1 One-pot bioconversion of algae biomass into terpenes for advanced biofuels and bioproducts

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Under robust algae growth conditions, algal carbohydrates and proteins typically comprise up to ~ 80% of the ash-free dry weight of microalgae biomass. Therefore, production of algal biofuel through comprehensive utilization of all algal components and the addition of high energy density fuel compounds with “fit for purpose” properties or high-value bioproducts will both diminish the process cost and improve the overall process feasibility. In this study, we firstly demonstrated the concept of a "one-pot" bioconversion of algal carbohydrate and protein into value-added terpene compounds as advanced biofuel and high value bioproducts to improve the overall process feasibility through the development of engineered microbial consortium. The consortium for caryophyllene production yielded the highest titer of total terpene, up to 507.4 mg/L, including 471 mg/L of sesquiterpene, 36.4 mg/L of monoterpene, and 124.4 mg/L of caryophyllene on algal hydrolysate from *Nannochloropsis sp.* Additionally, the consortium expressing chamigrene synthase produced 187 mg/L total terpene including 87 mg/L of monoterpene, 100 mg/L of sesquiterpene, and 62 mg/L chamigrene on hydrolysate from benthic polyculture biomass. Compared to the yields of terpene extracted from plant tissue, both consortia increased the terpene yield about 3~40 times, which makes it a promising alternative pathway for terpene production.

Key words: One-pot conversion, terpene, microbial consortium, algal biofuel, caryophyllene, chamigrene

55 Introduction

56 Rising demand for transportation fuels and the concerns with fossil fuel derived environmental
57 pollution as well as the green-house gas emission derived climate change have resulted in the
58 compelling need for alternative, sustainable energy sources(1). Algae-based biofuels have been
59 considered one of the promising alternatives to fossil fuels as they can overcome some of these issues
60 (2-4). The current state-of-the-art of algal biofuel technologies have primarily focused on biodiesel
61 production through prompting high algal lipid yields under the nutrient stress conditions. There has
62 been less emphasis on using algae-based carbohydrates and proteins as carbon sources for the
63 fermentative production of liquid fuel compounds or other high-value bioproducts(5-7).

64 Terpenes are a group of natural products with over 55,000 structurally similar chemical compounds.
65 Compared to biodiesel and other short- and medium-chain alcohols, these molecules contain near zero
66 oxygen content, have various biological functionalities (8-12) and have high energy density, making
67 them particularly attractive candidates as “drop-in” fuel candidates for aviation fuels(13-18). In this
68 study, we demonstrated the concept of "one-pot" bioconversion of algal carbohydrates and proteins
69 into terpenes as advanced biofuel compounds and the high value bioproducts (figure 1) through the
70 development of engineered microbial bioconversion consortium.

72 Figure 1.

73 Results and Discussion

74 Caryophyllene and chamigrene, natural bicyclic sesquiterpene (C15) compounds, are common
75 components present in the essential oils from various plants (19-22). A recent study suggested that the

76 blending of hydrogenated sesquiterpanes (in particular carophyllanes), which have a moderate cetane
77 number and only moderately high viscosity, with synthetic branched paraffins to raise cetane and
78 reduce viscosity, could produce biosynthetic fuels that meet applicable jet fuel and diesel
79 specifications(23). Therefore, caryophyllene and its isomers have been deemed to be among the top
80 three most promising candidates for jet fuel with high energy density (24). In our previous study, we
81 discovered and functionally characterized caryophyllene and chamigrene synthases from
82 endophytes(25). Furthermore, we demonstrated the feasibility of bioconversion of algal protein into
83 terpene through terpene biosynthesis reconstruction into mutant *E. coli* strain YH40. Based on the
84 previous studies, we developed a synthetic microbial consortium and investigated the production of
85 caryophyllene, chamigrene, and other terpene products in one-pot fermentation using algal hydrolysate
86 of microalgae monocultures from strain *Nannochloropsis sp* as well as natural benthic algal
87 assemblages cultivated from wastewater. To achieve this, the terpene biosynthesis pathway was
88 reconstructed into *E. coli* strain YH40(7), designated for the conversion of algal protein into
89 caryophyllene or chamigrene, and into *E. coli* strain DH1, designated for the conversion of algal
90 carbohydrate into caryophyllene or chamigrene, respectively, as described in previous studies(15). The
91 caryophyllene and chamigrene yields were investigated under three different combinations of
92 inoculum YH40-CI4A-CS/DH1-CI4A-CS at ratios of 2:1, 1:1, 0.5:1 as well as the single strains YH40-
93 CI4A-CS or DH1-CI4A-CS alone. As shown in figure 2 (B), when co-culture of the two strains
94 containing caryophyllene synthases were grown on algal hydrolysate from *Nannochloropsis sp*, at an
95 inoculum ratio 1:1 (consortia R1) the consortia produced the highest titer of total terpene, up to 507.4
96 mg/L, including 471 mg/L of sesquiterpene, 36.4 mg/L of monoterpene as well as 124.4 mg/L of
97 caryophyllene. Correspondingly, the consortia R1 consumed the highest amount of algal carbohydrates
98 and proteins, which accounted for 48.2% of total algal carbohydrates and 36% of total algal proteins in
99 the media, figure 2(C). Compared to the consortia R1, the consortia R2 and R0.5 consumed a
100 significantly lower fraction of the total algal biomass, with correspondingly lower concentrations of
101 terpenes. The strain YH40-CI4A-CS alone produced the least amount of total terpene (274.7 mg/L),
102 sesquiterpene (232.1 mg/L) and caryophyllene (14.4 mg/L) while DH1-CI4A-CS yielded 30% higher
103 sesquiterpene and total terpene than strain YH40-CI4A-CS as well as 4 times higher titer of
104 caryophyllene (75.2 mg/L). Compositional analysis of the *Nannochloropsis sp*. biomass indicated that
105 the biomass was 20% carbohydrates and 58% protein (data not shown). Based on this data, the highest
106 terpene yield that was achieved corresponded to ~42 mg total terpene/ g algae from consortia R0.5
107 with 37.4 mg sesquiterpene/ g algae and 6.6 mg caryophyllene/ g algae, as shown in figure 2(D).

