Production of the sesquiterpene bisabolene from one-carbon compounds in an engineered *Methanosarcina acetivorans*

Andrea Mentrup, Luca V. Scheitz, Theo Wallenfang[1] and Michael Rother (ORCID ID 0000-0002-2306-8783)[#]

Fakultät Biologie, Technische Universität Dresden, Dresden, Germany

1: present address: Max Delbrück Center for Molecular Medicine, Berlin, Germany

#Address correspondence to Michael Rother, michael.rother@tu-dresden.de

Corresponding Author: Michael Rother
Fakultät Biologie
Technische Universität Dresden
01062 Dresden, Germany
Phone: ++49-351-46342611
Fax: ++49-351-46337715
e-mail: michael.rother@tu-dresden.de

Keywords: *Methanosarcina*, isoprenoids, bisabolene, mevalonate pathway, engineering

Running Head: Bisabolene production in *Methanosarcina acetivorans*

Abstract

The isoprenoid bisabolene, one of the simplest monocyclic sesquiterpenes, is a natural plant product that, in addition to its biological function, serves as a precursor for many industrial products. Due to the low concentration of bisabolene and the long harvest cycle, industrial production of this isoprenoid in plants is economically challenging. Chemical synthesis of bisabolene also suffers from significant disadvantages, such as low yields, toxic side products and high costs. Archaea appear suitable producers of isoprenoids, as their membrane lipids consist of isoprenoid ethers, which are synthesized via a variant of the mevalonate pathway. Archaeal model species have versatile metabolic capacities, which makes them suitable candidates for biotechnological applications. Here, we engineered *Methanosarcina acetivorans* for production of α-bisabolene from one-carbon substrates by introducing a bisabolene synthase from *Abies grandis*. Expression of a codon-optimized bisabolene synthase gene in a *M. acetivorans* wild-type strain resulted in 10.6 mg bisabolene/ liter of culture. Overexpressing genes of the mevalonate pathway only slightly increased bisabolene yields, which, however, were reached much earlier during incubations than in the corresponding wild-type strain. The data presented argue for the suitability of *M. acetivorans* for the biotechnical production of certain isoprenoids.

Introduction

Isoprenoids are natural products composed of C5-isoprene units and are among the most diverse compounds synthesized by biological systems. With more than 35,000 known isoprenoids, these compounds vary in size, structure, and biological function. They play essential roles in various cellular processes, including regulation of gene expression, energy conservation, cell membrane structure, signaling, cell defense, and as vitamins (Chandran et al., 2011). Beyond their biological functions, isoprenoids have numerous biotechnological applications, as biofuels, insecticides, pharmaceuticals, colorants, flavorings, fragrances, and precursors for semisynthetic approaches (Lei et al., 2022). Depending on the type of substance required, there are various strategies for meeting the increasing demand for economically relevant isoprenoids. The first option is their extraction from plant material, which, however, faces various challenges. On the one hand, the isolation of isoprenoids produced by plants often requires substantial energy and resource consumption. On the other hand, the quality of the product fluctuates due to different environmental conditions (Immethun et al., 2013). Alternatively, isoprenoids can be produced by chemical synthesis from petrochemical raw materials. Beside the problem of finiteness of the resource, the complexity and thus the yield pose a problem. It also requires significant amounts of energy while producing toxic by-products (Chemler et al., 2006; Winter and Tang 2012). Thus, biotechnical approaches represent favorable tracks for the production of isoprenoids. Microorganisms exhibit rapid growth and can utilize inexpensive carbon sources to produce products without the generation of toxic by-products, thereby offering a cost-effective and environmentally benign production process (Chemler et al., 2006; Ajikumar et al., 2008).

Bisabolene is one of the simplest monocyclic sesquiterpenes (Souček et al., 1961) and is characterized by a remarkable diversity of applications in various industrial branches. Despite its low concentration in plants, which makes economically viable isolation from plant sources difficult, bisabolene has considerable industrial potential (Zhao et al., 2021). This low

availability in nature means that alternative production methods, such as biotechnological approaches, are becoming increasingly important. In the cosmetics and perfume industry, bisabolene is employed as a fragrance and flavoring agent due to its pleasant and balsamic-woody aroma (Wang et al., 2017). Another, particularly innovative, application of bisabolene is as a starting material for the biosynthesis of bisabolane, a potential alternative fuel to D2 diesel. This application illustrates the potential of bisabolene to contribute to the development of sustainable and renewable energy sources (Peralta-Yahya et al., 2011).

The MVA (mevalonate) and MEP (methylerythritol phosphate) pathways are two biochemical pathways for the synthesis of isoprenoids, which occur in many organisms. The MEP pathway (Zhao et al., 2013) is used by many bacteria, plants and algae to synthesize the isoprenoid precursor isopentenyl diphosphate (IPP) and its isomer dimethylallyl diphosphate (DMAPP). On the other hand, the MVA pathway is the classical pathway for the synthesis of isoprenoids in archaea, eukaryotes and some bacteria. The canonical eukaryotic MVA pathway's crucial steps include the enzyme acetoacetyl-CoA thiolase catalyzing the condensation of two acetyl-CoA molecules to form acetoacetyl-CoA. Subsequently, HMG-CoA synthase combines two acetoacetyl-CoA molecules to produce 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA), which is then reduced to mevalonate by HMG-CoA reductase (Miziorko 2011). Mevalonate is phosphorylated to mevalonate-5-diphosphate by mevalonate kinase. Mevalonate-5-diphosphate is decarboxylated to isopentenyl pyrophosphate (IPP) by mevalonate diphosphate decarboxylase. IPP, the principal isoprenoid building block, is isomerized to DMAPP. Both IPP and DMAPP serve as precursors for synthesizing more complex isoprenoids. Studies on *Methanocaldococcus jannaschii* and *Aeropyrum pernix* established modified versions of this pathway in the Archaea (Fig. 1) (Grochowski et al., 2006; Komeyama et al., 2023). Within this pathway the three enzymes phosphomevalonate dehydratase, anhydromevalonate phosphate decarboxylase, and isopentenyl phosphate kinase are used to catalyze the dehydration of mevalonate-5-phosphate to *trans*-anhydromevalonate 5-

92 phosphate, and its decarboxylation to isopentenyl phosphate, which is then phosphorylated to
93 IPP.

94 *Methanosarcina acetivorans* belongs to the methanogenic archaea, the only organisms
95 that produce methane (CH_4) as means of their energy metabolism. These archaea are essential
96 for the complete remineralization of organic material in oxygen-depleted environments
97 (Whitman et al., 2006). Species of the genus *Methanosarcina* are characterized by their
98 metabolic versatility. They can utilize a variety of one- and two-carbon substrates for growth,
99 including methanol, methylamines, methyl sulfides, acetate and carbon monoxide (CO), to
100 produce methane (Schöne and Rother 2018).The latter capacity might be a particular advantage
101 that makes *M. acetivorans* a good candidate for isoprenoid production because large amounts
102 of acetate are formed during growth on CO (Rother and Metcalf 2004). Since the isoprenoid
103 precursors IPP and DMAPP and acetate are generated from acetyl-CoA, catabolic levels of the
104 starting material should be available in the cell. Furthermore, archaea do not employ the MVA
105 pathway for the formation of secondary metabolites, but for the synthesis of their membrane
106 lipid precursors (Jain et al., 2014), requiring a comparably high turnover. Thus, the prospect of
107 *M. acetivorans* being able to produce IPP not only in trace amounts makes it a promising
108 candidate for isoprenoid production. Indeed, this organism produced up to 850 mg/L of isoprene
109 when isoprene synthase was heterologously synthesized (Aldridge et al., 2021).

