovered by centrifugation and washing of the intermediate phase containing organic phase and cell debris as well as extraction of the aqueous phase with n-dodecane. The intermediate phase was washed four times with n-dodecane, with still high amounts of product recovered from the strong cell debris containing the interface.

OD was measured at 600 nm using BioWave Cell Density Meter CO8000 (Biochrom WPA) and a 10 mm cuvette to calculate the number of cells for reproducible inoculation of experiments. The correlation was obtained by growing C. necator (pKR_hum) in septum flasks under autotrophic conditions (n = 8) followed by harvest of the whole flask contents, determination of liquid volume, washing pellets twice with the same volume of ddH₂O, and weighing the pellets with the moisture analyzer M 100 (Sartorius AG). A linear correlation forced through the coordinate system origin was obtained (Krieg et al., 2018).

Biomass (gCDWL⁻¹) = 0.43 × OD

Quantification of ammonium ions was quantified in the range of 0.2–20 mg L⁻¹ by using the Reflectoquant® system (Merck Chemicals GmbH) with the respective test stripes according to the manufacturer specifications.

The oxygen transfer rate (OTR) and carbon dioxide transfer rate (CTR) were calculated via the gas balancing method (supplementary information).

2.9 Measurement of transcription levels of the pKR-hum plasmid with RT-qPCR

For analyzing the expression of the pKR-hum genes using RT-qPCR, C. necator cells were harvested 1.5 h after induction (OD600 of ~0.8) from shake flasks cultivations in triplicates in minimal medium. As a control, uninduced cells were cultivated in parallel and harvested at the same time. The cells were pelleted by centrifugation at 10,000 × g at 4°C for 5 min and total RNA was isolated immediately with the RNaseasy Mini Kit (Qiagen) and stored at −20°C until quantitative reverse-transcription polymerase chain reaction (RT-qPCR) analysis. The primers and details of the RT-qPCR are listed in Table S6.

2.10 Statistical analysis for the autotrophic α-humulene production tests in the injection flasks

An unpaired two-tailed t-test was used to test the null hypothesis that there was no difference between the two conditions. A 99% or
95% confidence interval was used to reject the null hypothesis and thus determine the significance of single variables (p values < 0.01 or < 0.05, indicated in the results part).

2.11 Cloning of *M. xanthus* genes into *E. coli* expression vectors

The *Myxococcus xanthus* genes encoding the MVA-pathway enzymes *hmgS*, *hmgR*, *mvaK*, *mvaD*, and *mvaK2* were amplified by PCR from the plasmid pKr-Hum and cloned (ligation independent cloning) into a proprietary backbone vector (pGT1960) containing a medium copy origin of replication (p15A-ori; 15–20 plasmid copies/cell), an ampicillin resistance gene as selection marker and a constitutive promoter driving recombinant gene expression. Details are described in the supplementary information.

2.12 Testing of MVA genes in lycopene-producing *E. coli* cells

To test the activity of the MVA enzymes, two previously constructed lycopene producing *E. coli* strains were used: strain BL21.G45 with a lycopene biosynthetic pathway integrated into the genome and BL21.G171 carrying in the genome the lycopene pathway as well as a functional MVA lower portion pathway previously constructed using plant genes.

For testing the hmgS-hmgR enzymes, the plasmids pGT2148 and pGT2184 were transformed into BL21.G171 and for testing the mvaK-mvaD-mvaK2 the plasmids pGT2071 and pGT2225 were transferred into BL21.G45. As a negative control, an empty plasmid without MVA genes was used.

The cells expressing the MVA enzymes were grown in 2 mL FIT-B media (using glucose as a carbon source and supplemented with 100 mg L\(^{-1}\) ampicillin, media composition in Table S8). The cultivation was performed at 28°C and 350 rpm for 48 h, using a 24-Deepwell microtiter plate (Duetz System). The 2 mM mevalonate was added to the media to test the activity of the mvaK-mvaD-mvaK2 enzymes. After the incubation period, the cultures were centrifuged at 14,000 rpm for 2 min and the lycopene was extracted from the cell pellet with acetone. The concentration of lycopene in the acetone extracts was determined by measuring the extinction at 502 nm using a spectrophotometer (Evolution™ 201/220 UV-Vis-Spektrophotometer, Thermo Fisher Scientific).