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Figure 2.

For co-culture of the two engineered strains containing chamigrene on the hydrolysate of benthic algal assemblages, the experimental results showed that the terpene yield reached 187 mg/L total terpene at the 2:1 ratio (YH40-CI4A-CPS/DH1-CI4A-CPS), including 87 mg/L of monoterpene and 100 mg/L of sesquiterpene, and chamigrene was the major product accumulated up to 62 mg/L. The synthetic microbial consortia produced similar total terpene at the 1:1 and 0.5:1 ratios (YH40-CI4A-CPS/DH1-CI4A-CPS), which were ~150 mg/L of total terpene. The microbial consortium at ratio 1 yielded the highest concentration of sesquiterpene (113 mg/L) as well as chamigrene (80 mg/L) among

three consortia, while the monoterpene yield was the lowest (34.5 mg/L). The strains YH40-TS and DH1-TS alone produced only 26 and 43 mg/L of total terpene, respectively, indicating relatively inefficient bioconversion of algal biomass. Compared to a single bioconversion strain, the synthetic microbial consortia produced 2.5-6.2 times higher total terpene concentration, suggesting that both algal carbohydrate and protein can be more effectively converted in the single-pot process. In terms of algal carbohydrate and amino acid consumption, none of the synthetic consortia were able to completely consume the algal carbohydrates and amino acids. The 2:1 consortium ratio utilized the highest amount of algal biomass, corresponding to 36.8% of total carbohydrates and 31.3% of algal amino acids. The other two consortia ratios consumed similar amount of the total carbohydrates and algal amino acids, which were 10-15% less than the 2:1 consortium. Strain YH40—CI4A-CPS utilized approximately half of the algal amino acids in the medium but algal carbohydrate consumption was minimal (3.8% of total carbohydrate). Strain DH1-TS consumed both algal carbohydrates (37.8 % of total carbohydrate) and amino acids (23.3% of algal amino acids) in the medium. Compositional analysis indicated that carbohydrate and protein accounts for 74.2% of the mixed benthic biomass ash free dry weight (HydroMentia, Inc). Based on these data, the 2:1 consortium ratio produced the highest terpene yield at 30.5 mg terpene/ g algae while the 1:1 and 1:2 consortium ratios yielded 27.0 and 28.5 mg terpene/ g algae, respectively. The strain YH40—CI4A-CPS only produced 3.3 mg terpene/ g algae, which was lower than 8.7 mg terpene/ g algae yielded by strain DH1-CI4A-CPS, as shown in figure 3 (C). Compared to total terpene yield produced from the benthic polyculture biomass in our previous study, the consortium employing *Nannochloropsis sp.* monoculture produced more than 2- fold higher titer of total terpene. In the consortium used for bioconversion of the benthic polyculture biomass, the chamigrene synthase (JGI protein ID 322581) gene was expressed as the last enzyme in the terpene biosynthesis pathway. Compared to the multiple sesquiterpene produced by caryophyllene synthase in this study, chamigrene synthase only produces a single sesquiterpene (chamigrene) with a limited number of monoterpenes(15), which was likely a reason for the higher yield of total terpene from *Nannochloropsis sp.* Furthermore, the ash content of the benthic polyculture was more than 50% of total biomass, compared to 5.9% of *nannochloropsis sp.* (data not shown). The higher ash content of the benthic polyculture biomass resulted in higher ionic strength in the final algal hydrolysates (fermentation medium), which retarded the cell growth and compromised the terpene yield. Additionally, according to techno-economic analysis of the current state-of-the-art technologies for essential oil production, which are mainly based on water/solvent extraction, the extraction yield of essential oil ranged from 0.1% to 1% of plant tissue, corresponding to 1 mg-10 mg essential oil/ g plant tissue(26, 27) based on the relatively low concentration of essential oils in plant