110 Despite the fact that the biochemical and bioenergetic aspects of methanogenic energy
111 metabolism are well understood and that sophisticated tools for their genetic manipulation are
112 available for some model methanogens, their industrial application is currently limited to their
113 ability to produce methane, either in anaerobic digestion, or employing power-to-gas concepts
114 (Angenent et al., 2018; Enzmann et al., 2018; Contreras et al., 2022). In order to expand the
115 biotechnological utility of *M. acetivorans*, production of α-bisabolene in recombinant strains
116 was studied using different energy substrates and cultivation regimes.

117 **Materials and Methods**

118 *Strains, growth, and transformation*

119 *Escherichia coli* strains DH10B (Grant et al., 1990) and WM1788 (for propagating R6K
120 replicons) (Haldimann and Wanner 2001) were grown and transformed employing standard
121 conditions (Ausubel et al., 2003). *Methanosarcina acetivorans* strains, listed in Table 1, were
122 cultivated in high salt (HS) medium (Metcalf et al., 1996) containing trace elements and
123 vitamins (Sowers and Noll 1995). Either 26 mL glass (Balch) tubes (Ochs, Bovenden,
124 Germany) containing 10 mL or 5 mL of medium, or 100 ml serum bottles (Ochs) containing 50
125 mL of medium were used. 125 mmol/L methanol (Sigma-Aldrich, Munich, Germany) plus 40
126 mmol/L sodium acetate (Carl Roth, Karlsruhe, Germany), supplemented from anaerobic, sterile
127 stock, or 150 kPa carbon monoxide (CO) (Air Liquide, Düsseldorf, Germany), added through
128 sterile filters, served as energy substrates. For induction of the tetracycline-inducible promoter,
129 cultures were transferred two or three times into media containing 100 µmol/L tetracycline (tet)
130 (Carl Roth). Growth was monitored photometrically at 578 nm (OD_{578}) either directly (i.e.,
131 undiluted) in Balch tubes using a Genesys 20 spectrophotometer (Thermo Scientific,
132 Langenselbold, Germany) or using an Ultrospec 2000 spectrophotometer (Pharmacia Biotech,
133 Uppsala, Sweden) for measurements of diluted culture samples. In the presence of different
134 isoprenoids growth of *M. acetivorans* was monitored nephelometrically using a cell growth
135 quantifier (CGQ) (Aquila Biolabs GmbH, Baesweiler, Germany) (Bruder et al., 2016) in serum
136 bottles with 50 mL media as described (Richter et al., 2024). Bisabolene producing
137 *M. acetivorans* were cultivated overlaid with dodecane (10% v/v, Sigma-Aldrich) to extract the
138 isoprenoid from the medium.

139 *Methanosarcina acetivorans* was transformed as described (Oelgeschläger and Rother
140 2009). Markerless deletion of *ssu*, MA2965, or MA3852, concomitant with inserting *trans*-
141 genes was conducted as described for *hpt* (Pritchett et al., 2004).

142

143 *Generation of plasmids*

144 Plasmids used in this study are listed in Table 2. DNA fragments deriving from PCR
145 (oligonucleotides used are listed in Table 3) and used for cloning were sequenced by
146 Microsynth Seqlab using the BigDye Terminator Cycle Sequencing protocol.

147 Isoprenoid synthases to be produced in *M. acetivorans* were identified through a review
148 of the literature. The retrieved genes, trimmed of plastid localization signals, were codon-
149 optimized for *M. acetivorans* using Geneious Prime (v. 2019.2.3 Biomatters Ltd., Auckland,
150 New Zealand) using a custom codon usage table and subsequently optimized manually, to a
151 codon adaptation index of 0.6-0.8. The fusion of the tet-inducible promoter PmcrB(tetO1)
152 (Guss et al., 2008) and the respective isoprenoid synthase gene was obtained commercially. The
153 synthesis products were then moved to the *M. acetivorans/E. coli* shuttle vector pMR08
154 (Oelgeschläger and Rother 2009) via SphI and SpeI. In the case of AgBIS (Table 2), the
155 synthesized plasmid was first used as a template for PCR amplification with the primers
156 o_SphI_pmcrB_tetO1_for and o_SpeI_HindIII_AgBIS_rev (Table 3) to remove the His_6-tag
157 encoding sequence, before moving the fusion to pMR08.

158 The ten genes encoding mevalonate pathway enzymes from *M. mazei* were divided into three
159 modules, in order to facilitate cloning and to identify potential bottlenecks of carbon flow
160 through the pathway. Module MVA I consists of the genes MM_0870, MM_0871 and
161 MM_0335. MVA II consists of the genes MM_1526, MM_1525, MM_1524 and MM_1871.
162 MVA III consists of the genes MM_1762, MM_1763, MM_1764 (Fig. 1). In the case of MVA
163 I and MVA II, all genes were combined into a synthetic operon and the fusion product was
164 synthesized fused to a tet-inducible promoter. The synthesis product was then moved to pMssu
165 in the case of MVA I, and to the plasmid pMA_3852 in the case of MVA II via NotI. Since the
166 genes assembled in the MVA III module are present in a natural operon of *M. mazei*, the entire
167 operon was amplified by PCR from the *M. mazei** genome (Ehlers et al., 2005) using the
168 primers o_NdeI_Operon_MM1762_1764_for and o_Operon_MM1762_1764_BamHI_rev

169 (Table 3). The resulting PCR product was cloned into plasmid pJK028a via NdeI and BamHI,
170 thereby creating a PmcrB(tetO3) fusion. The fusion was PCR amplified using the primers
171 o_NotI_pmcrB_tetO3_Operon_MM1762_64_for and
172 o_pmcrB_tetO3_Operon_MM1762_64_NotI_rev, and the resulting PCR product was cloned
173 into plasmid pMA2965 via NotI.
174
175 *Analysis of bisabolene*
176 For the determination of bisabolene, the dodecane phase of the respective culture was removed
177 and centrifuged at 10,000 g for 5 min. 200 µL of the organic phase was transferred to an air-
178 tight vial and 2 µl was injected into a GC-2010 Plus gas chromatograph (Shimadzu Europa
179 GmbH, Duisburg, Germany) operated with nitrogen as the carrier gas at an inlet pressure of
180 124.6 kPa, and equipped with an Optima 5 column (Macherey Nagel, Dueren, Germany). The
181 temperature of the GC oven was initially maintained at 60 °C for 3 min and then increased to
182 280 °C at a rate of 12 °C per minute. This temperature was then held for 5 minutes. The injector
183 was held at 300°C and the temperature of the flame ionization detector was also set at 300°C.
184 Data were recorded and analyzed using lab solution software version 5.98 (Shimadzu Europa
185 GmbH, Duisburg, Germany). A bisabolene isomer mixture (Thermo Fisher scientific,
186 Darmstadt, Germany) was used to generate a calibration curve.
187

188 **Results and Discussion**

189 *Production of different monoterpenes in M. acetivorans*

190 In a previous study, *M. acetivorans* was genetically amended by introducing the gene for
191 isoprene synthase from *Populus alba*, which resulted in a strain producing the hemiterpene
192 isoprene (Aldridge et al., 2021). We wanted to extend on this finding and genetically engineer
193 *M. acetivorans* to synthesize larger isoprenoids. Through a review of the literature on
194 isoprenoid synthases from plants or bacteria for synthesis in *M. acetivorans*, we chose the
195 monoterpenes α-pinene, limonene, and linalool to be produced via α-pinene synthase from
196 *Pinus taeda*, limonene synthase from *Mentha spicata*, and linalool synthase from *Streptomyces*
197 *clavuligerus*, respectively. The codon usage of the *trans*-genes was adapted to that of
198 *M. acetivorans*, and the corresponding gene was fused with to a well-established strong
199 promoter established inducible by tet (Guss et al., 2008). The gene fusion was placed onto an
200 autonomously replicating vector, and the resulting plasmid was subsequently transferred into
201 *M. acetivorans* WWM73, used as a "wild type" in this study. Transformants obtained were
202 analyzed for the presence of the respective isoprenoids. However, none of the isoprenoids in
203 question could be detected, regardless whether gene expression had been induced by tet, or not.
204 Also, extraction of the isoprenoids from the medium by growing the cultures in the presence of
205 an overlay of paraffin oil or dodecane (10%, v/v), which should also have a concentrating effect,
206 was attempted. However, in none of the cultures could the respective isoprenoid be detected.