3 RESULTS AND DISCUSSION

3.1 Theoretical comparison of autotrophic and heterotrophic production of \(\alpha\)-humulene in *C. necator*

*C. necator* can grow on different substrates (e.g., fructose or mixtures of \(\text{H}_2/\text{O}_2/\text{CO}_2\)). To assess which of the systems is currently economically feasible, a model-based comparison was carried out. For the theoretical evaluation of the autotrophic versus the heterotrophic production of \(\alpha\)-humulene with *C. necator* the target production was set to 10 t a\(^{-1}\) for both production types. As the maximal yield of \(\alpha\)-humulene, the maximal respective literature value for P(3)HB production of 2.42 g L\(^{-1}\) h\(^{-1}\) was considered, which when accounted for the same carbon flux, corresponds to a \(\alpha\)-humulene production of 1.5 g L\(^{-1}\) h\(^{-1}\) (7.3 mM h\(^{-1}\)) (Kim et al., 1994). For the autotrophic production, lower optical densities (Sydow, Krieg, et al., 2017) and therefore lower P(3)HB titers are described. The highest literature value for P(3)HB is 1.55 g L\(^{-1}\) h\(^{-1}\) (Tanaka et al., 1995), which corresponds to the productivity of 4.89 mM h\(^{-1}\) \(\alpha\)-humulene.

For the heterotrophic production, literature values for medium components and setpoints are applied (Table S1). The process is performed in a stirred-tank reactor in a batch mode with 80% of the maximal working volume. For the autotrophic production, an airlift reactor was chosen with 80% of the maximal working volume. Apart from the same process parameters, the use of gasses as carbon and energy sources requires an atmosphere of 16% CO\(_2\), 64% H\(_2\), and 20% O\(_2\) and an initial pressure of 1.5 bar (Krieg et al., 2018).

In the simulation of the heterotrophic production with SuperPro Designer, \(\alpha\)-humulene titers of 30 g L\(^{-1}\) with 7.5 g L\(^{-1}\) of biomass formation were simulated in a 10,000 L fermenter in the main operation step during the 24 h production period. This translated to 316 kg \(\alpha\)-humulene per bioreactor run (1546 mol). The time for the whole process including the precultures and the downstream processing was estimated to 334.5 h. For the simulation of the autotrophic production with SuperPro Designer, \(\alpha\)-humulene titers of 38.9 g L\(^{-1}\) were obtained, with 21.7 g L\(^{-1}\) of biomass formation in a 7935 L reactor within 40 h of cultivation. This equals to 310.7 kg \(\alpha\)-humulene per reactor run (1520 mol). The duration of the whole process from precultivation to the distilled \(\alpha\)-humulene is 339.1 h.

For both production modes setting up the next preculture in the preculture reactor directly after inoculation of the main reactor shortens the operation time by 218.5 h, leading to 35 production runs in 1 year and 11 t of \(\alpha\)-humulene annually. The main production parameters of the process were calculated and can be found in Table S2.

The heterotrophic process has higher total fixed costs than the autotrophic process due to the use of a stirred-tank reactor which is more complicated in its construction and maintenance than an airlift reactor, which does not contain any stirrer as the stirring is performed with air. Therefore, the payback period is 1.6 times longer for the heterotrophic process (2.13 a) compared with the autotrophic process (1.33 a, Table S2). However, after the payback period, the net gain of the heterotrophic process is higher than the net gain of the autotrophic process due to slightly higher productivity and makes it therefore more profitable in the long run. Nevertheless, both processes are comparable and can be considered industrially relevant. We would like to emphasize that this is only a rough estimation and that more detailed modeling is needed, once the production strain has been optimized.
3.2 Production in a heterotrophic fed-batch system

The α-humulene production process in the DASGIP parallel reactor system was divided in three different phases. In the first phase, the batch phase, the C. necator strain was grown until induction. The second phase was the fed-batch phase with different feed rates \( f \) (Figure 1) of a mixed fructose/ammonium sulfate solution. In the third phase, this feed was replaced with only fructose feed.