tissue(28). Compared to the extraction yield of essential oil from plant tissue, the engineered strains in this study increased the terpene yield about 3~40 times, which makes it a promising alternative pathway for terpene production.

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160 **Figure 3**

161 Conclusion

162 Algae-based biofuels production has primarily focused on biodiesel production through
 163 transesterification of algal lipids. Under robust algal biomass accumulation conditions, carbohydrate
 164 and proteins typically comprise up to ~80% of the ash-free dry weight of algae biomass. Therefore, a
 165 comprehensive process for bioconversion of algal carbohydrates and proteins to high energy density
 166 fuels and value-added bioproducts should significantly improve the algal fuel process feasibility. In
 167 this study, we demonstrated simultaneous bioconversion of algal carbohydrates and proteins to
 168 terpenes which are attractive candidates for high energy density aviation fuels and other intermediate
 169 to high value bio-based chemicals applications. Using an engineered microbial consortium, greater
 170 than 30% of the carbohydrates and proteins from both a wastewater-based mixed algal feedstock and
 171 monoculture of strain *Nannochloropsis* sp were converted to terpenes, including both monoterpenes

172 and sesquiterpenes. This microbial consortium concept for comprehensive utilization of algal biomass
173 offers a versatile path forward for the production of fuels and active bioproducts from algae.
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175 Material and Methods

176 Strains and Plasmids

177 The *E.coli* strain DH1 was obtained from Joint BioEnergy Institute (JBEI). The mutant *E.coli* strain
178 YH40 (BW25113/F' [traD36, proAB+, lacIqZΔM15]ΔglnA, ΔgdhAΔluxSΔlsrA) was generously
179 provided by Professor James C Liao from University of California, Los Angeles (UCLA). The plasmid
180 pBbE1a-MEV_{up} containing the terpene biosynthesis pathway(29), the plasmid pBbE1a-GPPS, and
181 pBbE7k-TS were constructed in our previous study(15, 25). The plasmids containing the whole
182 terpene biosynthesis pathway were co-transformed into strains DH1 and YH40, respectively.

183 Terpene production from a microbial consortium on algal hydrolysates

184 Algal biomass samples from both sources were pretreated according to protocols from the National
185 Renewable Energy Laboratories and hydrolyzed with 2 mg/mL Pronase (Promega, CA) following the
186 manufacturer's protocol. The pretreated and hydrolyzed algal biomass was sterilized through filtration.
187 *E. coli* strains DH1 and YH40 each containing the terpene biosynthesis pathway were cultured into
188 15ml of LB medium as described in the previous study. The overnight cultures were centrifuged and
189 the cell pellets were re-suspended into 4 ml of pretreated algal hydrolysate. Various ratios (2:1, 1:1,
190 1:2) of engineered YH40 to DH1 were inoculated into the algal hydrolysate at a final concentration of
191 10% v/v. The culture were incubated at 37°C, 220 rpm and induced with 1 mM IPTG once the OD
192 reached 0.8. The flasks were cap-sealed and cultured for another 72 hours at 25°C, 180 rpm for terpene
193 production. Analytical samples were taken at the initial and end point of fermentation. The
194 concentrations of total carbohydrate and amino acids were determined according to the established
195 colorimetric protocols. The terpene profile and concentration was determined as described in the
196 previous study(15, 25).

198 Experimental replication and statistical treatment

199 All the fermentation experiments were performed in triplicate. The data presented in the figures
200 were the mean values and the errors were calculated as the standard deviation of the triplicates.
201

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Caption of Figures

213 Figure 1: Cartoon depiction of “one-pot” bioconversion of algal hydrolysate into terpenes.

214 Figure 2: Comprehensive conversion of algal carbohydrates and proteins into caryophyllene and other
215 terpenes using a synthetic microbial consortium on algal hydrolysate of *Nannochloropsis* sp. A:
216 Caryophyllene biosynthesis pathway construct, B: concentration of caryophyllene and other terpenes,
217 C: algal carbohydrate and protein consumption of the microbial consortia, D: caryophyllene and other
218 terpene yields based on the substrate consumption.