207 The exact reason why none of the monoterpenes was produced was not thoroughly
208 investigated and, thus, remains unclear. Still, considering the antimicrobial property of
209 isoprenoids (Wang et al., 2019), together with a certain "leakiness" of the inducible promoter
210 employed (Guss et al., 2008), it is possible that the producing phenotype was selected against
211 and quickly lost. To gather some general evidence for this notion, the antimicrobial activity of
212 several isoprenoids on *M. acetivorans* was investigated. To this end, different isoprenoids, α-
213 pinene, linalool, geraniol, terpineol, borneol, and bisabolene, were added to the medium at

214 varying concentrations (0.1 mmol/L, 0.5 mmol/L, 1 mmol/L, and 2.5 mmol/L) and growth of
215 *M. acetivorans* on methanol plus acetate was assessed by means of CGQ measurements (see
216 Materials and Methods, (Fig. 2). α-pinene completely inhibited growth of *M. acetivorans*
217 already at 0.5 mmol/L (Fig. 2A), indicating a profound antimicrobial effect on the organism. A
218 similar effect was observed using 0.5 mmol/L geraniol, with only minimal cell proliferation
219 detectable (Fig. 2B). Linalool (Fig. 2C), terpineol (Fig. 2D), and borneol (Fig. 2F) required 1
220 mmol/L to be present for the same effect. Notably, bisabolene exhibited the least severe
221 antimicrobial effect on *M. acetivorans*. In the presence of 2.5 mmol/L, *M. acetivorans* still
222 grew, albeit at a slower rate than without it (Fig. 2E). Thus, all six isoprenoids tested had a
223 negative impact on the growth of *M. acetivorans*, with bisabolene exerting the least pronounced.
224 Whether this effect is (partly) due to the solubility of the respective isoprenoid, was not
225 investigated here.

226 Inspired by these results, we decided to introduce a bisabolene synthase into
227 *M. acetivorans* to produce this sesquiterpene as a proof of principle for a more complex
228 isoprenoid. After introduction of an episomal vector containing the gene for α-bisabolene
229 synthase from *Abies grandis* (and lacking the N-terminal signal peptide) under the control of a
230 tet-inducible promoter into *M. acetivorans*, the resulting strain WWM73/pAgBIS was
231 cultivated with or without tet induction. On methanol plus acetate, 0.5 ± 0.2 mg/L culture
232 bisabolene was obtained (Fig. 3). Cultivation on CO led to a notable increase in bisabolene
233 production, reaching 1.7 ± 0.6 mg/L (Fig. 3). The difference between the two C-sources might
234 be due to the fact that *M. acetivorans* produces acetyl-CoA only for anabolic purposes on
235 methanol plus acetate, but as a catabolic intermediate when growing on CO (Schöne et al.,
236 2022). Our data indicated the suitability of *M. acetivorans* as production host for the isoprenoid
237 bisabolene. Still, the amounts of bisabolene produced were not competitive to known bacterial
238 production systems. Thus, we aimed to optimize the strain and the cultivation procedure.
239

240 *Increasing bisabolene production in M. acetivorans*

241 First, we were interested in optimizing induction and growth conditions of WWM73/pAgBIS.
242 After transferring the WWM73/pAgBIS strain twice into tet-containing media maximum
243 bisabolene yield was achieved, as was previously shown for a PmcrB(tetO1)-*uidA* fusion (Guss
244 et al., 2008). Thus, this procedure was adopted for all subsequent experiments. Next, the
245 question was addressed if growth and bisabolene production are coupled, as many
246 biotechnological systems rely on separating growth phase and production phase (Klamt et al.,
247 2018). To this end, tet-induced WWM73/pAgBIS was grown on methanol plus acetate to
248 stationary phase and further incubated (Fig. 4). Since each culture had to be sacrificed for each
249 measurement, due to sample preparation from the dodecane overlay, each time point is
250 represented by three independent cultures. Bisabolene production continued after the culture
251 reached stationary phase and increased even further, when methanol and acetate was fed (Fig.
252 4A, arrows). Employing this "fed-batch" approach, bisabolene yields could be increased around
253 sixfold (compared to the beginning of the stationary phase, day 4, Fig. 4A) to 10.6 ± 0.6 mg/L
254 after 23 days, when the experiment was terminated. During growth on CO WWM73/pAgBIS
255 produced a maximum of 7.5 ± 2.8 mg/L bisabolene, which was achieved already after 9 days
256 (Fig. 4B), a concentration reached only after 19 days when methanol plus acetate was the energy
257 source (Fig. 4A). Addition of CO led to considerable cell lysis, probably due to the acetate and
258 formate produced (Rother and Metcalf 2004) and had, therefore, no beneficial effect on
259 bisabolene production in *M. acetivorans*. Comparing the yield of bisabolene obtained here with
260 published values, the *M. acetivorans* "wild type" produces from methanol plus acetate approx.
261 20 times more than a non-optimized *Yarrowia lipolytica* wild-type strain (carrying a bisabolene
262 synthase gene) produces (from waste cooking oil) (Zhao et al., 2021) and about twice as much
263 as a *Synechocystis* wild-type strain (carrying a bisabolene synthase gene) produces (from CO_2)
264 (Rodrigues and Lindberg 2021).

265 The experiments conducted thus far do not suggest that the cellular level of AgBIS might
266 be limiting the bisabolene yield. Conversely, both the difference in bisabolene yield (Fig. 3)
267 and in rate of production (Fig. 4) suggests that the cellular level of acetyl-CoA can become rate-
268 limiting depending on the growth substrate. The availability of the isoprenoid precursors IPP
269 and DMAPP, i. e., the flux of intermediates through the MVA pathway, seemed another
270 attractive avenue to further increase bisabolene production. We therefore aimed to overproduce
271 enzymes of the MVA pathway in *M. acetivorans*. To minimize unwanted recombination events,
272 the genes from the closely related *M. mazei* Gö1 were used for this purpose. The complete
273 MVA pathway comprises nine enzymes, which require a total of ten genes for their production
274 (Fig. 1, MM_ numbers). To facilitate cloning, the metabolism was organized into three
275 "modules," and the coding genes were integrated markerlessly (Pritchett et al., 2004) into one
276 of three established chromosomal integration sites: *ssu* (MVA I), MA3852 (MVA II), and
277 MA2965 (MVA III) (Guss et al., 2005; Sattler et al., 2024). In order to regulate gene expression,
278 the “weakest” tet-inducible promoters, PmcrB(tetO3) or PmcrB(tetO4) (Guss et al., 2008) were
279 placed in front of each construct. The double-integrated strains MVA12, MVA13, and MVA23,
280 as well as the triple-integrated strain MVA231, were generated analogously by re-using the
281 integration constructs after their segregation. All double- or triple-integrated strains containing
282 module III carried a single mutation within gene MM_1763 ($D_{31}N$), which, however, was
283 predicted to not interfere with the activity of the corresponding gene product. Subsequently, the
284 self-replicating plasmid pAgBIS was introduced into these MVA background strains. After the
285 generation of the strains overexpressing enzymes of the MVA pathway, their bisabolene
286 production was determined under tet-induced conditions in cultures grown on methanol plus
287 acetate. None of the strains produced bisabolene to significantly increased concentrations
288 compared to the “wild type” (Fig. 5). Only the strain with all enzymes of the MVA pathway
289 from *M. mazei* (MVA231) with 3.2 +/- 0.6 mg/L showed a somewhat increased amount of
290 bisabolene if compared to the “wild-type” strain. In conclusion, the overproduction of the MVA