The growth of C. necator did not show any lag phase (Figure 1a). The induction was performed 14 h after inoculation. The α-humulene production started directly after induction and the addition of 20% n-dodecane as the second phase for in situ product removal (ISPR). There was a correlation between the production of α-humulene and the biomass formation of C. necator during the linear growth phase, which was regulated by the linear feed. After switching the feed, the cell growth did not stop due to the ammonia accumulation in the medium. At the end of the process, more than 1 g L \( \text{NH}_4^+ \) was detected in the fermentation broth. Especially during the feeding with a fructose-only feed, fructose was nondetectable in the fermentation broth. Oxygen limitation did not occur at any time during the fermentation as shown by the OTR signal, since the dissolved oxygen (DO) signal was regulated at 20% by an increase in stirring and gassing (Figure 1b). The biological respiration was controlled by the fructose availability, indicated by an increase in OTR and CTR after an increase in feeding rate of the fructose stock solution. After 144 h, a decrease in biological activity was detected, indicated by a slow but steady decrease in OTR and CTR. Simultaneously, an increase in foam production was observed, which led to the assumption that increased cell lysis of old cells was observed. In addition, we could observe a formation of interphase between the organic and the aqueous phase containing cell debris. This further confirmed our cell lysis hypothesis. By daily addition of antifoam, the amount of foam in the reactor was reduced to a minimum (spiking not shown). The antifoam had no obvious impact on the cell metabolism, as there was no response in CTR and OTR. The respiratory quotient (RQ) was between 0.9 and 1.0 during the whole process time (Figure 1b). The value means that the consumption of oxygen matched the production of carbon dioxide, which is typical for an aerobic fermentation process on sugars (Bendtsen, 2000). Final
concentrations of α-humulene of 10 mM were observed with productivity of 0.027 mmol L\(^{-1}\) h\(^{-1}\).

During the whole process time, 156 and 160 g fructose were consumed by \textit{C. necator} (pKR-hum) in reactor 1 and 2, respectively. These amounts per reactor volume correspond to 7857 and 8374 mmol C L\(^{-1}\). By measuring the optical density of the culture, the α-humulene concentration, and the carbon dioxide concentration in the exhaust gas stream, a carbon balance was set up. In total, 8952 and 8374 mmol C L\(^{-1}\) were found in the products. The majority was found in carbon dioxide, followed by biomass with approximately 25% of the carbon recovered. Despite the high titers of 10 mM, only 2% of the total carbon from the substrate source can be found in the target compound α-humulene (Table 1). With means of MVA expression optimization, this value could be improved in the future. The low MVA performance is the reason for underperforming with the modeled yields and productivity values since the assumption of the same carbon flux towards α-humulene production as towards P(3)HB relied on an unlimited and balanced flux through the MVA pathway without any bottlenecks. Comparison of heterotrophic α-humulene production with literature shows that the titers achieved in this cultivation are higher than reported in other publications (Figure 2).

To the best of our knowledge, the achieved α-humulene titers are the highest reported to date. With future improvements of the limitations caused by the expression of the MVA pathway, the titers could be further improved towards an industrially relevant α-humulene production with \textit{C. necator}. Therefore, we had a closer look at the current MVA performance in \textit{C. necator}.

### 3.3 Production of α-humulene in \textit{C. necator} with the MVA pathway

\textit{C. necator} carrying the pKR-hum plasmid and variations thereof (Krieg et al., 2018) was cultivated autotrophically in injection flasks to see the differences in α-humulene production between the pKR-hum plasmids and the controls. The full plasmid pKR-hum was compared to the α-humulene synthase deletion mutant pKR-hum ΔzssI, the MVA pathway deletion mutant pKR-hum ΔMVA and an empty plasmid control pKRrha (Table 2).