219 Figure 3: Comprehensive conversion of algal carbohydrate and protein into chamigrene and other
220 terpenes using a synthetic microbial consortium on algal hydrolysate of benthic polyculture biomass. A:
221 Chamigrene biosynthesis pathway construct, B: concentration of chamigrene and other terpenes, C:
222 algal carbohydrate and protein consumption and total terpene yields based on the substrate
223 consumption.

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- 232 1. Lynd LR, van Zyl WH, McBride JE, Laser M. Consolidated bioprocessing of cellulosic biomass: an update.
233 Current Opinion in Biotechnology. 2005;16(5):577-83.
- 234 2. Moody JW, McGinty CM, Quinn JC. Global evaluation of biofuel potential from microalgae. Proceedings of
235 the National Academy of Sciences of the United States of America. 2014;111(23):8691-6.
- 236 3. Razeghifard R. Algal biofuels. Photosynthesis Research. 2013;117(1-3):207-19.
- 237 4. Luque R. Algal biofuels: the eternal promise? Energy and Environmental Science. 2010;3:254-7.
- 238 5. El-Mashad HM. Biomethane and ethanol production potential of *Spirulina platensis* algae and enzymatically
239 saccharified switchgrass. Biochemical Engineering Journal. 2015;93:119-27.
- 240 6. Babujanathanam R, Kavitha P. Simultaneous Saccharification and Fermentation of Dilute Acid Pretreated
241 Red Algae (*Gelidiella acerosa*) for Bioethanol Production. Energy Sources Part a-Recovery Utilization and
242 Environmental Effects. 2014;36(12):1305-14.
- 243 7. Huo Y-X, Cho KM, Rivera JGL, Monte E, Shen CR, Yan Y, et al. Conversion of proteins into biofuels by
244 engineering nitrogen flux. Nature Biotechnology. 2011;29(4):346-U160.
- 245 8. Zhang Z, Guo S, Liu X, Gao X. Synergistic Antitumor Effect of alpha-pinene and beta-pinene with Paclitaxel
246 against Non-small-cell Lung Carcinoma (NSCLC). Drug research. 2015;65(4):214-8.
- 247 9. Rufino AT, Ribeiro M, Sousa C, Judas F, Salgueiro L, Cavaleiro C, et al. Evaluation of the anti-inflammatory,
248 anti-catabolic and pro-anabolic effects of E-caryophyllene, myrcene and limonene in a cell model of
249 osteoarthritis. European journal of pharmacology. 2015;750C:141-50.
- 250 10. Kovac J, Simunovic K, Wu Z, Klancnik A, Bucar F, Zhang Q, et al. Antibiotic Resistance Modulation and
251 Modes of Action of (-)-alpha-Pinene in *Campylobacter jejuni*. Plos One. 2015;10(4).
- 252 11. Han L, Yang L, Liu B, Cheng X. Trans-caryophyllene suppresses tumor necrosis factor (TNF alpha)-
253 induced inflammation in human chondrocytes. European Food Research and Technology. 2014;239(6):1061-6.
- 254 12. Guo K, Mou X, Huang J, Xiong N, Li H. Trans-Caryophyllene Suppresses Hypoxia-Induced
255 Neuroinflammatory Responses by Inhibiting NF-kappa B Activation in Microglia. Journal of Molecular
256 Neuroscience. 2014;54(1):41-8.
- 257 13. Strobel G. The story of mycodiesel. Current Opinion in Microbiology. 2014;19:52-8.
- 258 14. Riyaz-Ul-Hassan S, Strobel G, Geary B, Sears J. An Endophytic *Nodulisporium* sp from Central America
259 Producing Volatile Organic Compounds with Both Biological and Fuel Potential. Journal of Microbiology and
260 Biotechnology. 2013;23(1):29-35.
- 261 15. AM Gladden CAT, Connie Gao, Greg O'Bryan, Amy J Powell, Adam M Scheer, Kevin Turner, Weihua Wu,
262 Eizadora T Yu. Tailoring Next-Generation Biofuels and Their Combustion in Next-Generation Engines. Sandia
263 Report. 2013:2013-10094.
- 264 16. Strobel G, Singh SK, Riyaz-Ul-Hassan S, Mitchell AM, Geary B, Sears J. An endophytic/pathogenic *Phoma*
265 sp from creosote bush producing biologically active volatile compounds having fuel potential. Fems
266 Microbiology Letters. 2011;320(2):87-94.
- 267 17. Strobel GA, Knighton B, Kluck K, Ren Y, Livinghouse T, Griffin M, et al. The production of myco-diesel
268 hydrocarbons and their derivatives by the endophytic fungus *Gliocladium roseum* (NRRL 50072) (vol 154, pg
269 3319, 2008). Microbiology-Sgm. 2010;156:3830-3.
- 270 18. Griffin MA, Spakowicz DJ, Gianoulis TA, Strobel SA. Volatile organic compound production by organisms
271 in the genus *Ascomycorhiza* and a re-evaluation of myco-diesel production by NRRL 50072. Microbiology-Sgm.
272 2010;156:3814-29.
- 273 19. Malingré T HH, Batterman S, Bos R, Visser J. The essential oil of *cannabis sativa*. Planta Medica.
274 1975;28(1):56-61.
- 275 20. Kpadonou Kpoviessi BG1 LE, Kpoviessi DS, Gbaguidi F, Yehouenou B, Quetin-Leclercq J, Figueredo G,
276 Moudachirou M, Accrombessi GC. Chemical variation of essential oil constituents of *Ocimum gratissimum* L.
277 from Benin, and impact on antimicrobial properties and toxicity against *Artemia salina* leach. Chemistry &
278 biodiversity. 2012;9(1):139-50.
- 279 21. Rodrigues FF1 OL, Rodrigues FF, Saraiva ME, Almeida SC, Cabral ME, Campos AR, Costa JG. Chemical
280 composition, antibacterial and antifungal activities of essential oil from *Cordia verbenacea* DC leaves.
281 Pharmacognosy research. 2012;4(3):161-5.