291 pathway had only a small, if any, positive effect on bisabolene production, which lends support
292 to the argument that the availability of IPP and DMAPP is not a critical bottleneck during
293 bisabolene synthesis under the experimental conditions employed. Since acetyl-CoA might
294 already be limiting flux through the MVA pathway with methanol plus acetate as substrate,
295 increasing the amounts of downstream enzymes would not have much of an effect. Another
296 potential reason for this observation could be a limitation in the conversion of IPP and DMAPP
297 to geranyl pyrophosphate (GPP) and/or farnesyl pyrophosphate (FPP), essential intermediates
298 for bisabolene synthesis (Fig. 1). To at least tentatively address this question, the MVA231
299 strain carrying pAgBIS was cultivated on CO in the presence of tet and the amount of
300 bisabolene determined. At the end of the strain's growth (day 7) it had produced 8.8 +/- 1.2
301 mg/L bisabolene (Fig. 6), which is nearly twofold more than the "wild-type" under the same
302 condition (compare to Fig. 4B), which argues for a beneficial effect of the MVA-encoding
303 *trans*-genes. Notably, the MVA231 strain carrying pAgBIS continued to produce bisabolene
304 (unlike the "wild type"), to a maximal concentration (without feeding CO again) of 10.1 ±1.1
305 mg/L. Possibly, in this strain carbon could flow better through the MVA pathway towards
306 bisabolene than towards acetate, leading to delayed cell lysis compared to the "wild type".
307 Additional re-feeding CO resulted in 12.2 ±2.2 mg/L, the highest bisabolene concentration
308 achieved (Fig. 6), and similar to that achieved on methanol plus acetate (Fig. 4). However, as it
309 took less than half the incubation time (11 versus 23 days, compare Fig. 4A and Fig. 6),
310 increasing the cellular level of acetyl-CoA (via growth on CO) and of MVA pathway enzymes
311 (via expressing *M. mazei* genes), substantially improved bisabolene production in
312 *M. acetivorans*.

313 In summary, the optimized *M. acetivorans* strain MVA231 on CO produced the highest
314 amount of bisabolene in the shortest time under the experimental culture conditions used, with
315 about 12 mg bisabolene/L culture. *M. acetivorans* in terms of titer still cannot compete with
316 other highly engineered microorganisms such as *Y. lipolytica* (>280 mg bisabolene/L culture)

317 (Zhao et al., 2021) or *E. coli* (>900 mg bisabolene/L culture) (Peralta-Yahya et al., 2011). Still,
318 the fact that our engineered host can use a waste stream gas (e. g., off-stream gas of the steel
319 industry) instead of complex, and expensive, organic compounds (like fats and sugar), not only
320 promises a higher value added due to lower cost of the feedstock, but also a (small) step towards
321 a more circular, and, therefore, more sustainable biotechnological application.
322

323 **Acknowledgments**

324 This work was supported by a grant from the Bundesministerium für Bildung und Forschung
325 via the MethanoPEP project to M.R. (grant no 031B0851A). The funders had no role in study
326 design, data collection and interpretation, or the decision to submit the work for publication.
327

328 **Author contribution**

329 M.R. conceived and supervised the study, A.M. and M.R. designed experiments, A.M., L.V.S.,
330 and T.W. acquired the data, all authors analyzed and interpreted the data, A.M. drafted the
331 manuscript, all authors revised the manuscript.
332

333 **References**

334 Ajikumar, P.K., Tyo, K., Carlsen, S., Mucha, O., Phon, T.H., and Stephanopoulos, G. (2008)
335 Terpenoids: Opportunities for biosynthesis of natural product drugs using engineered
336 microorganisms. *Mol Pharmaceutics* **5**: 167-190.
337 Aldridge, J., Carr, S., Weber, K.A., and Buan, N.R. (2021) Anaerobic production of isoprene
338 by engineered *Methanosarcina* spp. archaea. *Appl Environ Microbiol* **87**: e02417-02420.
339 Angenent, L.T., Usack, J.G., Xu, J., Hafenbradl, D., Posmanik, R., and Tester, J.W. (2018)
340 Integrating electrochemical, biological, physical, and thermochemical process units to expand
341 the applicability of anaerobic digestion. *Bioresour Technol* **247**: 1085-1094.

342 Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidmann, J.G., Smith, J.A., and Struhl,
343 K., Eds. (2003). Current Protocols in Molecular Biology. New York, J. Wiley & Sons, Inc.
344 Bruder, S., Reifenrath, M., Thomik, T., Boles, E., and Herzog, K. (2016) Parallelised online
345 biomass monitoring in shake flasks enables efficient strain and carbon source dependent growth
346 characterisation of *Saccharomyces cerevisiae*. *Microb Cell Fact* **15**: 127.
347 Chandran, S.S., Kealey, J.T., and Reeves, C.D. (2011) Microbial production of isoprenoids.
348 *Process Biochem* **46**: 1703-1710.
349 Chemler, J.A., Yan, Y., and Koffas, M.A.G. (2006) Biosynthesis of isoprenoids,
350 polyunsaturated fatty acids and flavonoids in *Saccharomyces cerevisiae*. *Microb Cell Fact* **5**:
351 20.
352 Contreras, G., Thomsen, J., Pfitzer, M., Hafenbradl, D., Kostner, D., Holtmann, D., Schmitz,
353 R.A., Rother, M., and Molitor, B. (2022) New perspectives for biotechnological applications of
354 methanogens. *Curr Res Biotechnol* **4**: 468-474.
355 Ehlers, C., Weidenbach, K., Veit, K., Deppenmeier, U., Metcalf, W.W., and Schmitz, R.A.
356 (2005) Development of genetic methods and construction of a chromosomal *glnK*(1) mutant in
357 *Methanosarcina mazei* strain Gö1. *Mol Genet Genomics* **273**: 290-298.
358 Enzmann, F., Mayer, F., Rother, M., and Holtmann, D. (2018) Methanogens: biochemical
359 background and biotechnological applications. *AMB Express* **8**: 1.
360 Grant, S.G., Jessee, J., Bloom, F.R., and Hanahan, D. (1990) Differential plasmid rescue from
361 transgenic mouse DNAs into *Escherichia coli* methylation-restriction mutants. *Proc Natl Acad*
362 *Sci USA* **87**: 4645-4649.
363 Grochowski, L.L., Xu, H., and White, R.H. (2006) *Methanocaldococcus jannaschii* uses a
364 modified mevalonate pathway for biosynthesis of isopentenyl diphosphate. *J Bacteriol* **188**:
365 3192-3198.
366 Guss, A.M., Mukhopadhyay, B., Zhang, J.K., and Metcalf, W.W. (2005) Genetic analysis of
367 *mch* mutants in two *Methanosarcina* species demonstrates multiple roles for the methanopterin-