![FIGURE 2](image)

**TABLE 1** Carbon balancing of the heterotrophic α-humulene production

| Component   | Reactor 1 carbon percentage | Reactor 2 carbon percentage |
|-------------|-----------------------------|-----------------------------|
| CO\(_2\) in offgas | 72.3                        | 73.7                        |
| biomass     | 25.7                        | 24.3                        |
| α-humulene  | 2.0                         | 2.0                         |

**TABLE 2** Comparison of different genetic constructs in terms of autotrophic α-humulene production in injection flasks

| Condition | 1 | 2 | 3 | 4 | 5 |
|-----------|---|---|---|---|---|
| Plasmid Induction | pKR-hum | pKR-hum | pKR-hum | pKR-hum | pKR-hum |
| pKR-hum | pKR-hum | pKR-hum | pKR-hum | pKR-hum | pKR-hum |
| pKR-hum | pKR-hum | pKR-hum | pKR-hum | pKR-hum | pKR-hum |
| pKR-hum | pKR-hum | pKR-hum | pKR-hum | pKR-hum | pKR-hum |
| pKR-hum | pKR-hum | pKR-hum | pKR-hum | pKR-hum | pKR-hum |
| pKR-hum | pKR-hum | pKR-hum | pKR-hum | pKR-hum | pKR-hum |
| pKR-hum | pKR-hum | pKR-hum | pKR-hum | pKR-hum | pKR-hum |
| pKR-hum | pKR-hum | pKR-hum | pKR-hum | pKR-hum | pKR-hum |
| pKR-hum | pKR-hum | pKR-hum | pKR-hum | pKR-hum | pKR-hum |
| pKR-hum | pKR-hum | pKR-hum | pKR-hum | pKR-hum | pKR-hum |
| pKR-hum | pKR-hum | pKR-hum | pKR-hum | pKR-hum | pKR-hum |
| pKR-hum | pKR-hum | pKR-hum | pKR-hum | pKR-hum | pKR-hum |
| pKR-hum | pKR-hum | pKR-hum | pKR-hum | pKR-hum | pKR-hum |
| pKR-hum | pKR-hum | pKR-hum | pKR-hum | pKR-hum | pKR-hum |
| pKR-hum | pKR-hum | pKR-hum | pKR-hum | pKR-hum | pKR-hum |
| pKR-hum | pKR-hum | pKR-hum | pKR-hum | pKR-hum | pKR-hum |
| pKR-hum | pKR-hum | pKR-hum | pKR-hum | pKR-hum | pKR-hum |
| pKR-hum | pKR-hum | pKR-hum | pKR-hum | pKR-hum | pKR-hum |
| pKR-hum | pKR-hum | pKR-hum | pKR-hum | pKR-hum | pKR-hum |
| pKR-hum | pKR-hum | pKR-hum | pKR-hum | pKR-hum | pKR-hum |
| pKR-hum | pKR-hum | pKR-hum | pKR-hum | pKR-hum | pKR-hum |
| pKR-hum | pKR-hum | pKR-hum | pKR-hum | pKR-hum | pKR-hum |
| pKR-hum | pKR-hum | pKR-hum | pKR-hum | pKR-hum | pKR-hum |
| pKR-hum | pKR-hum | pKR-hum | pKR-hum | pKR-hum | pKR-hum |
| pKR-hum | pKR-hum | pKR-hum | pKR-hum | pKR-hum | pKR-hum |
| pKR-hum | pKR-hum | pKR-hum | pKR-hum | pKR-hum | pKR-hum |
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| pKR-hum | pKR-hum | pKR-hum | pKR-hum | pKR-hum | pKR-hum |
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| pKR-hum | pKR-hum | pKR-hum | pKR-hum | pKR-hum | pKR-hum |
| pKR-hum | pKR-hum | pKR-hum | pKR-hum | pKR-hum | pKR-hum |
| pKR-hum | pKR-hum | pKR-hum | pKR-hum | pKR-hum | pKR-hum |