- 282 22. Meccia G RL, Velasco J, Díaz T, Usubillaga A, Arzola JC, Ramos S. Chemical composition and
283 antibacterial activity of the essential oil of *Cordia verbenacea* from the Venezuelan Andes. *Natural product*
284 *communications*. 2009;4(8):1119-22.
- 285 23. Benjamin G. Harvey WWM, and Thomas A. Koontz. High-Density Renewable Diesel and Jet Fuels
286 Prepared from Multicyclic Sesquiterpanes and a 1-Hexene-Derived Synthetic Paraffinic Kerosene. *Energy Fuels*.
287 2015;29(4):2430-6.
- 288 24. Nakano C, Kim H-K, Ohnishi Y. Identification of the First Bacterial Monoterpene Cyclase, a 1,8-Cineole
289 Synthase, that Catalyzes the Direct Conversion of Geranyl Diphosphate. *Chembiochem*. 2011;12(13):1988-91.
- 290 25. Weihua Wu WT, Craig A. Taatjes, Jorge Alonso-Gutierrez, Taek Soon Lee, John M. Gladden. Rapid
291 Discovery and Functional Characterization of Terpene Synthases from Four Endophytic Xylariaceae. *PLoS One*.
292 2016;11(2):e0146983.
- 293 26. Jonathan Moncada JAT, Carlos A. Cardona. Techno-economic and environmental assessment of essential
294 oil extraction from Oregano (*Origanum vulgare*) and Rosemary (*Rosmarinus officinalis*) in Colombia. *Journal of*
295 *Cleaner Production*. 2015:1-10.
- 296 27. HY. Gong WL, GY. LV, Xiaoying Zhou,* . Analysis of essential oils of *Origanum vulgare* from six
297 production areas of China and Pakistan. *Revista Brasileira De Farmacognosia-Brazilian Journal of*
298 *Pharmacognosy*. 2014;24:25-32.
- 299 28. Iijima Y D-RR, Fridman E, Gang DR, Bar E, Lewinsohn E, Pichersky E. The biochemical and molecular
300 basis for the divergent patterns in the biosynthesis of terpenes and phenylpropenes in the peltate glands of three
301 cultivars of basil. *Plant Physiology*. 2004;136(3):3724-36.
- 302 29. Alonso-Gutierrez J, Chan R, Batth TS, Adams PD, Keasling JD, Petzold CJ, et al. Metabolic engineering of
303 *Escherichia coli* for limonene and perillyl alcohol production. *Metabolic Engineering*. 2013;19:33-41.

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305 **Contributions**

306 W.W.H conceived and designed the study, performed the experiments, collected and analyzed the data, wrote
307 and revised the manuscript. R.W.D supervised the study, and revised the manuscript. All authors read and
308 approved the manuscript.