368 dependent C-1 oxidation/reduction pathway and differences in H_2 metabolism between closely
369 related species. *Mol Microbiol* **55**: 1671-1680.
370 Guss, A.M., Rother, M., Zhang, J.K., Kulkarni, G., and Metcalf, W.W. (2008) New methods
371 for tightly regulated gene expression and highly efficient chromosomal integration of cloned
372 genes for *Methanosarcina* species. *Archaea* **2**: 193-203.
373 Haldimann, A., and Wanner, B.L. (2001) Conditional-replication, integration, excision, and
374 retrieval plasmid-host systems for gene structure-function studies of bacteria. *J Bacteriol* **183**:
375 6384-6393.
376 Immethun, C.M., Hoynes-O'Connor, A.G., Balassy, A., and Moon, T.S. (2013) Microbial
377 production of isoprenoids enabled by synthetic biology. *Front Microbiol* **4**: 75.
378 Jain, S., Caforio, A., and Driessen, A.J.M. (2014) Biosynthesis of archaeal membrane ether
379 lipids. *Front Microbiol* **5**: 641.
380 Klamt, S., Mahadevan, R., and Hädicke, O. (2018) When do two-stage processes outperform
381 one-stage processes? *Biotechnol J* **13**: 1700539.
382 Komeyama, M., Kanno, K., Mino, H., Yasuno, Y., Shinada, T., Ito, T., and Hemmi, H. (2023)
383 A [4Fe-4S] cluster resides at the active center of phosphomevalonate dehydratase, a key enzyme
384 in the archaeal modified mevalonate pathway. *Front Microbiol* **14**: 1150353.
385 Lei, L., Wang, X.J., and Tang, S.C. (2022) Novel taxanes in development: Hopes or hypes?
386 *Crit Rev Oncol Hematol* **176**: 103727.
387 Metcalf, W.W., Zhang, J.K., Apolinario, E., Sowers, K.R., and Wolfe, R.S. (1997) A genetic
388 system for Archaea of the genus *Methanosarcina*: liposome-mediated transformation and
389 construction of shuttle vectors. *Proc Natl Acad Sci USA* **94**: 2626-2631.
390 Metcalf, W.W., Zhang, J.K., Shi, X., and Wolfe, R.S. (1996) Molecular, genetic, and
391 biochemical characterization of the *serC* gene of *Methanosarcina barkeri* Fusaro. *J Bacteriol*
392 **178**: 5797-5802.

393 Oelgeschläger, E., and Rother, M. (2009) *In vivo* role of three fused corrinoid/methyl transfer
394 proteins in *Methanosarcina acetivorans*. *Mol Microbiol* **72**: 1260-1272.
395 Peralta-Yahya, P.P., Ouellet, M., Chan, R., Mukhopadhyay, A., Keasling, J.D., and Lee, T.S.
396 (2011) Identification and microbial production of a terpene-based advanced biofuel. *Nat*
397 *Commun* **2**: 483.
398 Pritchett, M.A., Zhang, J.K., and Metcalf, W.W. (2004) Development of a markerless genetic
399 exchange method for *Methanosarcina acetivorans* C2A and its use in construction of new
400 genetic tools for methanogenic archaea. *Appl Environ Microbiol* **70**: 1425-1433.
401 Richter, M., Sattler, C., Schöne, C., and Rother, M. (2024) Pyruvate-dependent growth of
402 *Methanosarcina acetivorans*. *J Bacteriol* **206**: e00363-00323.
403 Rodrigues, J.S., and Lindberg, P. (2021) Metabolic engineering of *Synechocystis* sp. PCC 6803
404 for improved bisabolene production. *Metab Eng Commun* **12**: e00159.
405 Rother, M., and Metcalf, W.W. (2004) Anaerobic growth of *Methanosarcina acetivorans* C2A
406 on carbon monoxide: An unusual way of life for a methanogenic archaeon. *Proc Natl Acad Sci*
407 *USA* **101**: 16929-16934.
408 Sattler, C., Richter, M., and Rother, M. (2024) Physiological barriers for glucose utilization in
409 *Methanosarcina acetivorans*. *bioRxiv*, doi: 10.1101/2024.08.21.609014.
410 Schöne, C., Poehlein, A., Jehmlich, N., Adlung, N., Daniel, R., von Bergen, M., Scheller, S.,
411 and Rother, M. (2022) Deconstructing *Methanosarcina acetivorans* into an acetogenic
412 archaeon. *Proc Natl Acad Sci USA* **119**: e2113853119.
413 Schöne, C., and Rother, M. (2018) Methanogenesis from carbon monoxide. *In* Biogenesis of
414 Hydrocarbons Handbook of Hydrocarbon and Lipid Microbiology. Stams A.J., and Sousa D.Z.
415 (Eds.). Springer, Cham, doi:10.1007/978-3-319-53114-4_4-1.
416 Souček, M., V., H., and Šorm, F. (1961) On terpenes. CXVIII. Constitution of parthenolide.
417 *Collect Czech Chem Commun* **26**: 803-810.

418 Sowers, K.R., and Noll, K.M. (1995) Techniques for anaerobic growth. *In* Methanogens.
419 Sowers K.R., and Schreier H.J. (Eds.). Cold Spring Harbor Laboratory Press, Plainview, NY.
420 Vol. 2, pp. 15-47.
421 Wang, C.-Y., Chen, Y.-W., and Hou, C.-Y. (2019) Antioxidant and antibacterial activity of
422 seven predominant terpenoids. *Int J Food Prop* **22**: 230-238.
423 Whitman, W.B., Bowen, T.L., and Boone, D.R. (2006) The Methanogenic Bacteria. *In* The
424 Prokaryotes-A Handbook on the Biology of Bacteria. Dworkin M. et al. (Eds.). Springer, New
425 York. Vol. 3, pp. 165–207.
426 Winter, J.M., and Tang, Y. (2012) Synthetic biological approaches to natural product
427 biosynthesis. *Curr Opin Biotechnol* **23**: 736-743.
428 Zhao, L., Chang, W.C., Xiao, Y., Liu, H.W., and Liu, P. (2013) Methylerythritol phosphate
429 pathway of isoprenoid biosynthesis. *Annu Rev Biochem* **82**: 497-530.
430 Zhao, Y., Zhu, K., Li, J., Zhao, Y., Li, S., Zhang, C., Xiao, D., and Yu, A. (2021) High-
431 efficiency production of bisabolene from waste cooking oil by metabolically engineered
432 *Yarrowia lipolytica*. *Microb Biotechnol* **14**: 2497-2513.

433

434 **Tables**

435 Table 1: *M. acetivorans* strains used in this study.

Strain	Genotype	Reference
WWM73	Δ*hpt*::PmcrB-*tetR*-ΦC31-int-attP	(Guss et al., 2008)
MVA I	Δ*ssu*::PmcrB(tetO1)-MM0870-MM0871-MM0335	This study
MVA II	ΔMA3852::PmcrB(tetO3)-MM1526-MM1525-MM1524-MM1871	This study
MVA III	ΔMA2965::PmcrB(tetO3)-MM1762-MM1763-MM1764	This study
MVA12	MVA I, ΔMA3852::PmcrB(tetO3)-MM1526-MM1525-MM1524-MM1871	This study
MVA13	MVA I, ΔMA2965::PmcrB(tetO3)-MM1762-MM1763($D_{31}N$)-MM1764	This study
MVA23	MVA II, ΔMA2965::PmcrB(tetO3)-MM1762-MM1763($D_{31}N$)-MM1764	This study
MVA231	MVA23, Δ*ssu*::PmcrB(tetO1)-MM0870-MM0871-MM0335	This study