**TABLE 2** Comparison of different genetic constructs in terms of autotrophic α-humulene production in injection flasks

| Condition | 1 | 2 | 3 | 4 | 5 |
|-----------|---|---|---|---|---|
| Plasmid Induction | pKR-hum | pKR-hum | pKR-hum | pKR-hum | pKR-hum |
| pKR-hum | pKR-hum | pKR-hum | pKR-hum | pKR-hum | pKR-hum |
| pKR-hum | pKR-hum | pKR-hum | pKR-hum | pKR-hum | pKR-hum |
| pKR-hum | pKR-hum | pKR-hum | pKR-hum | pKR-hum | pKR-hum |
| pKR-hum | pKR-hum | pKR-hum | pKR-hum | pKR-hum | pKR-hum |
| pKR-hum | pKR-hum | pKR-hum | pKR-hum | pKR-hum | pKR-hum |
| pKR-hum | pKR-hum | pKR-hum | pKR-hum | pKR-hum | pKR-hum |
| pKR-hum | pKR-hum | pKR-hum | pKR-hum | pKR-hum | pKR-hum |
| pKR-hum | pKR-hum | pKR-hum | pKR-hum | pKR-hum | pKR-hum |
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| pKR-hum | pKR-hum | pKR-hum | pKR-hum | pKR-hum | pKR-hum |
| pKR-hum | pKR-hum | pKR-hum | pKR-hum | pKR-hum | pKR-hum |
| pKR-hum | pKR-hum | pKR-hum | pKR-hum | pKR-hum | pKR-hum |
| pKR-hum | pKR-hum | pKR-hum | pKR-hum | pKR-hum | pKR-hum |
| pKR-hum | pKR-hum | pKR-hum | pKR-hum | pKR-hum | pKR-hum |
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In C. necator, the genes hmgR and mvaD from M. xanthus showed rather limited expression levels (Figure 3) and are likely to limit performance of the implemented MVA pathway.

To check whether this limitation is host-dependent, a series of experiments were conducted in the host E. coli. Moreover, M. xanthus MVA enzymes were evaluated in comparison to alternative candidate enzymes from plants to check for optimization potential.

For this purpose, the M. xanthus genes from plasmid pKR-hum were cloned into a plasmid backbone suitable for expression in E. coli. Two plasmids were generated, one with the so-called upper portion (UP) genes of the MVA pathway (pGT2148, hmgS, and hmgR) and a second one with the lower portion (LP) genes (pGT2225, mvaK, mvaD, and mvaK2). As α-humulene synthase is poorly active in E. coli (Figure 2), the functionality of these constructs was tested in a system employing lycopene-producing E. coli-strains to provide an easy detectable terpene product build from the building blocks provided by the MVA pathway (Figure 4).

For the testing of UP genes, the lycopene-producing E. coli strain BL21.G171 was used, which also carries plant lower portion genes (LP2) integrated into the E. coli genome. The results of this experiment are summarized in Figure 4a. Without expression of the MVA pathway, BL21.G171 was able to produce 1.3 mg gCDW⁻¹ lycopene. Feeding of this strain with external mevalonate results in an over 400% (to 6.6 mg gCDW⁻¹) increase in lycopene due to the presence/activity of LP genes. This demonstrates the maximum capacity for lycopene synthesis for the applied mevalonate amount. Further increases in lycopene yield can be achieved when even higher mevalonate concentrations are applied (up to 10×; data not shown), indicating that neither the capacities of the LP nor of the lycopene biosynthesis are limiting under the conditions tested.