436

437 Table 2: Plasmids used in this study[a].

Plasmid	Genotype and/or construction	Reference
pWM321	*M. acetivorans/E. coli* shuttle vector	(Metcalf et al., 1997)
pMR08	pWM321, PmcrB-*tetR*	(Oelgeschläger and Rother 2009)
pJK028a	ϕC31-*attB* vector (for genomic integration) with PmcrB(tetO3) promoter fusion to *uidA*	(Guss et al., 2008)
pMssu	Platform vector for chromosomal integration into the *ssu* locus (MA0063-MA0065)	(Sattler et al., 2024)
pMA2965	Platform vector for chromosomal integration into MA2965	(Sattler et al., 2024)
pMA3852	Platform vector for chromosomal integration into MA3852	(Sattler et al., 2024)
pUC57-BsaI-free	General cloning	Biocat GmbH, Heidelberg, Germany
pUC57_tetO1_Pt_APS	Codon optimized α-pinene synthase gene from *Pinus taeda* fused to PmcrB(tetO1) promoter	Biocat

pUC57_tetO1_Ms_LMS	Codon optimized limonene synthase gene of *Mentha spicata* fused to PmcrB(tetO1) promoter	Biocat
pUC57_tetO1_Sc_LIS	Codon optimized linalool synthase gene of *Streptomyces clavuligerus* fused to PmcrB(tetO1) promoter	Biocat
pUC57/tetO1_AgBIS_His_6	Codon optimized bisabolene synthase gene (with 3' His_6-tag encoded) of *Abies grandis* fused to PmcrB(tetO1) promoter	Biocat
pUC57_tetO4_MVA I	Artificial operon of the *M. mazei* genes MM_0335, MM_0870 and MM_0871 fused to PmcrB(tetO4) promoter	Biocat
pUC57_tetO3_MVA II	Artificial operon of the *M. mazei* genes MM_1526, MM_1525, MM_1524 and MM_1871 fused to PmcrB(tetO3) promoter	Biocat
pJK028a_MVA III	MM_1762, MM_1763 and MM_1764 from *M. mazei* (PCR amplified) fused to PmcrB(tetO3)	This study
pJK028a_MVA III(D_{31}N MM1763)	MM_1762, MM_1763 and MM_1764 from *M. mazei* (PCR amplified) fused to PmcrB(tetO3)	This study
pAPS	PmcrB(tetO1)-APS fusion (pUC57_tetO1_Pt_APS) moved to pMR08	This study
pLMS	PmcrB(tetO1)-LMS fusion (pUC57_tetO1_Ms_LMS) moved to pMR08	This study
pLIS	PmcrB(tetO1)-LIS fusion (pUC57_tetO1_Sc_LIS) moved to pMR08	This study
pAgBIS	PmcrB(tetO1)-AgBIS fusion PCR amplified, moved to pMR08	This study
pMssu_MVAI	PmcrB(tetO4)_MVA I fusion (pUC57_tetO4_MVA I)moved to pMssu	This study
pMA3852_MVA II	PmcrB(tetO3)_MVA II fusion pUC57_tetO3_MVA II) moved to pMA3852	This study
pMA2965_MVA III	PmcrB(tetO3)_MVA III fusion (pJK028a_MVA III) moved to pMA2965	This study

pMA2965_MVA III $D_{31}N$ MM1763	PmcrB(tetO3)_MVA III $D_{31}N$ MM1763 fusion (pJK028a_MVA III $D_{31}N$ MM1763) moved to pMA2965	This study

438 a: sequences are available upon request.

439

440

441

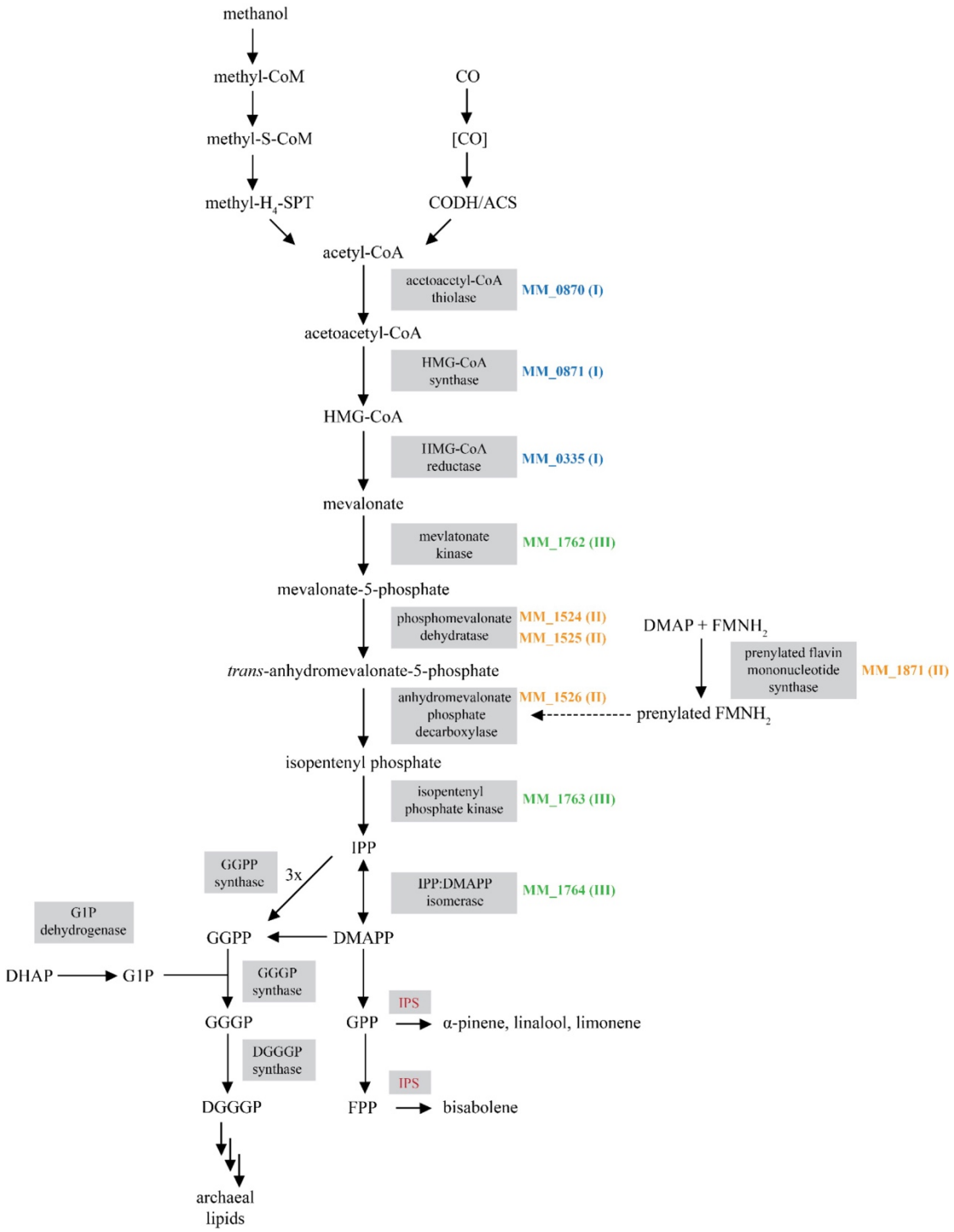
442 Table 3: Oligonucleotides used in this study.

Oligonucleotide	Sequence (5'→3')
o_SphI_pmcrB_tetO1_for	AAAGCATGCTTCATTTATCGGAGAACAC
o_SpeI_HindIII_AgBIS_rev	AAAACTAGTAAGCTTAAAGCGGAAGCG
o_NdeI_Operon_MM1762_1764_for	TTTCATATGGTTTCATGTTCTGCGC
o_Operon_MM1762_1764_BamHI_rev	AAAGGATCCTTAAAGAGCGTTCC
o_NotI_pmcrB_tetO3_Operon_MM1762_64_for	TTTGCGGCCGCGCATGCTTCATTTATCG
o_pmcrB_tetO3_Operon_MM1762_64_NotI_rev	TTTGCGGCCGCCTTAAAGAGCGTTCC

443

444

445 **Figures**



446 Figure 1: Scheme of the formation of archaeal membrane lipids composed of isoprene subunits
447 linked to glycerol-1-phosphate via ether bonds. The isoprene precursors IPP and DMAPP are
448 derived from acetyl-CoA (generated for anabolic purposes from methanol and during
449 catabolism from CO) via the mevalonate (MVA) pathway. The enzymes involved are boxed.
450 To enhance the endogenous MVA pathway, the corresponding genes from *M. mazei* (see MM_

numbers) were integrated into the genome of *M. acetivorans* in three modules (MVAI: blue numbers; MVAII: orange numbers; MVAIII: green numbers). CoA, coenzyme A; HMG, 3-hydroxy-3-methyl-glutaryl; DMAP, dimethylallyl phosphate; $FMNH_2$, reduced flavin mononucleotide; IPP, isopentenyl pyrophosphate; DMAPP, dimethylallyl pyrophosphate; GPP, geranyl pyrophosphate; GGPP, geranylgeranyl diphosphate; FPP, farnesyl pyrophosphate; DH1P, dihydroxyacetone phosphate; G1P, *sn*-glycerol-1-phosphate; GGGP, 3-*O*-geranylgeranyl-*sn*-glyceryl-1-phosphate; DGGGP, 2,3-bis-*O*-geranylgeranyl-*sn*-glyceryl-1-phosphate; IPS, isoprenoid synthase.

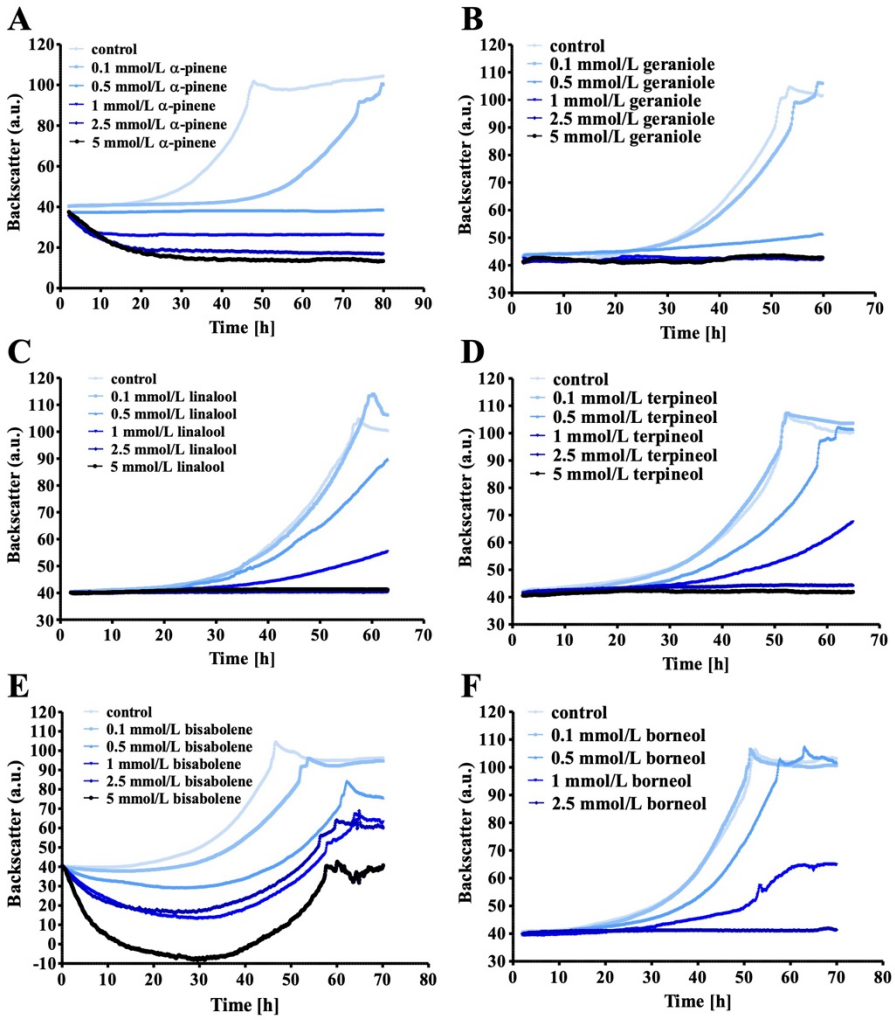
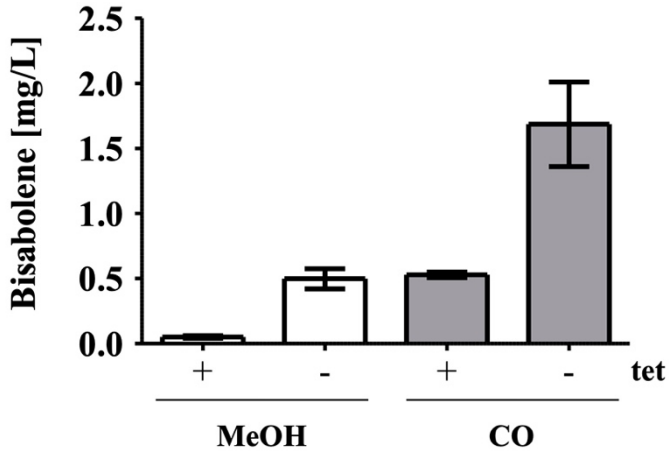


Figure 2: Growth of *M. acetivorans* in the presence of isoprenoids. Strain WWM73 cultivated on HS medium with methanol and acetate. Linalool (A), terpineol (B), geraniol (C), α-pinene (D), borneol (E) and bisabolene (F) were added in concentrations of 0.1 mmol/L, 0.5 mmol/L, 1 mmol/L or 2.5 mmol/L, respectively. A preculture was inoculated to an OD_{578} of 0.05 into

463 medium containing the different isoprenoids. The cultures were then incubated at 37 °C and
464 shaken at 130 rpm. Cell growth was measured nephelometrically at 515 nm using backscatter
465 measurement in the Cell Quantifier (from AquilaBiolabs). The diagrams indicate mean values
466 and their standard deviations (as error bars) from experiments performed in (at least) triplicates.

467



468 Figure 3: Bisabolene formation in *M. acetivorans* WWM73/pAgBIS. Gas chromatographic
469 analysis of the organic dodecane phase of cultures grown on methanol plus acetate or on CO,
470 for three days in the presence (+) or absence (-) of 100 µmol/L tet. The bars represent mean
471 values and their standard deviations (as error bars) from experiments performed in (at least)
472 triplicates.