Co-expression of UP genes shall lead to cellular production of mevalonate also indicated by increased lycopene yields. Use of UP from M. xanthus (UP1), however, has only very little effect (7% increase; 1.43 mg gCDW⁻¹) on the lycopene yield, whereas plant enzyme based UP2 lead to a strong increase (+110%; 2.73 mg gCDW⁻¹) compared with the control (without feeding). Together with the data obtained in C. necator this leads to the conclusion that M. xanthus hmgR enzyme is indeed a major factor strongly limiting the overall activity of the MVA pathway. The exact reasons for the poor activity of this particular enzyme are not known yet. Likely reasons are poor recombinant expression due to false codon-usage and/or RNA instability, protein misfolding to unfavorable cellular microenvironments rendering the enzyme inactive, or the fact that enzyme naturally has a poor activity. This remains to be determined. The plant enzyme UP performed much better and offers an alternative that can be used
for optimization. However, the activity of UP2 is still not capable of achieving the lycopene production levels mediated by mevalonate feeding (Figure 4a). Thus, UP2 still suffers from limited performance delivering mevalonate levels that are not sufficient to take full advantage of the available synthesis capacities of the implemented LP, finally resulting in lower product yields. A replacement of the current hmgR enzyme with the plant-based version tested in this manuscript would be only one cornerstone of a possible optimization project for MVA enhancement in C. necator.

Possible limitations of the M. xanthus LP (LP1) were investigated in comparison to a plant enzyme-based LP (LP2) in a second approach (Figure 4b) using plasmid-based expression cassettes for these LPs together with mevalonate feeding to the lycopene-producing E. coli strain BL21.G45. This strain is identical to BL21.G171 but lacks the genome integrated LP2 enzyme cascade.

Without MVA feeding all strains gave rise to almost identical levels of lycopene, indicating the presence of the LP genes as such has no detectable impact. Upon feeding, significant increases in lycopene yields are detectable for LP1 and LP2, with plant-based LP2 performing slightly better. In presence of M. xanthus LP the cells produced 68% more lycopene than the control strain lacking LP genes or without the addition of mevalonate, whereas LP2 lead to 88% higher product yield upon mevalonate feeding. As proven by the results in Figure 4a, lycopene synthesis capacity/sink strength is not limiting in the chosen test system. Strikingly, the LP2 in this experimental setup showed significantly lower performance than the genome integrated copy of the same LP2 enzyme cassette used in Figure 4a. It has been concluded that the higher recombinant expression of the LP2 genes (due to the higher gene dosage caused by the plasmid system) negatively impacted LP activity. Achieving proper expression levels of these genes is key to gain maximum activity. This fact needs to be considered in future optimization work.

4 | CONCLUSION

In this study, we provided evidence that C. necator is a very promising host organism for heterotrophic and autotrophic production of terpenes at similar yearly production amounts and costs in silico. With 2 g L$^{-1}$ in a heterotrophic fed-batch system, the produced α-humulene titers are the highest titers reported up to date. However, the full biosynthetic potential has not been gained yet, as has been shown by the modeling data and the expression data of the MVA pathway. The implementation of the MVA pathway to boost biosynthesis had limited success in improving the product yields. Experimental data show that this is most likely due to the poor expression and poor activity of the hmgR enzyme from M. xanthus used in these experiments. However, a replacement of the hmgR enzyme would be only one step in the optimization work, that needs to be performed in the future, as other limitations within the upper portion of the MVA revealed. The correct balancing of enzyme expression would be an additional topic to improve the MVA activity. Furthermore, the tested alternative enzymes from plants showed much higher activity and can serve as a basis for future optimization work to unravel the full potential of heterotrophic terpene production in C. necator.

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CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

AUTHOR CONTRIBUTIONS

Sofia Milker: Conceptualization, methodology, investigation, and validation of all experiments and modeling, writing—original draft, review & editing. Anne Sydow: Performing fermentations, writing parts of original
draft. **Ingrid Torres-Monroy**: Conceptualization, methodology, and investigation of limitation in pathways, writing parts of original draft. **Guido Jach**: Conceptualization, and methodology in pathways optimization, supervision, writing of manuscript, review & editing. **Frederik Faust**: Conceptualization, and performing of modeling, writing parts of original draft. **Lea Krantz**: Conceptualization, and performing of modeling, writing parts of original draft. **Ljubov Tkatschuk**: Conceptualization, and performing of modeling, writing parts of original draft. **Dirk Holtmann**: conceptualization, methodology, supervision of the complete work, writing, editing.

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