473

474

475

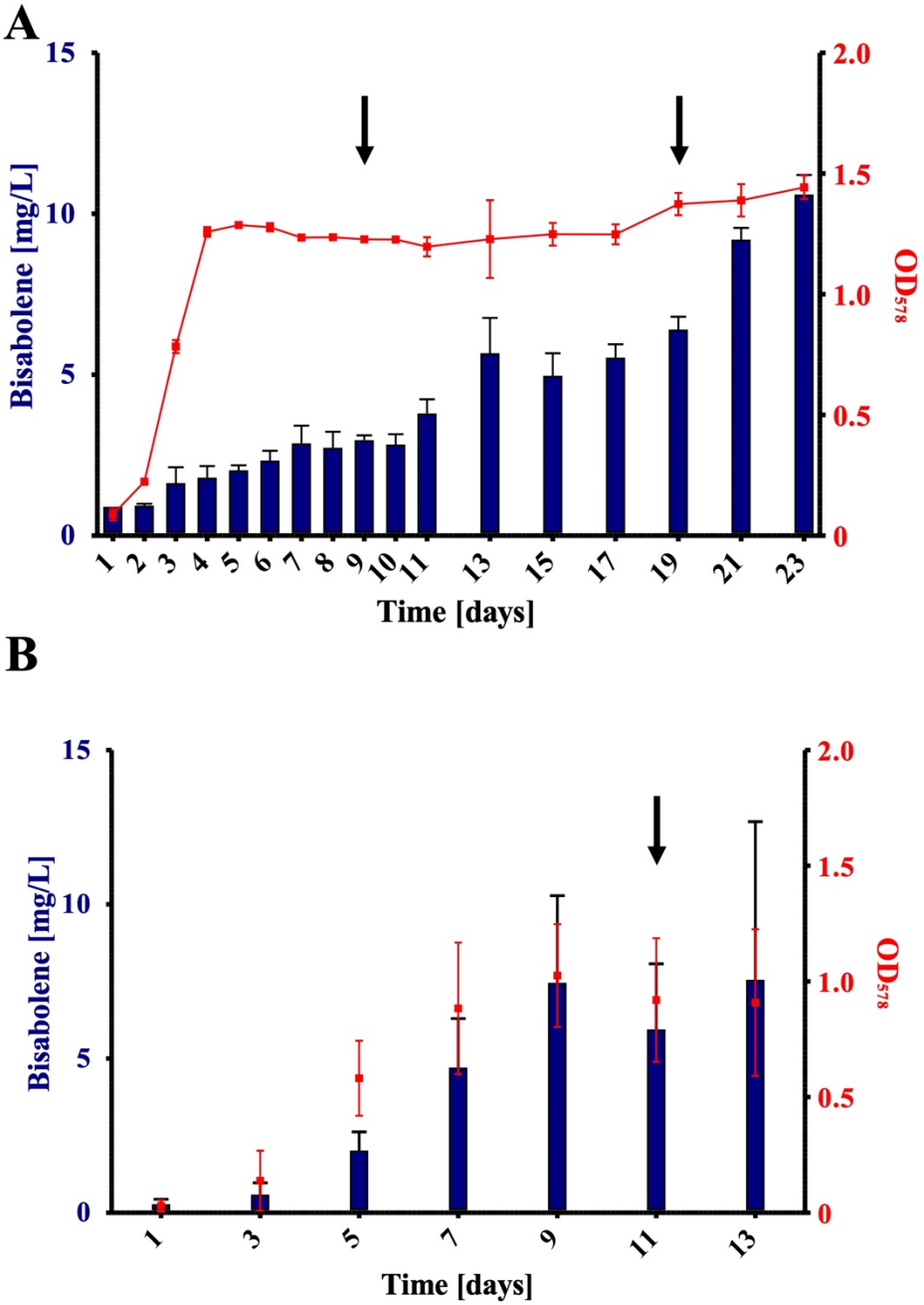
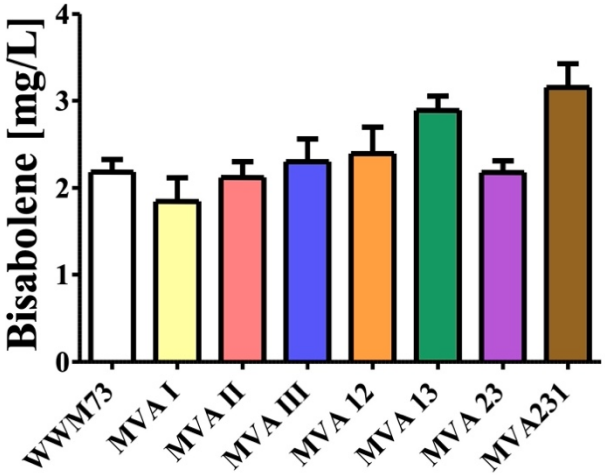
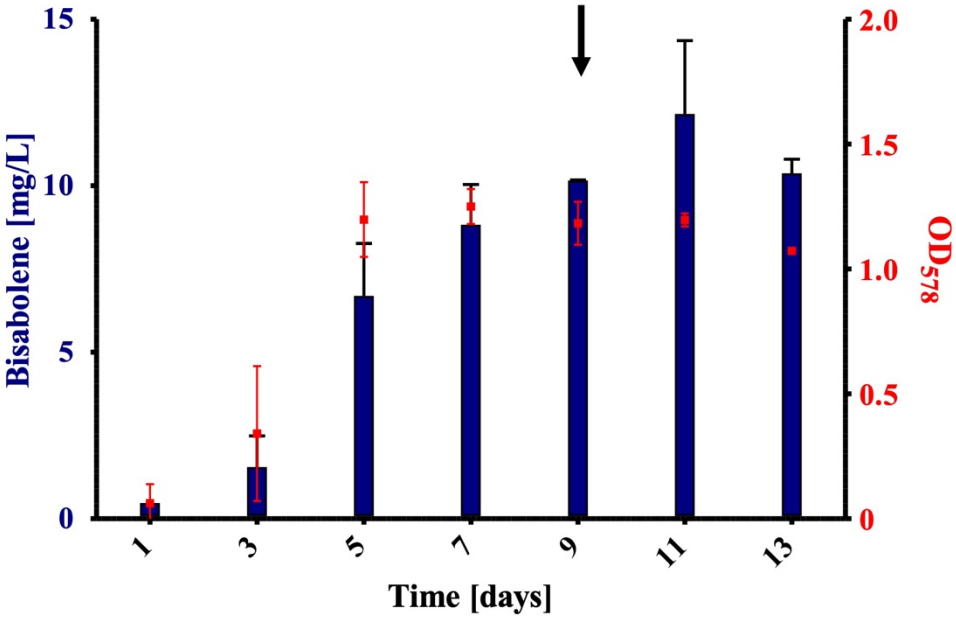


Figure 4: Correlation between cell mass and bisabolene yield. Cultures of *M. acetivorans* WWM73/pAgBIS were cultured on methanol plus acetate (A) or on CO (B) in the presence of 100 µM tet. Cultures were overlaid with dodecane (10% v/v) to extract the bisabolene from the medium. Bisabolene in the organic phase was quantified gas chromatographically (see Materials and Methods) (left y-axis). Growth (right y-axis) was determined photometrically at 578 nm (OD_{578}). The arrows indicate the addition of 125 mmol/L methanol and 40 mmol/L acetate (A) or 150 kPa CO (B). Shown are mean values and their standard deviations (as error bars) from experiments performed in triplicates.



484 Figure 5: Effect of *M. mazei* MVA pathway enzymes on bisabolene production in *M.*
485 *acetivorans*. Strains WWM73, MVA I, II, III, 12, 13, 23 and 231 carrying pAgBIS were
486 cultured in HS medium with methanol plus acetate in the presence of 100 µmol/L tet and
487 overlaid with dodecane (10% v/v)) for eight days. Bars represent mean values and their
488 standard deviations (as error bars) from experiments carried out in triplicates.



489 Figure 6: Correlation between cell mass and bisabolene yield for MVA231/pAgBIS. Cultures
490 of MVA231/pAgBIS were cultured with CO in the presence 100 µmol/L tet. Cultures were
491 overlaid with dodecane (10% v/v) to extract the bisabolene from the medium. Bisabolene in the
492 organic phase was quantified gas chromatographically (see Materials and Methods) (left y-
493 axis). Growth (right y-axis) was determined photometrically at 578 nm (OD_{578}). The arrow

494 indicates the addition 1.5 atm CO. Shown are mean values and their standard deviations (as

495 error bars) from experiments performed in triplicates.

496

497